国立公害研究所研究報告　第65号
Research Report from the National Institute for Environmental Studies, Japan, No. 65, 1984.

Studies on Effects of Air Pollutant Mixtures on Plants

Part 1

環境庁　国立公害研究所
THE NATIONAL INSTITUTE FOR ENVIRONMENTAL STUDIES

ERRATA

Research Report from the National Institute for Environmental Studies No. 65
Studies on Effects of Air Pollutant Mixtures on Plants Part 1

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Preface

The toxic effects of sulfur dioxide, nitrogen dioxide and ozone on plants have been extensively studied at the institute by conducting a special research program since 1976. The results of the first three years program were published in the Research Report No. 11 (1981) entitled "Studies on the Effects of Air Pollutants on Plants and Mechanisms of Pyhtotoxicity".

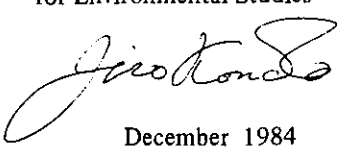
In the first program, most studies were concerned in the effects of the single air pollutant. However, plants are usually exposed to the mixed air pollutants in the urban area and few results have been reported on the effects of mixed air pollutants on plants. For clear understanding of the effects of the mixed pollutants, the second three years research program "Studies on Effects of Air Pollutant Mixtures on Plants" have been conducted from 1979 to 1982.

Mixed gas showed either additive, synergistic or antagonistic effect of the single gases. The sensitivity of plants to mixed pollutants was changed by species and by combination of the pollutants. The mechanism of phytotoxicity was studied from physiological, biochemical and micrometeorological standpoints. These results are collec-ed in this report. The detailed description of the facilities in which the experiments are conducted is also included. The extensive studies should be continued to reach the complete understanding of the mechanism of phytotoxicity.

The previous report No. 11 (1981) seems to call attention among biologists as well as environmental scientists. We appreciate that the useful suggestions and discussion are given to the report.

It is hoped that this report is also of some use for scientists who are interested in the toxic effects of atmospheric pollutants.

Jiro Kondo, Eng. D.
Director of the National Institute
for Environmental Studies



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Effects of Air Pollutants on Transpiration Rate in Relation to Abscisic Acid Content

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The effects of air pollutants singly or in mixture on transpiration rate were examined using several plant species. The transpiration rate of tomato and peanut plants which contain large amount of ABA was rapidly decreased by exposure to 0.5 ppm O_3, while the rate of radish, broad bean, spinach and corn plants with a little ABA gradually declined or began to decrease after a lag period of 20-30 min. The fumigation with 8 ppm NO_2 gave the results similar to O_3 fumigation. The decline of transpiration rate of tomato caused by a short term exposure to 2 ppm SO_2 and 8 ppm NO_2 singly was completely restored within 24 h after the termination of the fumigation, whereas the rate decreased by O_3 was not restored at all. Fumigation with 0.05 ppm O_3 and 0.1 ppm SO_2 singly had no effect on transpiration rate of tomato. Exposure to 0.05 ppm O_3 and NO_2 at the concentrations above 2 ppm in mixture pronouncedly decreased the transpiration rate, while 0.1 ppm SO_2 alleviated the inhibitory effect of 4 ppm NO_2.

SO_2 fumigation for 30-40 min had little effect on ABA content in leaves of any species of plants tested, while O_3 fumigation decreased the content in broad bean leaves, suggesting that the change of transpiration rate during the fumigation did not result from the change of ABA content in leaves.

Key words: Abscisic acid, Nitrogen dioxide, Ozone, Stomata, Sulfur dioxide, Transpiration.

Air pollutants are absorbed by land plants mainly through stomata. It has been often observed that the degree of plant injury caused by SO_2 fumigation was closely correlated with the amount of absorbed SO_2 or the rate of SO_2 absorption (Thomas & Hill, 1935; Thomas, 1961; Taylor, 1973; Caput & Belot, 1978; Omasa *et al.*, 1981). Injury due to O_3 fumigation has been reported to be reduced by treatment with ABA which is known to close stomata (Adedipe *et al.*, 1973). These results suggested some relationship between the plant damage and stomatal density and/or aperture size. However, there has been no experimental evidence for this suggestion until a recent date. Rapid stomatal movement induced by fumigation with

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Abbreviation: ABA, abscisic acid.

air pollutants would be also concerned in the injury. We have already reported that SO_2-resistant plants had high contents of ABA and rapidly closed the stomata in response to SO_2 fumigation (Kondo & Sugahara, 1978; Kondo *et al.*, 1980). In the present study, we examined the stomatal sensitivity to O_3 and NO_2 in relation to ABA content. In addition, the stomatal behavior after the termination of fumigation and the effect of the mixed air pollutants on stomatal movement were also investigated.

Materials and Methods

Plant materials

Tomato (*Lycopersicon esculentum* Mill cv. Fukuju No. 2), peanut (*Arachis hypogaea* L. cv. Chibahandachi), radish (*Raphanus sativus* L. cv. Minowase) and corn (*Zea mays* L. cv. Yellow Dent-corn) plants were grown at 25 ± 0.5°C with a relative humidity of 70 ± 5% in an environment-controlled glass house under natural light conditions for about 6-7, 7-8, 5 and 4 weeks, respectively. Broad bean (*Vicia faba* L. cv. Otafuku) and spinach (*Spinacia oleracea* L. cv. New Asia) plants were grown at 22 ± 0.5 and 20 ± 0.5°C during the day and 17 ± 0.5 and 15 ± 0.5°C at night for about 6-7 and 5 weeks, respectively. Tobacco (*Nicotiana tabacum* L. cv. Samsun NN) plants were grown for about three months after sowing in a greenhouse at 20-28°C. Potting soil, nutrition and irrigation were as described previously (Kondo & Sugahara, 1978).

Fumigation of plants with air pollutants

The test plants grown in the glass house were transferred to a growth cabinet (170 X 230 X 190 cm) at 9:00-10:00 A. M. for fumigation of SO_2, NO_2 and O_3. The plants were preconditioned for 1 to 2 h in the cabinet at 25 ± 0.5°C (22 ± 0.5 and 20 ± 0.5°C in the cases of broad bean and spinach, respectively) with a relative humidity of 75 ± 3% under light intensity of about 400 $\mu E\ m^{-2}\ s^{-1}$ PPFD at leaf level. The light source was 24 metal halide lamps (400W; Yoko Lamp, Toshiba). The concentrations of SO_2, NO_2 and O_3 in the cabinet rose to the desired levels in 5 to 10 min after the start of introduction of the gases, and controlled below ±0.04% of the fixed concentrations. The concentrations of SO_2, NO_2 and O_3 in the cabinet fell to 50% of the equilibrated level within 4, 5 and 4 min after the stop of gas supply. Mean wind velocity in the cabinet was 0.22 m/s.

Measurement of transpiration rate

To investigate stomatal movement caused by air pollutants, the change of transpiration rate was measured. The transpiration rate of test plants was obtained from the rate of decrease in the weight of the pot containing a plant. The pot was covered with vinyl sheet to prevent evaporation of water from the soil surface. After the fumigation experiments was the leaf area measured with a leaf area meter and the transpiration rate per leaf area was determined.

Extraction and measurement of ABA

Approximately 3 to 5 g of leaves of all ages were excised from various leaf positions of the test plants and quickly weighed. Immediately after weighing were the leaves immersed in ice cold 60 ml of methanol-ethyl acetate-acetic acid (50:50:1, v/v) containing 20 mg/l 2, 6-di-*tert*-butyl-4-methylphenol (Lovey, 1977), homogenized in a homogenizer (Polytron, Kinematica) and allowed to stand overnight at 4°C. The homogenate was then centrifuged for 10

min at 7,000x*g* at 4°C. The extraction was repeated, and the extracts were combined and concentrated in an evaporator at 40°C to the aqueous phase. The aqueous solution was diluted with distilled and deionized water up to 50 ml, then partitioned three times against equal volumes of *n*-hexane at pH 2.5 and thereafter against equal volumes of dichloromethane three times at pH 9.0 and then three times at pH 2.5 (Ciha, *et al.*, 1977). The acidic dichloromethane extracts were combined and evaporated to dryness. ABA in the dried extract was purified by thin layer chromatography, methylated with diazomethane and analyzed by gas liquid chromatography as described previously (Kondo & Sugahara, 1978). Each sample was measured three times and the mean obtained. Values in Table 1 and 2 are averages of the quantities of two or three samples.

Table 1 Effect of SO_2 fumigation on ABA content in plant leaves

	ABA (ng/g fr. wt)[a]	
	Non-fumigated	Fumigated
Tomato	338 ± 36	340 ± 27
Peanut	195	185
Tobacco	108	99
Radish	16 ± 2	20 ± 6
Corn	2 ± 0	4 ± 1

Test plants were exposed to 2 ppm SO_2 for 30-40 min.
[a]Average of three samples ± SD or average of two samples.

Table 2 Effect of O_3 fumigation on ABA content in broad bean leaves

	ABA (ng/g fr. wt.)[a]
Non-fumigated	8.0 ± 1.3
O_3 fumigated	4.8 ± 1.7

Broad bean plants were exposed to 0.5 ppm O_3 for 30-40 min.
[a]Average of three samples ± SD.

Results and Discussion

Effect of 0.5 ppm O_3 on transpiration rate of six species of plants is shown in Fig. 1. The transpiration rates of tomato and peanut plants containing large amounts of ABA (Table 1) began markedly to decrease within 10 min after the commencement of the fumigation, while radish, broad bean, spinach and corn plants with low ABA contents (Table 1 and 2) showed gradual decrease in transpiration rate or began to decrease in the rate after lag periods of 20-30 min. The fumigation with 8 ppm NO_2 gave the similar results (Fig. 2). These results coincided with the case of 2 ppm SO_2 fumigation, except that the rate of corn plant was rapidly

decreased by SO_2 fumigation (Kondo & Sugahara, 1978; Kondo *et al.*, 1980). The effect of concentration of SO_2 on the change in transpiration rate of tomato is shown in Fig. 3. The lower the concentration was, the smaller the suppressive effect of SO_2 was, but the rapidity of the decrease in the transpiration rate was identical among three concentrations of SO_2 tested. Next, we examined the restoration of transpiration rate after the termination of fumigation (Fig. 4). Tomato plants were exposed to 2 ppm SO_2, 8 ppm NO_2 or 0.5 ppm O_3 for 30 min, then the gas supply was stopped. Transpiration rate of tomato plants exposed to SO_2, NO_2 and O_3 decreased within 30 min of fumigation and only slightly restored during 2 h after the termination of fumigations. After 24 h, the transpiration rate depressed by SO_2 and NO_2 was completely restored, but the rate decreased by O_3 was not restored till 24 h after the termination. The change of transpiration rate of corn plant due to SO_2 fumigation was also shown in Fig. 4. The rate was decreased by 2 ppm SO_2 to 57% of the initial level, then gradually restored after the discontinuance of fumigation and reached 94% of the initial level 2 h later. The decline of transpiration rate caused by short term exposures to SO_2 and NO_2 might mean temporary avoidance from air pollutants, while the decline caused by O_3 would be a result of irreversible damages.

Fig. 5 shows the effect of NO_2 addition on transpiration rate of tomato plants being exposed to low concentration of SO_2 or O_3, which alone exerted no effect on the rate. Tomato plants were continuously exposed to 0.05 ppm O_3 or 0.1 ppm SO_2 and besides exposed to NO_2 of which the concentration was raised successively every 40 min from 0.5 to 4 ppm. The transpiration rate decreased with rise of NO_2 concentration whether NO_2 fumigation was performed singly or in mixture with O_3. The suppressive effect was larger in combination of NO_2 with O_3 than in NO_2 alone. Sometimes even at a low concentration of NO_2, the transpiration rate rapidly declined. This rapid decrease in the rate was enhanced by 0.1 ppm SO_2. But at higher concentrations of NO_2 (3 and 4 ppm), the transpiration rate became higher in the mix-

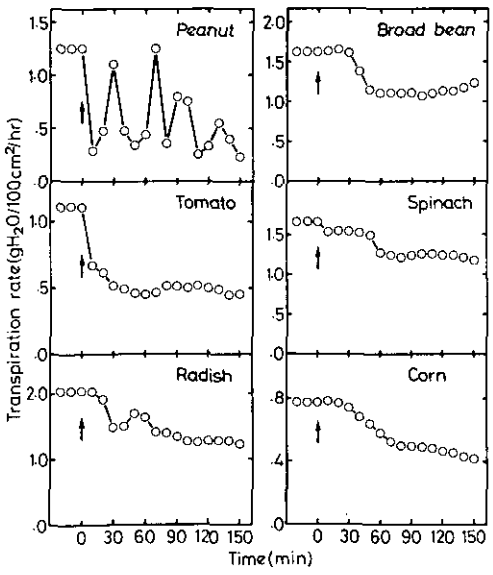

Fig. 1 Changes in transpiration rate with O_3 fumigation

Test plants were preconditioned for about 2 h in the growth cabinet prior to 0.5 ppm O_3 fumigation. O_3 gas was introduced into the cabinet at 0 time indicated by the arrow.

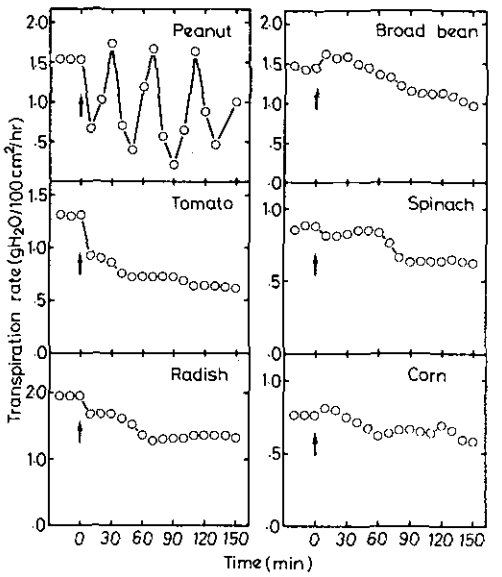

Fig. 2 Changes in transpiration rate with NO_2 fumigation

Test plants were preconditioned for about 2 h in the growth cabinet prior to 8 ppm NO_2 fumigation. NO_2 gas was introduced into the cabinet at 0 time indicated by the arrow.

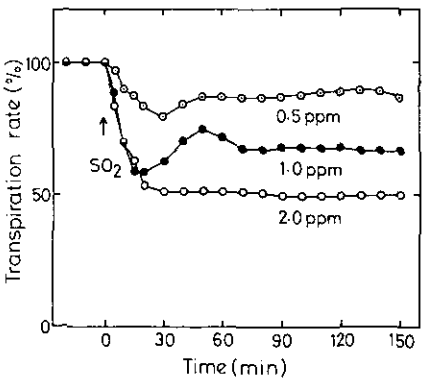

Fig. 3 Effect of SO_2 concentration on transpiration rate of tomato plant

SO_2 gas was introduced at 0 time indicated by the arrow and maintained at 0.5, 1.0 or 2.0 ppm.

ture than in NO_2 alone. Namely, SO_2 alleviated the effect of high concentrations of NO_2. Therefore, larger amount of NO_2 might be absorbed by the plant through stomata in the mixture than in NO_2 alone at high NO_2 concentration. NO_2 in the present range of concentration, 0.05 ppm O_3 and 0.1 ppm SO_2 each usually caused little visible injury to tomato leaves, but the fumigation with the mixture of NO_2 and 0.1 ppm SO_2 wilted most tomato leaves. The present result is consistent at low NO_2 concentration with previous reports which have described the stomatl closure by the mixture of SO_2 and NO_2 (Ashenden, 1979; Amundson & Weinstein,

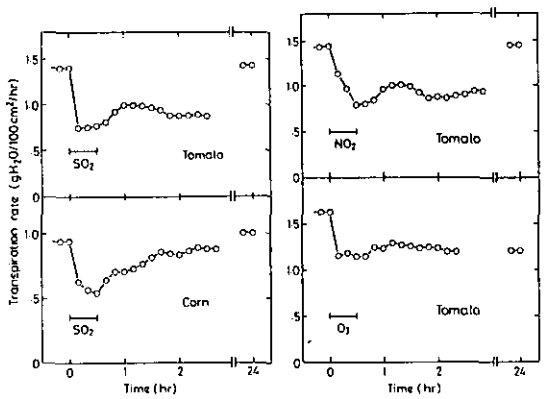

Fig. 4 Changes in transpiration rate of tomato and corn plants after the termination of fumigation with SO_2, NO_2 *or* O_3

Tomato and corn plants were exposed to 2 ppm SO_2, 8 ppm NO_2 or 0.5 ppm O_3 for 30 min and then the fumigation was stopped.

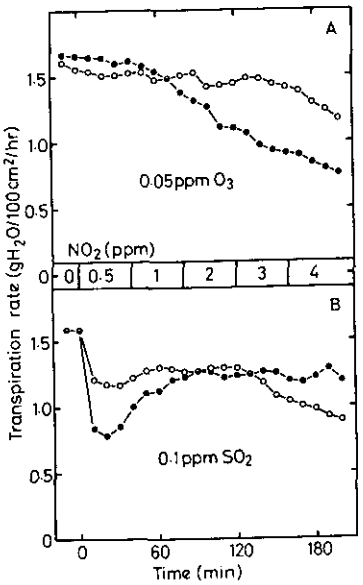

Fig. 5 Effects of O_3 (A) and SO_2 (B) on the changes in transpiration rate caused by NO_2 fumigation

Tomato plants were exposed to 0.05 ppm O_3 or 0.1 ppm SO_2, and then NO_2 was introduced at 0 time and maintained at the indicated concentrations. NO_2 concentration was successively raised at the interval of 40 min. o, NO_2 alone; •, mixture of NO_2 and 0.05 ppm O_3 (A) or 0.1 ppm SO_2 (B).

1981).

The effect of SO_2 fumigation on ABA content in leaves was examined. ABA content was not changed by SO_2 fumigation for 30-40 min in tomato, peanut, tobacco, radish and corn plants (Table 1). O_3 fumigation for 30-40 min reduced ABA content in broad bean leaves

(Table 2). Therefore, it might be concluded that the decrease in transpiration rate observed from 20-30 min after the start of fumigation with SO_2, NO_2 and O_3 does not result from the increase in ABA content in leaves. Long term fumigation with these pollutants probably causes damage to stomatal function.

The results obtained in the present experiments suggest that ABA could act as a controlling factor for protection of plants from damages induced not only by SO_2 but also by NO_2 or O_3. However, it remains to be solved whether ABA plays some important roles as the protecting substance under mixed air pollution or not.

Acknowledgment

We wish to thank the members of the Engineering Division of this institute for their adept operation of the growth cabinet and the cultivation and provision of the plant materials.

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大気汚染物質暴露による蒸散速度変化とアブサイシン酸含有量との関係

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0.5ppm オゾン（O_3）及び8 ppm 二酸化窒素（NO_2）暴露による植物の蒸散変化を，アブサイシン酸（ABA）含有量の多いトマト，落花生及びABA含有量の少ないダイコン，ソラマメ，ホウレンソウ，トウモロコシを実験材料として調べた。トマトと落花生の蒸散速度はO_3暴露により急速に低下したのに対し，ダイコン，ソラマメ，ホウレンソウ，トウモロコシの蒸散速度は暴露開始後徐々に低下した。NO_2暴露の場合も同様の結果が得られた。2 ppm 二酸化硫黄（SO_2）と8 ppm NO_2により低下したトマトの蒸散速度は暴露停止後回復したが，0.5ppm O_3による蒸散低下は回復しなかった。0.05ppm O_3がNO_2と同時に与えられると，NO_2による蒸散低下は促進されたが，0.1ppm SO_2はNO_2の影響を軽減した。

30－40分間のSO_2暴露は植物葉のABA含有量にはほとんど影響を与えなかったが，O_3暴露によりソラマメ葉のABA含有量は低下した。これらの結果より大気汚染物質暴露時の蒸散速度の変化がABA含有量の変化によるものではないことが示唆された。

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Res. Rep. Natl. Inst. Environ. Stud., Jpn., No. 65, 1984.

Effect of Sulfite on Stomatal Aperture Size in *Vicia* Epidermal Peels

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The stomatal aperture size in *Vicia* epidermal strips was reduced by sulfite. Stomatal sensitivity to sulfite was very high at low pH. Sulfite addition to the incubation medium caused a rapid stomatal closure at pH 4.0, and it inhibited stomatal opening induced by light at pH 6.0. Sulfite treatment hardly changed K^+ content in the strips, while it notably reduced malate content, even if KIDA was used in place of KCl or pyruvate was added. Sulfite remarkably decreased ATP content in the strips. Mechanism of decrease of malate content caused by sulfite was discussed.
Key words: ATP, Malate, pH, Potassium, Stomata, Sulfite, *Vicia*.

The transpiration rate of the plants with a high ABA content was rapidly decreased by SO_2 fumigation (Kondo & Sugahara, 1978). Based on this finding, we proposed the idea that ABA would act as a controlling factor for stomatal closure induced by SO_2 fumigation and consequently for resistance to SO_2 injury. However, corn and sorghum containing only a little ABA also decreased in the transpiration rate following SO_2 fumigation (Kondo *et al.*, 1980), suggesting that SO_2 itself closed stomata without ABA-dependent regulation in some plants. SO_2 absorbed by plant leaves through stomata is transformed into sulfite and/or bisulfite ions on the wet surface of guard cells and in cytoplasmic fluid, resulting in the proton generation. Therefore, the effects of SO_2 on the stomatal movement must be derived from sulfite or bisulfite ions and/or from lowering of pH. The stomatal closure by sulfite has been reported by some workers (Zelitch & Walker 1964; Taylor *et al.*, 1981; Rao & Anderson, 1983).

Stomatal movements are caused by changes in guard cell turgor arising from the movement of K^+ and H^+ with electroneutrality being maintained by movement of Cl^- or internal production of malate (Raschke, 1979). The enzymes involved in malate formation, PEP

Abbreviation: ABA, abscisic acid; KIDA, potassium iminodiacetate; MES, 2-(N-morpholino)-ethanesulfonic acid; PEP, phosphoenolpyruvate.

carboxylase and NADP-malate dehydrogenase, in epidermis of pea leaves were suppressed by SO_2 fumigation (Rao *et al.*, 1983). It has been already reported that PEP carboxylase and malate dehydrogenase were strongly inhibited by sulfite treatment in competition with CO_2 (Ziegler, 1973, 1974; Mukerji & Yang, 1974). These results suggest that sulfite treatment would reduce malate content and in turn K^+ content in guard cells. In the present experiments, we investigated the effects of sulfite on stomatal aperture and contents of K^+ and malate in epidermal peels from *Vicia faba* leaves.

Materials and Methods

Plant materials

Vicia faba L. cv. Otafuku was grown for about 5 to 6 weeks at 22 ± 0.5°C in the day time and 17 ± 0.5°C at night with a relative humidity of 70 ± 5% in an environment-controlled glass house under natural light conditions. The preparation of epidermal peels was done in the same manner as described previously (Kondo *et al.*, 1980). Epidermal strips were peeled with tweezers from the abaxial surface of secondly and thirdly young leaves of fully expanded ones, immersed in distilled water and sonicated for 2 min with a 20-KC Ultrasonic disruptor (Branson Sonifier 185) to eliminate mesophyll contamination and epidermal cytoplasm except for guard cells (Durbin & Graniti, 1975), then washed with fresh deionized water. Microscopic observations confirmed that no mesophyll cells adhered to the sonicated peels.

Measurement of stomatal aperture size in epidermal strips

The sonicated epidermal strips were transferred to 10 ml of buffer solution containing 10 mM KCl, 0.1 mM $CaCl_2$ and varying concentrations of Na_2SO_3 with 0.1 mM EDTA to suppress sulfite oxidation in vials unless otherwise stated. One-tenth strength of McIlvaine's buffers or 10 mM MES-NaOH buffer, pH 6.0, were used. DCMU dissolved in ethanol was added to the incubation medium to make the final concentrations of DCMU and ethanol of 50 μM and 1%, respectively. The vials were placed in a water bath kept at 25°C, and illuminated at about 1200 $\mu E\ m^{-2}\ s^{-1}$ with a 300W tungsten lamp (Eye Lamp, Iwasaki Electric Co., Ltd.). The light was passed through a 5-cm-thick water layer. After 3-h incubation, stomata in epidermal strips were microscopically observed and photographed, and then the inner width of the stomatal aperture measured. Values represented in the figures and tables are averages of measurements of about 30 to 50 stomata with standard errors.

Extraction and measurement of K^+ in epidermal strips

The epidermal strips were trapped on nylon mesh (NXX 13, NBC Industries) after 3-h incubation under various conditions and washed with deionized water. Cations were extracted from the epidermis for 1 h in 5% analytical grade HNO_3 at about 100°C, then the peels rinsed twice with fresh 5% HNO_3. The extract solution and rinsings were combined and the fresh HNO_3 solution added to make a total of 15 ml. After extraction, the epidermal strips were dried in an oven overnight at 70°C and weighed. Concentration of K^+ in the solution was measured with an atomic absorption spectrophotometer (Hitachi 170-50A). The content of K^+ in the strips was calculated on the basis of dry weight.

Extraction and measurement of malate in epidermal strips

The epidermal strips were killed in boiling 90% ethanol after 3-h incubation, and malate was extracted from the epidermis with the same solvent at 60°C for 30 min. The extraction was repeated further 2 times with the fresh solvent, then the extracts combined and evaporated to dryness. After extraction, the epidermal strips were dried overnight at 70°C and weighed. The content of malate in the extract was determined by the enzymic assay after Möllering (1974). The extract was solubilized in a reaction mixture containing 1.6 ml of 0.1 M 3-amino-1-propanol buffer, pH 10.0, 0.20 ml of 0.5 M glutamate (pH 10.0), 0.15 ml of 60 mM β-NAD and 3.6 units of glutamic-oxaloacetic transaminase (Boehringer & Söhne G.m.b.H., Manheim, Germany). To this reaction mixture was added 36 units of malate dehydrogenase (Boehringer & Söhne G.m.b.H.), and the mixture incubated at 25°C. The amount of malate was estimated from the increase in absorbance at 340 nm after incubation with malate dehydrogenase for 1 h. The content of malate in the epidermal peels was calculated on the basis of the dry weight. The epidermal peels gave 3–4 mg dry weight for each sample.

ATP content

ATP was determined by the luciferine-luciferase method (Strehler, 1974). ATP in epidermal strips was extracted with 1.5 ml of ice-cold 1 M $HClO_4$ by standing at 0°C for 1 h. The extracted ATP solution was neutralized by adding an appropriate volume of 2 M KOH, and after centrifugation, the supernatant was used for ATP determination. The measurement was performed after Shimazaki *et al.* (1983).

Results

Effect of sulfite on stomatal aperture size

Fig. 1 shows the effect of 1 mM Na_2SO_3 on stomatal aperture size at pH 3.0 to 7.0. Aperture size remained unchanged over a pH range of 3.0 to 7.0 in the absence of sulfite, while it was remarkably reduced by sulfite at low pH, especially at pH 3.0 and 4.0. But sulfite had no effect on the aperture size at pH 7.0. As shown in Fig. 2, only high concentrations of sulfite, 10 and 100 mM, decreased the size at pH 7.0, while sulfite even at 10 μM produced a marked stomatal closure at pH 4.0. The suppressed stomatal aperture was not recovered by removal of sulfite from the incubation medium (data not shown).

Effect of sulfite on potassium and malate content

Fig. 3 shows the effect of 1 mM Na_2SO_3 on K^+ content in epidermal strips at various pH. K^+ content was small at low pH, 3.0 to 5.0, and high at pH 6.0 and 7.0. Sulfite treatment gave no effect on the content at any pH. In Fig. 4, the effect of sulfite at various concentrations on stomatal aperture was compared with its effect on K^+ content at pH 6.0. The stomatal aperture size was reduced at the concentrations above 1 mM, while K^+ content decreased only at the highest concentration tested, 10 mM. Thus, the aperture size did not associate with K^+ content. Fig. 5 shows the effect of 1 mM Na_2SO_3 on malate content in the epidermal strips at pH 3.0 to 7.0. Malate content considerably decreased with lowering of pH. Malate content was reduced by sulfite to 0, 0, 29 and 63% of the content without sulfite treatment at pH 3.0, 4.0, 5.0 and 6.0, respectively, whereas sulfite caused an increase in malate content at pH 7.0.

Time course of sulfite effect on stomatal aperture size

Stomatal aperture size began to increase 1 h after the commencement of illumination at

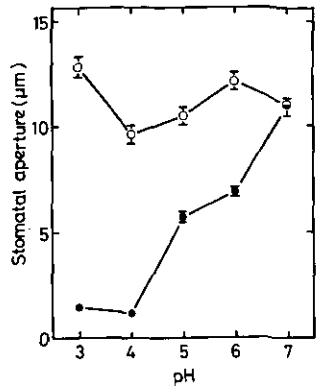

Fig. 1 Effect of sulfite on stomatal aperture size at various pH

Sonicated epidermal strips were incubated in light for 3 h at 25°C in one-tenth strength of McIlvaine's buffers containing 10 mM KCl, 0.1 mM $CaCl_2$ and 0.1 mM EDTA with or without 1 mM Na_2SO_3. Vertical bars indicate the range of standard error. –○–, without Na_2SO_3; –●–, with Na_2SO_3.

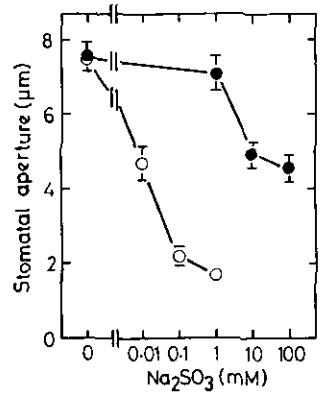

Fig. 2 Effect of sulfite concentration on stomatal aperture size

Incubation of the strips was performed as in Fig. 1 except for pH 4.0 and 7.0 used and sulfite concentration varied. –○–, pH 4.0; –●–, pH 7.0.

both pH 4.0 and 6.0. The stomata began to close within 20 min after the start of application of 1 mM Na_2SO_3 at pH 4.0 whether sulfite was added immediately after the commencement of illumination or 2 h later (Fig. 6). On the other hand, at pH 6.0, 10 mM sulfite inhibited the stomatal opening induced by illumination, i.e., the aperture size was constant during the incubation with sulfite.

Relationship between stomatal aperture size and malate content

When KIDA was used in place of KCl at pH 6.0, malate content markedly increased as shown by Raschke and Schnabl (1978), though stomatal aperture did not change (Table 1). Treatment with 10 mM Na_2SO_3 in KIDA medium decreased the malate content as well as the

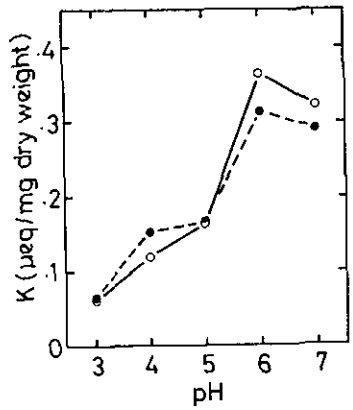

Fig. 3 Effect of sulfite on potassium content

Incubation of the strips was performed as in Fig. 1. —○—, without Na_2SO_3; —●—, with Na_2SO_3.

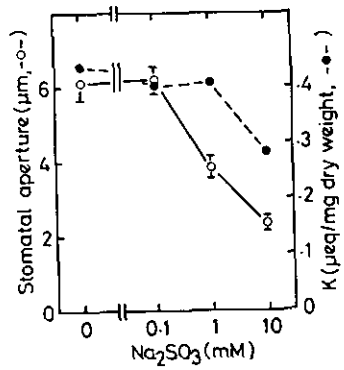

Fig. 4 Comparison of effects of sulfite on stomatal aperture size and potassium content

Incubation of the strips was performed as in Fig. 2 except for pH 6.0 used. Vertical bars indicate the range of standard error.

aperture size to the same level as the sulfite treatment in KCl medium. Addition of malate and pyruvate alleviated the inhibitory effect of sulfite (Table 2), though these substances could not recover the suppressed aperture size which had been caused by sulfite (data not shown). Pyruvate treatment increased malate content. However, sulfite treatment decreased the content in the strips to an identical level whether pyruvate was added to the incubation medium or not.

Effect of DCMU on stomatal aperture

DCMU at 50 μM decreased the aperture size by 30% of control at pH 6.0, but did not enhance the inhibition caused by 10 mM sulfite which reduced the aperture size by 60%. (Table 3).

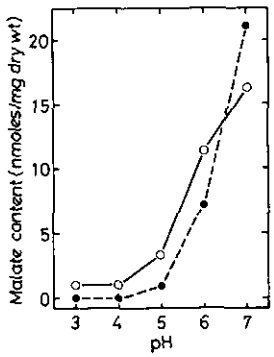

Fig. 5 Effect of sulfite on malate content at various pH

Incubation of the strips was performed as in Fig. 1. –o–, without Na_2SO_3; –•–, with Na_2SO_3.

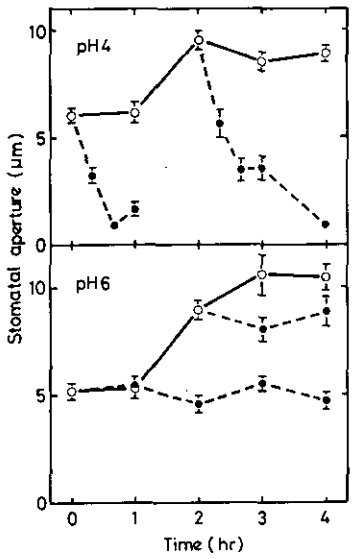

Fig. 6 Time course of effect of sulfite on stomatal aperture size

Sonicated epidermal strips were immersed in incubation medium indicated in the legend of Fig. 1 except for Na_2SO_3 and EDTA omitted. Light illumination was started at time 0. Na_2SO_3 in EDTA solution was added to the medium at time 0 or 2 h after the start of illumination to make sulfite concentration of 1 and 10 mM at pH 4.0 and 6.0, respectively, and to make EDTA concentration of 0.1 mM. Vertical bars indicate the range of standard errors. –o–, without Na_2SO_3; –•–, with Na_2SO_3.

Table 1 Effect of sulfite on stomatal aperture size and malate content in epidermal strips incubated in the medium containing KCl or KIDA

		Stomatal aperture[a] (μm)	Malate content (μmol/g dry wt.)
KCl	$-Na_2SO_3$	10.60 ± 0.76 (39)	11.0
	$+Na_2SO_3$	4.96 ± 0.64 (41)	1.6
KIDA	$-Na_2SO_3$	9.56 ± 0.70 (35)	18.5
	$+Na_2SO_3$	4.62 ± 0.43 (42)	1.7

Sonicated epidermal strips were incubated in light for 3 h at 25°C in 10 mM MES-Na buffer, pH 6.0, containing 10 mM KCl or KIDA and 0.1 mM EDTA with or without 10 mM Na_2SO_3.

[a] Mean ± S.E. Numbers in parentheses represent number of samples measured.

Table 2 Effect of addition of malate and pyruvate on stomatal aperture size and malate content

		Stomatal aperture[a] (μm)	Malate content (μmol/g dry wt.)
Control	$-Na_2SO_3$	10.47 ± 0.32 (44)	8.5
	$+Na_2SO_3$	3.87 ± 0.40 (41)	1.5
Malate	$-Na_2SO_3$	10.00 ± 0.58 (49)	–
	$+Na_2SO_3$	6.66 ± 0.72 (34)	–
Pyruvate	$-Na_2SO_3$	12.17 ± 0.49 (49)	16.7
	$+Na_2SO_3$	6.88 ± 0.61 (41)	1.7

Sonicated epidermal strips were incubated in light for 3 h at 25°C in 10 mM MES-Na buffer, pH 6.0, containing 10 mM KCl and 0.1 mM EDTA with or without 10 mM Na_2SO_3, 10 mM malate and 10 mM pyruvate.

[a] Mean ± S.E. Numbers in parentheses represent number of samples measured.

Table 3 Effect of DCMU on stomatal aperture size

		Stomatal aperture[a] (μm)
$-Na_2SO_3$	−DCMU	8.61 ± 0.19 (45)
	+DCMU	6.03 ± 0.31 (48)
$+Na_2SO_3$	−DCMU	3.69 ± 0.34 (43)
	+DCMU	3.46 ± 0.33 (48)

Sonicated epidermal strips were incubated in light for 3 h at 25°C in 10 mM MES-Na buffer, pH 6.0, containing KCl, 0.1 mM $CaCl_2$ and 1% ethanol with or without 10 mM Na_2SO_3 and 50 μM DCMU.

[a] Mean ± S.E. Numbers in parentheses represent number of samples measured.

Table 4 Effect of sulfite on ATP content

	ATP content (μmol/g dry wt.)
$-Na_2SO_3$	129
$+Na_2SO_3$	3

Incubation of the strips were performed in light for 3 h at 25°C using 10 mM MES-Na buffer, pH 6.0, containing 10 mM KCl, 0.1 mM $CaCl_2$ and 0.1 mM EDTA with or without 10 mM Na_2SO_3.

Effect of sulfite on ATP content

Treatment with 10 mM Na_2SO_3 for 3 h remarkably lowered ATP content in the epidermal strips (Table 4).

Discussion

Stomatal movements are caused by changes in water potential, mainly osmotic potential, in guard cells. Osmotic potential in guard cells is regulated by the movement of K^+ and Cl^- or internal production of malate (Raschke, 1979). Sulfite strongly suppressed the stomatal opening in *Vicia* epidermal peels at pH 6.0 and caused a rapid stomatal closure at pH 4.0 (Fig. 6). We could not find the close correlation between the stomatal aperture size and K^+ content. On the other hand, sulfite reduced malate content in the strips, indicating a close correlation between stomatal aperture size and malate content. It has been reported that sulfite inhibited PEP carboxylase (Ziegler, 1973; Mukerji & Yang, 1974) and NADP-malate dehydrogenase (Ziegler, 1974) involved in malate formation. PEP carboxylase extracted from the epidermal strips of *Commelina* was inhibited by malate, oxaloacetate and bisulfite and besides stomatal opening was also suppressed by these substances (Raghavendra, 1980), suggesting that stomatal opening is regulated by PEP carboxylase activity or malate content. In the present study, addition of pyruvate to the incubation medium increased the stomatal aperture size and malate content, while KIDA in place of KCl increased malate content but could not increase the stomatal aperture. In both cases, sulfite strongly decreased the stomatal aperture size and malate content. These results also suggest some relationship between the stomatal aperture size and malate content.

Rao and Anderson (1983) found that sulfite had no effect on PEP carboxylase extracted from *Pisum* epidermal strips. They also showed that sulfite completely inhibited light activation of PEP carboxylase and NADP-malate dehydrogenase when it was added to epidermal strips. From these results, they assumed that sulfite inhibits the light modulation of key enzymes in guard cells. It has been recently reported that sulfite inhibited photosystem II in spinach leaves (Shimazaki *et al.*, 1984). DCMU inhibited photosystem II in guard cells of *Vicia* (Shimazaki *et al.*, 1982), but gave only a small inhibitory effect on stomatal aperture (Table 3) compared with sulfite effect. Therefore, it seems unlikely that sulfite effect is only due to the inhibition of photosystem II and/or inhibition of light modulation of key enzymes which might be resulted from the photosystem inhibition. Sulfite inhibited NAD-malate dehydrogenase as well as NADP-malate dehydrogenase (Rao & Anderson, 1983), resulting in a strong inhibition of malate formation and in turn the intense inhibition of stomatal opening.

Sulfite extremely depressed ATP content in the strips (Table 4). Guard cells have a high respiratory activity and ATP was produced mainly due to oxidative phosphorylation in guard cells (Shimazaki *et al.*, 1983). Phenylmercuric acetate, a potent inhibitor of stomatal opening (Pallaghy & Fischer, 1974), inhibited the reoxidation of reduced Q by photosystem I and did respiration, and extremely decreased ATP content in guard cells (Shimazaki *et al.*, 1982, 1983). Sulfite might exert the inhibitory effect on respiration as well as photosystem in guard cells, though no marked effect of sulfite on respiration has been reported.

Effects of sulfite on stomata at pH 4.0 appears to be different from the effects at pH 6.0 (Fig. 6). Osmotic potential in guard cells may be higher at pH 4.0 than that at pH 6.0, because the contents of K^+ and malate were much lower at pH 4.0 than those at pH 6.0. However, the stomatal aperture size was almost identical over pH 4.0 to 7.0. These results suggest that cell wall of guard cells is loosened at low pH as shown by Jinno and Kuraishi (1982). Sulfite might cause the change in cell wall extensibility.

The decreased transpiration rate caused by SO_2 fumigation in corn plants was restored by termination of the fumigation (Kondo & Sugahara, 1984). On the other hand, the reduced stomatal aperture size induced by sulfite in *Vicia* epidermis could not be restored by removal of sulfite from the incubation medium. These results indicate that the sulfite effect on stomata in *Vicia* epidermis may not be exactly identical with the SO_2 effect on corn transpiration. To clarify the mechanism of the reversible inhibition of corn transpiration by SO_2 fumigation, further studies are required.

Acknowledgment

We wish to thank the members of the Engineering Division of this institute for their adept operation of the growth cabinet and the cultivation and provision of the plant materials.

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ソラマメ葉の剝離表皮中の気孔開度に対する亜硫酸の影響

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亜硫酸処理によりソラマメ葉の剝離表皮中の気孔開度は減少した。特に低 pH で亜硫酸に対する気孔の感受性が高かった。pH 4 では亜硫酸処理により急速に気孔は閉鎖し，pH 6 では亜硫酸は光による気孔開孔を阻害した。表皮中の K^+ 含有量はほとんど亜硫酸処理の影響を受けなかったが，リンゴ酸含有量は亜硫酸処理により顕著に減少した。ATP 含有量も亜硫酸により著しく減少した。亜硫酸によるリンゴ酸含有量低下の機作について考察した。

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Res. Rep. Natl. Inst. Environ. Stud., Jpn., No. 65, 1984.

Inhibition of Photosynthesis by Sulfite and Uptake of [^{35}S]-Sulfite in Mesophyll Protoplasts Isolated from *Vicia faba* L.

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Photosynthetic O_2 evolution as well as $^{14}CO_2$ fixation in mesophyll protoplasts isolated from *Vicia faba* leaves were strongly decreased by the preincubation of the protoplasts with 10 mM Na_2SO_3 in pH region below 6.0 even if no Na_2SO_3 was added to the assay mixture, whereas both activities were not affected by the incubation with Na_2SO_3 at pH above 6.0. The lower the pH of incubation medium containing Na_2SO_3 was, the larger the amount of sulfite was accumulated within the protoplasts. Protoplasts incubated with Na_2SO_3 at pH 5.0 were intact as judged by the observation under microscope and by the vital staining with Evans blue. Chloroplasts isolated from *Vicia* mesophyll protoplasts sustained more than 80% of the photosynthetic activity of original protoplasts at a maximum pH of 8.4–8.6. Unlike the case of protoplasts, photosynthetic O_2 evolution of chloroplasts isolated from the protoplasts was inhibited by Na_2SO_3 to the similar level over pH range examined (7.4–9.0).

Key words: Intact chloroplast, Mesophyll protoplast, pH, Photosynthesis, Sulfite uptake, *Vicia faba*

Exposure to SO_2, a major atmospheric pollutant, reduces the rate of net photosynthesis in many species of plants. Numerous studies have been reported on the physiological and biochemical effects of SO_2 on the photosynthetic processes (Malhotra & Hocking, 1976; Hällgren, 1978). SO_2 affects stomatal movement (Kondo & Sugahara, 1978) and consequently decreases the CO_2 exchange rate in whole leaves. To clarify the mechanisms of toxic effect of SO_2 on the photosynthetic processes in mesophyll cells, isolated free cells and protoplasts would be advantageous because of the absence of the stomatal responses (Paul & Bassham, 1978).

SO_2 absorbed by leaves through stomata dissolves in water on the wet surfaces of mesophyll cells, resulting in the formation of HSO_3^-, SO_3^{2-}, and H^+. Therefore the effect of SO_2 fumigation on plant leaves should be also observed in single cells incubated with sulfite (bisulfite) at an acidic pH. Paul and Bassham (1978) have reported that the addition of sulfite (bisulfite) caused no inhibition of photosynthetic $^{14}CO_2$ fixation in *Papaver* mesophyll cells at

pH 8.0. This suggests that the pH around the mesophyll cells as well as sulfite (bisulfite) would be an important factor for the SO_2 toxicity.

In the present study, we investigated the effects of Na_2SO_3 on photosynthetic activities of isolated *Vicia* mesophyll protoplasts at various pHs of incubation medium, and the uptake of sulfite by protoplasts using [^{35}S]-sulfite to examine the relationship between the inhibitory effect of Na_2SO_3 on photosynthesis in the protoplasts and the uptake of sulfite by protoplasts.

Materials and Methods

Plant materials

Broad bean (*Vicia faba* L. cv. Otafuku) plants were grown for 4–7 weeks in potting soil at 20 ± 0.5°C during the day and 15 ± 0.5°C at night with a relative humidity of 70 ± 5% under natural light condition. In the rainy season, about 4 weeks old plants grown as described above were further cultivated for additional 1–2 weeks in a growth cabinets under 14 h of light period at 20 ± 0.5°C and 10 h of dark period at 15 ± 0.5°C with a relative humidity of 70 ± 3%. The light source in the growth cabinet was 24 metal halide lamps (400W: Yoko Lamp, Toshiba) giving a photosynthetically active radiation (PAR) of 430–580 $\mu E\ m^{-2}\ s^{-1}$ at leaf level.

Isolation of mesophyll protoplasts

First to third youngest leaves fully developed were used for the isolation of mesophyll protoplasts. Approximately 20 leaflets (fresh weight of 12–15 g) were cut from plants in the morning and lower epidermis was removed by tweezers. The stripped leaflets were cut into 3–5 pieces and subjected to vacuum infiltration with a 50 ml of digestion medium containing 0.5% (w/v) Macerozyme R-10, 2.0% (w/v) Cellulase Onozuka R-10 (Yakult Pharmaceutical Industry), 0.5% (w/v) potassium dextran sulfate (Meito Sangyo), 0.2% (w/v) BSA, 1 mM $CaCl_2$, and 0.6 M mannitol (pH 5.5) in a 200-ml Erlenmeyer flask. The flask was shaken for 3–5 min (about 80 excursion min^{-1} with 4.5 cm of stroke) and the broken cells and most of spongy cells released were discarded by decantation. Leaf pieces were further incubated for complete digestion with a 50 ml of the renewed digestion medium by shaking of 45 excursions min^{-1} for about 1.5 h. Digestion was carried out at 28°C under illumination of about 400 $\mu E\ m^{-2}\ s^{-1}$ PAR (300W: Eye Lamp, Iwasaki Electric). Protoplast formation was checked by microscopic observation. After the digestion was terminated, released protoplasts were passed through 58-μm nylon net and washed twice with a medium consisting of 0.6 M mannitol and 1 mM $CaCl_2$ by centrifugation. Isolated mesophyll protoplasts were suspended in a medium of 0.6 M mannitol and 1 mM $CaCl_2$ in an ice bath before use.

Estimation of protoplast viability

Impermeability of Evans blue to the protoplasts was used for the estimation of protoplast intactness (Kanai and Edwards 1973). Protoplast suspension was mixed with equal volume of 0.25% (w/v) Evans blue, 3 mM $CaCl_2$, and 0.6 M mannitol. After 10 min, the exclusion of the dye by protoplasts was examined for intactness.

Determination of the volume and number of protoplasts

The protoplast volume was estimated from the diameter measured from the photograph of light microscopy. The protoplast number was counted with a Coulter counter^{TM} (Model TAII,

Coulter Electronics).

Incubation of protoplasts with Na_2SO_3

Protoplasts were incubated in sulfite solution containing desired concentration of Na_2SO_3, 4 mM citric acid, 1 mM EDTA, 3 mM $CaCl_2$, and 0.6 M mannitol, of which pH was adjusted to 5.0 with NaOH. In the experiment of Fig. 5 and 6, the buffer was changed to 10 mM citric acid (pH 3.5–5.5), 10 mM MES (pH 5.5–7.0), and 10 mM HEPES (pH 7.0–8.0), and pH was adjusted with NaOH after Na_2SO_3 was dissolved. To minimize oxidation of sulfite, the solution was prepared immediately before use in each experiment. After 3-min incubation with Na_2SO_3 at various pHs, same volume of aqueous solution (pH 8.3) consisting of 0.1 M HEPES-NaOH, 1 mM EDTA, 3 mM $CaCl_2$, and 0.6 M mannitol was added to the protoplast suspension. Protoplasts were collected by centrifugation and used for the subsequent experiments.

Measurement of sulfite uptake by protoplasts

The incubation of protoplasts with $Na_2{}^{35}SO_3$ (0.73 μCi μmol^{-1}) was carried out as described above. After the incubation, protoplast suspension was subjected to silicon oil centrifugation with a Microfuge^{TM} B (Beckman) for 30 s to separate protoplasts from suspending medium. Microfuge tube (400 μl, polyethylene) contained 20 μl of 2.5 N NaOH at the bottom, 70 μl of silicon oil (CR 50, Wacker Chemie) at the middle, and 250 μl of protoplast suspension at the top layer. Immediately after centrifugation, 20 μl of 2.5 N NaOH was added to the top layer of the tube to prevent generation of $^{35}SO_2$ from $Na_2{}^{35}SO_3$, thus the diffusion of $^{35}SO_2$ to the bottom layer through silicon layer was negligible at all pHs of the experiments. The centrifuged tube was stored overnight in a freezer. The tube was cut immediately above the bottom layer and the tip containing sedimented protoplasts was shaken vigorously in a 300 μl of 0.1 N NaOH. Two-hundreds μl of this suspension was mixed with 800 μl of Soluene-350 (Packard) and 10 ml of Aquasol-2 (New England Nuclear) in a vial, and the radioactivity was determined with a Liquid Scintillation Counter (LSC) (3255: Packard) after chemiluminescence had disappeared. The correction of quenching was made by the method of external standard channels ratio. To determine the amount of radioactivity carried into the bottom layer together with the protoplasts from the top layer of the centrifuged tube but not absorbed by the protoplasts, the transfer of [^{14}C]-mannitol added to the suspending medium from the top to the bottom layer was measured using the same protoplast preparations. Then the radioactivity of ^{35}S in the bottom layer was corrected for the net absorption of ^{35}S by the protoplasts.

Assay of photosynthetic activities of protoplasts

Light-dependent O_2 evolution was measured with a Rank Brothers O_2 electrode at 25°C. Reaction mixture was composed of 50 mM HEPES, 1 mM EDTA, 10 mM $NaHCO_3$, 0.6 M mannitol, and NaOH to make pH 8.0. The stirrer was manipulated at a low speed of revolution in order to prevent disintegration of protoplasts. After O_2 uptake in the dark had been steady, O_2 evolution was started by illumination of 800 μE m^{-2} s^{-1} PAR (300W: KP-10S Projector Lamp, Kondo). The difference of O_2 exchange rate between in the light and in the dark was measured. The *Vicia* mesophyll protoplasts isolated as described above usually retained the photosynthetic O_2 evolution of 80–150 μmol mgchl^{-1} h^{-1}. Maximum rate of O_2 evolution was obtained at light intensity of more than 500 μE m^{-2} s^{-1} PAR, at $NaHCO_3$ concentration of more than 1.5 mM, and at pH 8.0 (Sakaki & Kondo, 1981). Addition of 5 μM DCMU completely inhibited O_2 evolution.

For the assay of photosynthetic $^{14}CO_2$ fixation, protoplasts were incubated at 25°C in the reaction mixture of the same composition as that for the assay of O_2 evolution. Two min after

the start of illumination (800 $\mu E\ m^{-2}\ s^{-1}$), $NaH^{14}CO_3$ was added to the suspension at a final specific activity of 0.08 $\mu Ci\ \mu mol^{-1}$. Protoplasts were incubated for further 4 min in the light. The reaction was terminated by transferring 200 μl of the protoplast sample to the 800 μl of methanol in scintillation vials. The vial was mixed with 200 μl of conc. HCl and dried under a tungsten lamp to remove unfixed $^{14}CO_2$. Acid-stable ^{14}C fixed was dissolved in 0.5 ml of distilled water and then in 10 ml of Bray's scintillator (Bray, 1960) to be determined by LSC.

Isolation of chloroplasts from protoplasts

Chloroplasts were prepared from the mesophyll protoplasts according to the method of Rathnam and Edwards (1976) except for the composition of the isolation medium. The protoplasts were suspended in the medium composed of 50 mM MES, 1 mM $MgCl_2$, 2 mM KH_2PO_4, 5 mM sodium pyrophosphate, 5 mM DTT, 2 mM sodium isoascorbate, 0.1% (w/v) BSA, 2% (w/v) PVP-10 (Sigma), 0.33 M sorbitol, and KOH to make pH 6.5. Then the suspension was passed through a 20-μm nylon net to release the chloroplasts. After sedimentation by centrifugation at 600 x g for 90 s, chloroplasts were suspended in a medium containing 50 mM HEPES, 2 mM EDTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 0.5 mM KH_2PO_4, 5 mM sodium pyrophosphate, 0.33 M sorbitol, and KOH to make pH 7.6. Microscopic observation revealed that no protoplast was present in the chloroplast suspension. The chloroplasts isolated were more than 95% intact according to the method of Lilley *et al.* (1975).

Photosynthetic assay of chloroplasts

The method for the measurement of photosynthetic O_2 evolution was identical with those for protoplasts except for the composition of assay mixture, which is 50 mM Tricine, 2 mM EDTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 0.5 mM KH_2PO_4, 5 mM sodium pyrophosphate, 5 mM $NaHCO_3$, 0.33 M sorbitol, and KOH to make pH between 7.4 and 9.0.

Chlorophyll determination

Chlorophyll content was measured by the method of Arnon (1949).

Radioactive chemicals

$Na_2{}^{35}SO_3$, $NaH^{14}CO_3$, and [^{14}C]-mannitol were obtained from New England Nuclear.

Results

Fig. 1 shows the effects of Na_2SO_3 on the photosynthetic O_2 evolution of isolated *Vicia* mesophyll protoplasts. The activity was little affected by 10 mM Na_2SO_3 added to the assay mixture (pH 8.0). However, the evolution rate remarkably decreased when protoplasts had been preincubated with 10 mM Na_2SO_3 at acidic pHs before assay (Fig. 1, curve D, E). Incubation with Na_2SO_3 not only at 25°C but also at 4°C caused similar effects on the photosynthetic activities of protoplasts. Isolated *Vicia* mesophyll protoplasts stored at 4°C in darkness retained the photosynthetic activity for more than 8 h without loss, but those stored at 25°C in the dark lost the activity to 50–83% of the initial level during 3 h (see Huber and Edwards, 1975). Thus the incubation of protoplasts with Na_2SO_3 was carried out at 4°C in the subsequent experiments. The photosynthetic $^{14}CO_2$ fixation was also unaffected by the addition of Na_2SO_3 to the assay mixture (pH 8.0), but the fixation rate was strongly suppressed when protoplasts had been preincubated with Na_2SO_3 at pH 5.0 (Fig. 2). Treatment of protoplasts with 10 mM Na_2SO_3 at pH 5.0 reduced the photosynthetic O_2 evolution and $^{14}CO_2$ fixation

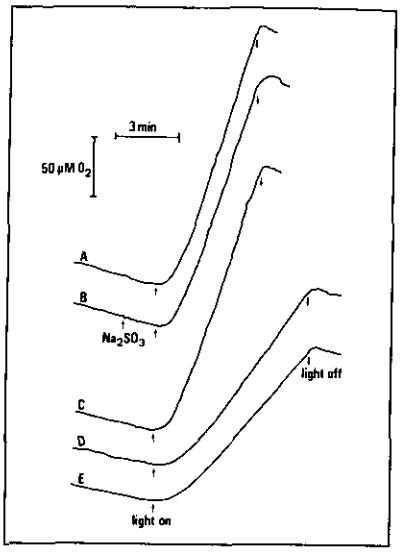

Fig. 1 Effects of Na_2SO_3 on the photosynthetic O_2 evolution of *Vicia* mesophyll protoplasts

Trace A; no addition, B; addition of 10 mM Na_2SO_3 to the assay mixture at the time indicated by the arrow, C – E; protoplasts being previously incubated with (E) and without (C) 10 mM Na_2SO_3 at pH 5.0, and with 10 mM Na_2SO_3 at pH 5.5 (D). Treatment of protoplasts with and without Na_2SO_3 was started 5 min after transfer of the protoplasts to the medium at 25°C from that at 4°C. Assay of photosynthesis in all protoplast preparations was started 20 min after the initiation of incubation at 25°C.

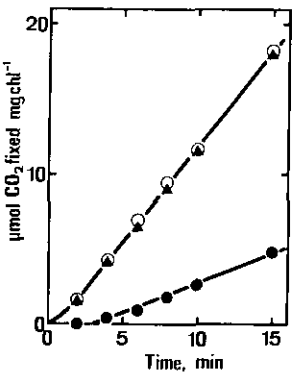

Fig. 2 Effects of Na_2SO_3 on the photosynthetic $^{14}CO_2$ fixation of mesophyll protoplasts

Photosynthesis of protoplasts was started at 0 time by the initiation of illumination. $NaH^{14}CO_3$ was added 2 min before the illumination.
(–○–); no addition, (–▲–); treated with 10 mM Na_2SO_3 in assay mixture for photosynthesis (pH 8.0), (–●–); temporarily preincubated with 10 mM Na_2SO_3 at pH 5.0.

to 45 and 41% of the initial level, respectively (Table 1). Incubation of protoplasts at pH 3.5–8.0 without Na_2SO_3 gave little effect on the photosynthetic activities. The extent of photosynthetic inhibition caused by Na_2SO_3 was different between the preparations of protoplasts, which was at least partly due to the growth condition and leaf age of plants used for the protoplast isolation (Sakaki & Kondo, 1981). Table 1 also shows the effects of 10 mM Na_2SO_3, K_2SO_3, and Na_2SO_4, and 20 mM NaCl at pH 5.0 on the O_2 evolution rate. Both Na_2SO_3 and K_2SO_3 reduced the activity to the similar level, whereas Na_2SO_4 and NaCl essentially had no effect. Thus the toxic species were sulfite (SO_3^{2-}), bisulfite (HSO_3^-), or hydrated sulfur dioxide ($H_2O{\cdot}SO_2$), the ratio of which greatly varies with the pH of solution. Sulfite inhibition of photosynthesis in *Vicia* protoplasts was not recovered by repeated washing with sulfite-free suspending medium (0.6 M mannitol and 1 mM $CaCl_2$) (Table 2). Photosynthetic activity of protoplasts reduced by Na_2SO_3 did not change for more than 6 h while protoplasts were suspended at 4°C under darkness.

By the incubation with Na_2SO_3 at an acidic condition, protoplasts had not been ruptured (Fig. 3). As shown in Table 3, the volume and number of the protoplasts after treatments with and without 10 mM Na_2SO_3 at pH 5.0 were little changed. Vital staining with Evans blue showed that more than 95% of the protoplasts in both the preparations were intact.

Fig. 4 shows the effect of the incubation with various Na_2SO_3 concentrations at pH 5.0 and 8.0 on the photosynthetic activities of protoplasts. The activity was greatly more sensitive to sulfite at pH 5.0 than at pH 8.0. It was suppressed to 54% of the original level by the

Table 1 Modification of the rates of O_2 evolution and $^{14}CO_2$ fixation of protoplasts by Na_2SO_3 and some other reagents

Expt No.	Preincubation	Addition in the assay mixture	O_2 evolution (μmol mgchl^{-1} h^{-1})	$^{14}CO_2$ fixation (μmol mgchl^{-1} h^{-1})
1.	None	None	90.8 ± 14.2	105.4 ± 3.8
	None	10 mM Na_2SO_3	86.4 ± 3.2	105.1 ± 3.0
	pH 5.0	None	92.8 ± 1.4	114.2 ± 2.2
	pH 5.0 with 10 mM Na_2SO_3	None	41.1 ± 1.4	42.9 ± 1.6
2.	None	None	134.3 ± 5.2	–
	pH 5.0	None	131.2 ± 2.9	–
	pH 5.0 with 10 mM Na_2SO_3	None	72.8 ± 4.8	–
	pH 5.0 with 10 mM K_2SO_3	None	72.4 ± 3.5	–
	pH 5.0 with 10 mM Na_2SO_4	None	129.1 ± 5.5	–
	pH 5.0 with 20 mM NaCl	None	131.8 ± 6.9	–
	pH 5.0 with 20 mM $NaNO_3$	None	116.2 ± 5.6	–

Means ± SD of 3 experiments were presented.

Table 2 Effect of washing on the photosynthetic activity reduced by Na_2SO_3

Treatment	O_2 evolution	
	μmol mgchl^{-1} h^{-1}	%
None	108.0	100
10 mM Na_2SO_3 at pH 5.0	44.4	41.1
washing, once	48.2	44.6
washing, three times	44.7	41.4

After treatment with 10 mM Na_2SO_3 at pH 5.0, protoplasts were washed with a medium containing 0.6 M mannitol and 1 mM $CaCl_2$. Then photosynthetic O_2 evolution was assayed as described in the Materials and Methods.

incubation with 2 mM Na_2SO_3 at pH 5.0, whereas at pH 8.0 it was decreased only to 89% by 30 mM Na_2SO_3.

Fig. 5 shows the effect of pH of suspending medium with Na_2SO_3 on the photosynthetic activities and sulfite uptake by the protoplasts. Photosynthetic activity was strongly susceptible to sulfite at pH below 6.0. The amount of sulfite taken up by the protoplasts was very small at pH above 6.0, whereas at pH region below 6.0 the lower the pH was, the more the amount of sulfite was taken up by the protoplasts. Fig. 6 illustrates the inhibition of photosynthesis versus

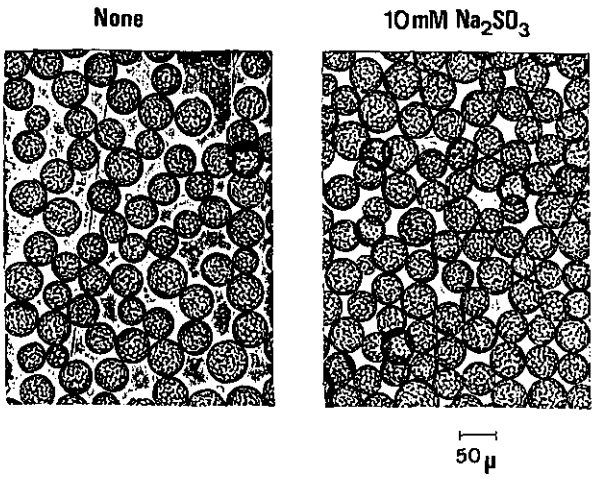

Fig. 3 Photographs of *Vicia* mesophyll protoplasts incubated at pH 5.0 with (right) and without (left) 10 mM Na_2SO_3.

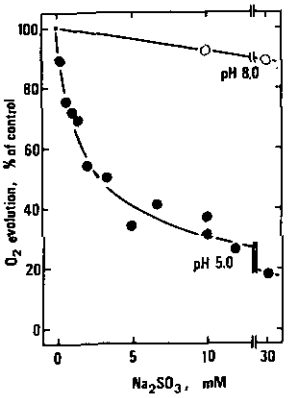

Fig. 4 Effect of Na_2SO_3 concentrations on the photosynthetic rate of protoplasts

The incubation of protoplasts with Na_2SO_3 at pH 5.0 and 8.0 and assay of photosynthesis at pH 8.0 were carried out as described in the Materials and Methods except that the incubation periods with Na_2SO_3 was 1 min.

Table 3 Incubation effect of *Vicia* mesophyll protoplasts with 10 mM Na_2SO_3 at pH 5.0 on their volume and number

Treatment	Volume (pl)	Number ($\times 10^6$ mgchl^{-1})	Calculated intracellular volume (ml mgchl^{-1})
None	51.9 ± 2.1 (106)	5.93 ± 0.14 (5)	0.308
Na_2SO_3, 10 mM	53.4 ± 2.1 (109)	6.20 ± 0.09 (5)	0.331

Mean ± SE (number of measurement).

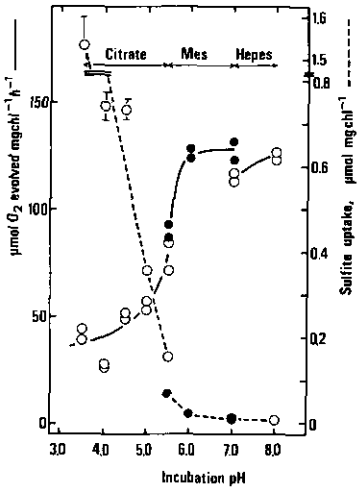

Fig. 5 pH Dependent inhibition of protoplast photosynthesis by Na_2SO_3 and uptake of sulfite by protoplasts

Both the assays of O_2 evolution and uptake of sulfite were measured with the same protoplast preparation. Uptake of ^{35}S was determined as described in the Materials and Methods. Means ± SD of 3 experiments are given in the study of sulfite uptake, and SD was included inside a circle at pH above 5.0.

the amount of sulfite incorporated into the protoplasts. When approximately 0.23 μmol mgchl^{-1} sulfite was incorporated into the protoplasts, the O_2 evolution was inhibited by 50%. Since the protoplast volume treated with 10 mM Na_2SO_3 was 0.331 ml mgchl^{-1} (Table 3), internal concentration of sulfite can be calculated to be 0.69 mM, assuming that the sulfite taken up by protoplasts is not metabolized and is distributed uniformly within the protoplasts.

In intact chloroplasts prepared from *Vicia* mesophyll protoplasts, the rate of CO_2-dependent O_2 evolution was maximum at pH 8.4–8.6 (Fig. 7). The rate of maximum O_2 evolution was more than 80% of that of the original protoplasts, though it varied according to the preparations of protoplasts. Addition of 1 mM Na_2SO_3 inhibited the photosynthesis of isolated chloroplasts by 60–70% at all pHs measured (Fig. 7). A half inhibition was obtained by the addition of approximately 0.55 mM of Na_2SO_3 (Fig. 8).

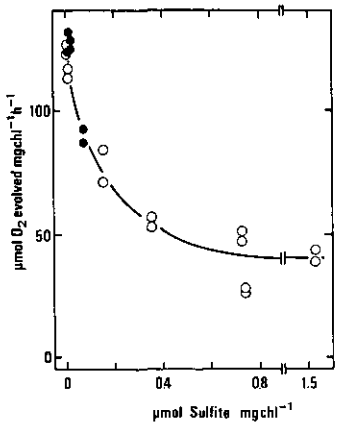

Fig. 6 Inhibition of photosynthesis in protoplasts as a function of internal sulfite concentrations

Symbols are the same as in Fig. 5.

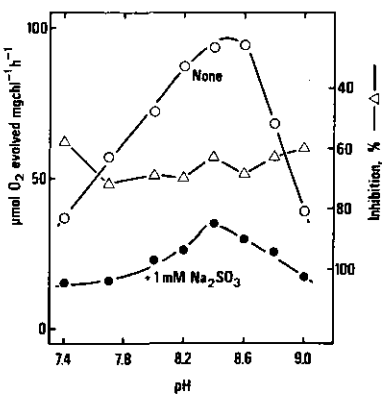

Fig. 7 pH Profile of photosynthetic O_2 evolution in intact chloroplasts prepared from *Vicia* protoplasts in the presence and absence of 1 mM Na_2SO_3

Photosynthetic O_2 evolution activity of the original protoplasts used for the isolation of chloroplasts was 117 μmol mgchl^{-1} h^{-1}.

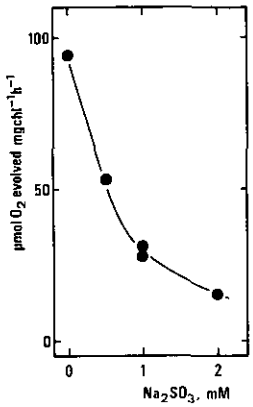

Fig. 8 Effects of Na_2SO_3 concentrations on the photosynthetic O_2 evolution of chloroplasts

Assay of photosynthesis was carried out at pH 8.6.

Discussion

Several workers have reported that sulfite was more toxic to plant cell metabolism at acidic pH of the incubation medium than at neutral and basic one. Hill (1971) and Puckett *et al.* (1973) showed that the photosynthetic activity in some lichen species was largely impaired by the incubation with sulfite or aqueous SO_2 at acidic pH, but less affected at weak acid and neutral conditions. Paul and Bassham (1978) reported that photosynthesis in *Papaver* free cells was rather enhanced by the addition of 20 mM sulfite at pH 8.0. In the present experiment with *Vicia* mesophyll protoplasts, pH dependency of photosynthetic response to Na_2SO_3 was consistent with those mentioned above, though the enhancement of photosynthesis by Na_2SO_3 was not observed at pH 8.0 (Fig. 1 and 2, Table 1). In the present study, we demonstrated that the inhibition of photosynthesis caused by Na_2SO_3 at an acidic condition could be ascribed to the large amount of sulfite incorporated into the protoplasts (Fig. 5 and 6). Sulfite present in the cytoplasm should attack directly the chloroplasts and other cell organella. Inhibition of photosynthetic activity by sulfite has already been observed in chloroplasts isolated from spinach (Libera *et al.*, 1973; Silvius *et al.*, 1975) and pea (Plesnićar & Kalezić, 1980) leaves. In intact chloroplasts isolated from *Vicia* protoplasts, photosynthesis was sensitive to Na_2SO_3 over pH region between 7.4 and 9.0 (Fig. 7). Na_2SO_3 concentration required for a half inhibition of photosynthesis was 0.55 mM in the chloroplasts, which approximated to 0.69 mM of the intracellular sulfite concentration calculated on the assumption that the sulfite incorporated into protoplasts was not metabolized and was uniformly distributed within the protoplasts (see Results). These results strongly suggest that the major toxicant in protoplasts is sulfite and/or bisulfite in cytoplasmic fluid rather than other toxic substances derived from sulfite in the cytoplasm.

We observed that the rate of sulfite uptake by protoplasts was different according as the pH of the incubation medium was changed (Fig. 5). Since the sulfite uptake proceeded at 4°C, metabolic energy would not be required in this process. Sulfite could be transported into chloroplast through the phosphate translocator (Hampp & Ziegler, 1977). However, the mechanism of transport through plant cell plasmamembrane has not been known yet. SO_2

dissolved in an aqueous solution transforms to $H_2O{\cdot}SO_2$, HSO_3^-, and SO_3^{2-} according to the solution pH. The dominant species at weak acid region is HSO_3^-, but the lower the pH is, the more the proportion of $H_2O{\cdot}SO_2$ becomes. SO_2 is known to be very soluble in organic solvent (Mudd, 1975), and therefore could easily diffuse through lipid bilayer of protoplast membranes. Thus it is conceivable that the sulfite would be incorporated into protoplasts mainly by simple diffusion as a non-ionic form. However, we cannot rule out the possibility that HSO_3^- could also penetrate into the protoplasts through plasmamembranes.

As discussed above, we suggested the possibility that the sulfite in the cytoplasm might be a major toxicant for inhibition of the photosynthesis of intracellular chloroplasts. The work on the mechanism of sulfite inhibition of chloroplast photosynthesis was reported by several investigators. Sulfite would suppress photosynthesis directly by means of the inhibition of ribulose-1,5-bisphosphate carboxylase (Ziegler, 1972) and photophosphorylation (Plesnićar & Kalezić, 1980; Cerović *et al.*, 1982), and indirectly by means of the inhibition of SH-enzymes, for example, NADP-glyceraldehyde-3-phosphate dehydrogenase, ribulose-5-phosphate kinase, and fructose-1,6-bisphosphatase, caused by H_2O_2 which was formed through O_2^- increased by sulfite (Tanaka *et al.*, 1982a, 1982b). On the other hand, sulfite is oxidized to sulfate enzymatically or photochemically (Asada & Kiso, 1973; Kondo *et al.*, 1980) and reduced to H_2S (Silvius *et al.*, 1976) in plant cells. Sulfate is less toxic to plant cells than sulfite, however, H_2S is reported to inhibit the photosynthetic electron transport (Oren *et al.*, 1979). Another possible mechanism of photosynthesis inhibition by sulfite is the decrease of intracellular pH. Since the cytoplasmic pH is around neutral (Smith & Raven, 1979), SO_2 passed through the protoplast membranes would be transformed to HSO_3^- and SO_3^{2-} resulting in the formation of H^+ in the cytoplasm. As shown in Fig. 7, photosynthetic rate in *Vicia* chloroplasts decreased sharply as the pH of the assay medium was apart from the maximum one for the photosynthesis. Thus the reduction of the cytoplasmic pH might influence the activity of photosynthesis. However, further study must be required to clarify the mechanism of the inhibition by sulfite.

Several workers reported that SO_2 affected membrane integrity in plant cells (Lüttge *et al.*, 1972), resulting in the massive leakage of K^+ and photosynthetically fixed products (Puckett *et al.*, 1974, 1977). However, plasmamembranes of protoplasts were still functional even after the sulfite treatment in our experimental conditions. Evans blue was still impermeable to protoplasts treated with sulfite. The protoplast volume after treatment with and without 10 mM Na_2SO_3 at pH 5.0 was same (Table 3). Both protoplast samples changed their volume in the same manner when incubated in various concentrations of mannitol solution (results not shown). Thus the sulfite gives a remarkable effect on the metabolic activity of protoplasts rather than the drastic damage of the membranes. However, further studies should be required to clarify whether sulfite affects the specific function of plant cell membranes, such as specific transport carriers, or not.

Acknowledgement

We are grateful to Dr. Y. Ohtsuki and Dr. F. Sakai of the institute for Plant Virus Research for their demonstration and valuable suggestions of the methods for mesophyll protoplast isolation. We wish to thank the members of the Engineering Division of this institute for their adept cultivation and provision of the plant materials.

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ソラマメ葉肉細胞プロトプラストの亜硫酸による光合成阻害と［^{35}S］-亜硫酸の取り込み

榊　剛[1]・近藤矩朗[1]

ソラマメ（*Vicia faba* L. cv. Otafuku）葉から単離した葉肉細胞プロトプラストを，pH6.0以下の酸性域において10mM Na_2SO_3で処理したところ，光合成酸素発生速度及び$^{14}CO_2$固定速度は大きく低下した。一方pH6.0以上でのNa_2SO_3処理ではどちらの活性も影響されなかった。プロトプラストをNa_2SO_3で処理するpHが低いほど，多くの量の亜硫酸がプロトプラスト内に蓄積された。pH5.0でNa_2SO_3処理したプロトプラストは，光学顕微鏡による観察や，エバンスブルーによる生体染色の結果からインタクトであった。プロトプラストから単離した葉緑体の光合成活性は，至適pHである8.4−8.6の下でプロトプラストの活性の80%以上を保持していた。葉緑体の光合成酸素発生速度は，プロトプラストの場合とは異なり，測定したpH（7.4−9.0）全域にわたってNa_2SO_3により同じ程度まで阻害された。

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Res. Rep. Natl. Inst. Environ. Stud., Jpn., No. 65, 1984.

Sulfite Inhibition of Uptake and Fixation of Inorganic Carbon in Mesophyll Protoplasts Isolated from *Vicia faba* L.

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Not only the rates of CO_2 fixation but also those of accumulation of inorganic carbon (C_{inorg}) were lowered in isolated *Vicia faba* mesophyll protoplasts by preincubation with 1 and 10 mM Na_2SO_3 at pH 4.5. The influx rates of C_{inorg} into protoplasts according to the external C_{inorg} concentrations were inhibited by Na_2SO_3 treatments. The initial rate of CO_2 fixation was compared with the C_{inorg} influx rate, indicating that the C_{inorg} influx rate may limit the fixation rate at low influx rates in protoplasts treated with 1 mM Na_2SO_3, and that the photosynthetic site(s) besides the site of C_{inorg} influx on cell membranes were injured by 1 and 10 mM Na_2SO_3.

The activity of *Vicia* carbonic anhydrase was inhibited by 1 mM Na_2SO_3 but not by 4 mM Na_2SO_4.

Key words: Carbonic anhydrase, Inoreganic carbon uptake, Mesophyll protoplasts, Photosynthesis, Sulfite, *Vicia faba*

Exposure to sulfur dioxide (SO_2), a major atmospheric pollutant, causes inhibition of photosynthesis in various species of plants. Extensive studies have been carried out to clarify the effect of SO_2 on photosynthetic processes using plant leaves, and isolated chloroplasts and enzymes (Malhotra & Hocking, 1976; Hällgren, 1978). We have already reported that photosynthesis of isolated *Vicia* mesophyll protoplasts was inhibited by the preincubation with Na_2SO_3 at an acidic condition, and that this inhibition was closely related with the amount of sulfite taken up by the protoplasts (Sakaki & Kondo, 1984). However, the mechanism of photosynthetic inhibition by intracellular sulfite remains to be determined. It was reported that not only CO_2 fixation on ribulose-1, 5-bisphosphate carboxylase, a key enzyme of photosynthetic CO_2 fixation (Ziegler, 1972), but also the fixation in the isolated spinach chloroplasts (Libera *et al.*, 1975) was inhibited by sulfite in a competitive manner with respect to C_{inorg}.

In this report, we studied the uptake and fixation of $C_{inorg.}$ in Na_2SO_3-treated protoplasts at various C_{inorg} concentrations to determine the action of sulfite on the photosynthetic

Abbreviations: CA, carbonic anhydrase; C_{inorg}, inorganic carbon.

processes of whole cell system. During the study, we found that the rate of C_{inorg} influx was extremely low in the protoplasts treated with Na_2SO_3 at an acidic condition. We also suggested the possibility that the rate of C_{inorg} influx limited that of photosynthetic CO_2 fixation in these protoplasts.

Materials and Methods

Plant growth and protoplast isolation

Broad bean (*Vicia faba* L. cv. Otafuku) plants were grown and mesophyll protoplasts were isolated as previously reported (Sakaki & Kondo, 1984). Isolated protoplasts were stored in a medium made up of 0.6 M mannitol and 1 mM $CaCl_2$ on ice under darkness.

Incubation of protoplasts with Na_2SO_3

Incubation of protoplasts with Na_2SO_3 and the termination of the incubation were performed at 4°C as described previously (Sakaki & Kondo, 1984) except that the incubation pH with Na_2SO_3 was 4.5 in place of 5.0. After sedimentation by standing, protoplasts were washed with 0.6 M mannitol by centrifugation and resuspended in the same medium before use.

Measurement of inorganic and fixed ^{14}C in protoplasts

Protoplasts (equivalent to approximately 0.1 mgchl ml^{-1}) were preincubated in an O_2 electrode chamber (Rank Brothers) at 25°C in 2-ml medium containing 50 mM HEPES, 1 mM EDTA, 0.6 M mannitol, and NaOH to make pH 8.0. The incubation medium had previously been prepared and stocked under CO_2-free condition. The protoplasts were illuminated (800 $\mu E\ m^{-2}\ s^{-1}$ PAR) before the termination of O_2 evolution to minimize the internal C_{inorg} pool. Then the accumulation of ^{14}C within the protoplasts was measured by silicon oil centrifugation methods. The microcentrifuge tube (400 μl; polyethylene) contained 20 μl of 2.5 N NaOH at the bottom, 70 μl of silicon oil (704: Dow Corning), the density of which was adjusted to 1.050 by hexane, at the middle, and 200 μl of CO_2-depleted assay medium with the same composition as above. Five min before the injection of protoplasts to the assay medium in the centrifuged tube, $NaH\ ^{14}CO_3$ was added to the assay medium and stand to allow the equilibrium among the species of $^{14}C_{inorg}$. During this period of time, neither decrease of radioactivity in the assay medium nor the diffusion of $^{14}CO_2$ to the bottom layer of the tube was detected. Fifty μl of CO_2-depleted protoplast suspension in an O_2 electrode chamber was transferred to a tube with the assay medium, mixed with a small glass rod and allowed for the accumulation of ^{14}C in protoplasts. During this procedure, the tube was illuminated (500 W; Eye Lamp, Iwasaki Electric) from above through 7 cm of water layer and 0.4 cm of infrared absorbing glass (HG; Obara Kogaku) giving a light intensity of 750-800 $\mu E\ m^{-2}\ s^{-1}$ PAR at the tube position. Assay was terminated by the start of centrifugation with a Microfuge ™B (Beckman) at the time indicated. Protoplasts were sedimented by centrifugation within 2 s. All tubes were centrifuged for 30 s. Immediately after centrifugation, 20 μl of 2.5 N NaOH was added to the top layer of the tube to prevent the diffusion of $^{14}CO_2$ to the bottom layer. After freezing the tube, the bottom layer containing sedimented protoplasts was cut and mixed in a 300 μl of 0.1 N NaOH. One-hundred μl of this suspension was measured for radioactivity (total $^{14}C_{inorg}$ accumulated) with Bray's scintillator (Bray, 1960) using a Liquid Scintillation Counter (3255; Packard). Another 100 μl was mixed with 20 μl of conc. HCl and dried to remove the unfixed $^{14}CO_2$. Acid-stable ^{14}C fixed was dissolved in 100 μl of distilled water and determined as described above (fixed $^{14}C_{inorg}$). The rate of $^{14}CO_2$ fixation in

Fig. 1 was determined from the amount of ^{14}C fixed during 40 s of the incubation period. The correction for the amount of $^{14}C_{inorg}$ carried into the bottom layer with the protoplasts but not absorbed by the protoplasts was made using [^{14}C]-mannitol as described previously (Sakaki & Kondo, 1984).

Assay of carbonic anhydrase

Isolated protoplasts (equivalent to 15 μgchl) were added to the ice-cold 5 ml of 50 mM sodium veronal-HCl (pH 8.3) containing indicated amount of Na_2SO_3 or Na_2SO_4. After 3 min, 5 ml of CO_2-saturated distilled water on ice was added to the mixture and the time required to change from pH 8.3 to 7.3 was measured with a glass electrode. During the assay, the reaction mixture was stirred on ice. The enzyme activity was determined with U=10 ($t_b/t_c - 1$), where U is the enzyme unit, and t_b and t_c are the time required for the pH change with boiled and unboiled protoplast medium, respectively (Rickli *et al.*, 1964).

Chlorophyll measurement

Chlorophyll content was determined by the method of Arnon (1949).

Radioactive chemicals

$NaH^{14}CO_3$ and [^{14}C]-mannitol were obtained from New England Nuclear.

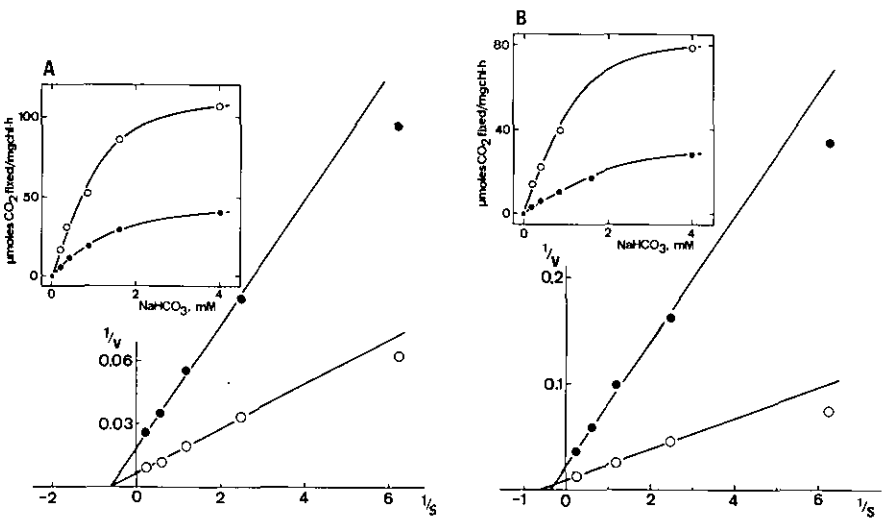

Fig. 1 The relation between the rate of photosynthetic $^{14}CO_2$ fixation in *Vicia* mesophyll protoplasts and $^{14}C_{inorg}$ concentrations added to the external medium, and the *Lineweaver-Burk* plots

The internal C_{inorg} pool of protoplasts had previously been depleted as described in Materials and Methods. A: protoplasts treated with 0 (○) and 1 mM (●) Na_2SO_3 at pH 4.5. V_{max} for 0 and 1mM Na_2SO_3-treated protoplasts was 156 and 54.9 μmol CO_2 fixed $mgchl^{-1}$ h^{-1}, respectively. K_m for both protoplast preparations was 1.6 mM $NaHCO_3$. B: protoplasts treated with 0 (○) and 10 mM (●) Na_2SO_3 at pH 4.5. In 0 and 10 mM Na_2SO_3-treated protoplasts, V_{max} was 111 and 46.5 μmol CO_2 fixed $mgchl^{-1}$ h^{-1}, and K_m was 1.7 and 2.8 mM $NaHCO_3$, respectively.

Results

Fig. 1 shows the effect of C_{inorg} concentration added to the incubation medium on the rate of photosynthetic $^{14}CO_2$ fixation in mesophyll protoplasts which were treated with 1 and 10 mM Na_2SO_3. The *Lineweaver-Burk* analyses show a non-competitive type of inhibition in the protoplasts treated with 1 mM Na_2SO_3 and a mixed type of inhibition in those treated with 10 mM Na_2SO_3. Libera *et al.* (1975) reported that CO_2 fixation in isolated spinach chloroplasts was inhibited by sulfite in a fully competitive manner with respect to C_{inorg} added to the medium, but such case was not observed in the present experiments.

Fig. 2 shows the time course of accumulation of inorganic and fixed ^{14}C in protoplasts, the internal C_{inorg} pool of which was previously depleted. Photosynthetic $^{14}CO_2$ fixation started immediately after the addition of $^{14}C_{inorg}$ without lag time and proceeded at a linear rate in both the non- and Na_2SO_3-treated protoplasts. Time-dependent accumulation of unfixed $^{14}C_{inorg}$ inside both protoplast samples appeared to be curvilinear at all external C_{inorg} concentrations. In contrast to the case with *Chlamydomonas* cells (Badger *et al.*, 1980) and isolated *Asparagus* mesophyll cells (Espie & Colman, 1982), C_{inorg} concentration in *Vicia*

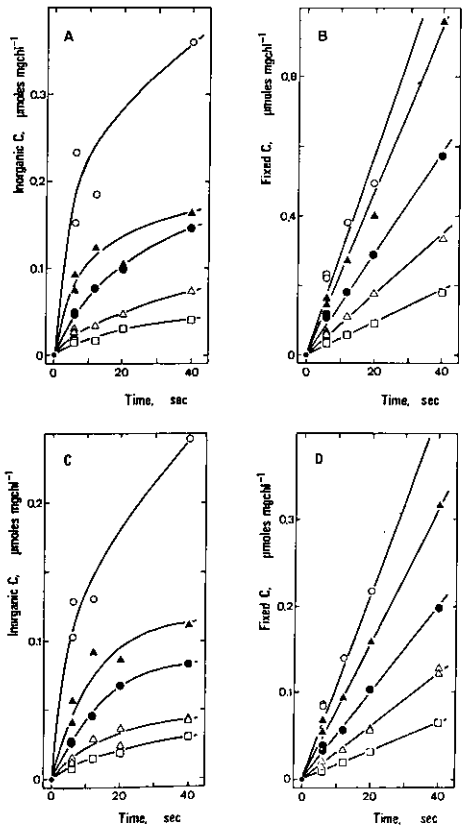

Fig. 2 Time-dependent accumulation of inorganic (A and C) and fixed (B and D) ^{14}C in the protoplasts treated with 0 (A and B) and 1 mM (C and D) Na_2SO_3 as a function of external C_{inorg} concentrations

–○–, 4.0; –▲–, 1.6; –●–, 0.84; –△–, 0.40; and –□–, 0.16 mM $NaH^{14}CO_3$.

mesophyll protoplasts did not reach a rapid equilibrium with the external medium. In both protoplasts, the larger the amount of external C_{inorg} concentrations added was, and the longer the incubation time of protoplasts with $^{14}C_{inorg}$ was, the more amount of $^{14}C_{inorg}$ was accumulated in the protoplasts. The influx rate of C_{inorg} was estimated from the initial rate of ^{14}C accumulation in the protoplasts, and the effects of Na_2SO_3 treatment on the rate of $^{14}C_{inorg}$ influx and that of fixation were examined (Fig. 3). The reaction time for the measurement of initial rate was only 6 s, thus the efflux of C_{inorg} from protoplasts is expected to be negligible. Fig. 3 clearly indicates that not only $^{14}CO_2$ fixation rate but also the $^{14}C_{inorg}$ influx one were severely suppressed by the Na_2SO_3 treatment over all external C_{inorg} concentrations. The decrease of both rates was larger with the treatment at 10 mM Na_2SO_3 than at 1 mM. Fig. 4 shows the relationship between the initial rate of influx and that of fixation of $^{14}C_{inorg}$ in both the protoplast preparations. In the non-treated protoplasts, the rate of photosynthetic ^{14}C fixation increased almost linearly with the increment of the C_{inorg} influx rate up to approximately 150 μmol mgchl^{-1} h^{-1}, and showed a tendency to saturate at the higher influx rate. The ratio of the C_{inorg} fixation rate to the influx one was 68-70 % at the region of low C_{inorg} influx rate. In the protoplasts treated with 1 mM Na_2SO_3, the ratio coincided with the non-treated protoplasts at the influx rate below 70 μmol mgchl^{-1} h^{-1}, in spite of the marked decrease of influx rate (see Fig. 3). The ratio was depressed by the treatment with 1 mM Na_2SO_3 at the higher influx rate. In the case of protoplasts treated with 10 mM Na_2SO_3, the ratio of C_{inorg} fixation rate to the influx one was small compared to the non-treated protoplasts over all C_{inorg} influx rates (Fig. 4).

Fig. 5 shows the effects of Na_2SO_3 and Na_2SO_4 on the CA activity of *Vicia* mesophyll protoplasts. The activity was inhibited by 1 mM Na_2SO_3 to 55 % of the original level, whereas it was not changed by 4 mM Na_2SO_4.

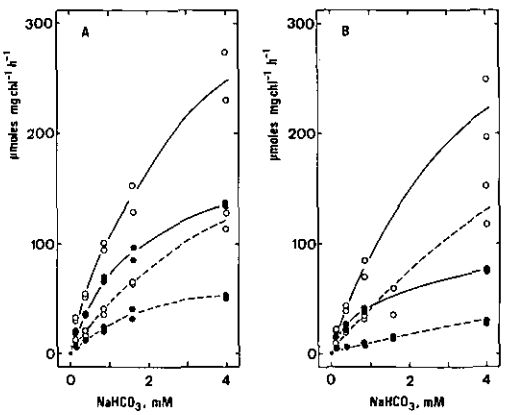

Fig. 3 Initial rates of total accumulation (○) and fixation (●) of ^{14}C in 1 (A) and 10 mM (B) Na_2SO_3-treated *Vicia* mesophyll protoplasts as affected by the external C_{inorg} concentrations

Solid lines, non-treated protoplasts; broken lines, Na_2SO_3-treated protoplasts.

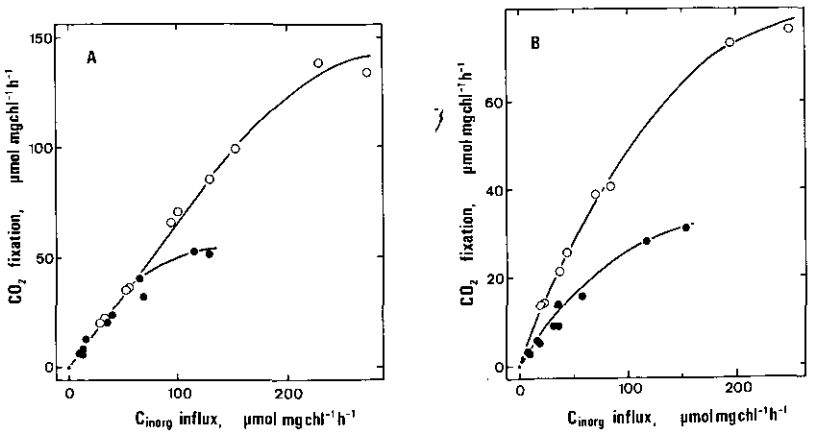

Fig. 4 Initial rate of ^{14}C fixation as a function of $^{14}C_{inorg}$ influx rate in 1 (A) and 10 mM (B) Na_2SO_3-treated protoplasts as determined from the results of Fig. 3

—o—, non-treated protoplasts; —●—, Na_2SO_3-treated protoplasts.

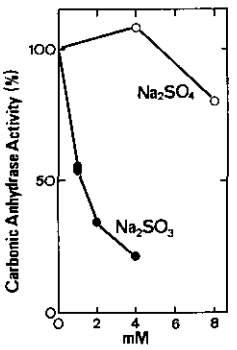

Fig. 5 Effects of Na_2SO_3 and Na_2SO_4 on CA activity in *Vicia* mesophyll protoplasts

CA activity without the chemicals was 970 U mgchl^{-1}.

Discussion

The results clearly indicate that the rate of C_{inorg} influx dependent on the external C_{inorg} concentrations was strongly depressed in *Vicia* mesophyll protoplasts by the treatment with Na_2SO_3 at an acidic condition (Fig. 3). The possible cause of the reduction of C_{inorg} influx is the inhibition of intracellular CA activity by sulfite. Several algal species lost CA activity when grown under especially high CO_2 condition (Graham *et al.*, 1970; Findenegg, 1976), and both influx rate and accumulation level of C_{inorg} were reduced in these cells (Badger *et al.*, 1980; Kaplan *et al.*, 1980). CA bound in either side of artificial membranes facilitates the

C_{inorg} transport (Broun *et al.*, 1970), and thus its action could be attributed to a part as the permease (Findenegg, 1974). Fig. 5 clearly shows that Na_2SO_3 inhibited CA activity more strongly than Na_2SO_4 did. We have already indicated that Na_2SO_3-treated protoplasts accumulated sulfite in the protoplasts (Sakaki & Kondo, 1984). Therefore the intracellular sulfite should suppress the CA activity and consequently the rates of C_{inorg} influx into the protoplasts. Another possibility is the reduction of intracellular pH (Werdan *et al.*, 1972) caused by Na_2SO_3 treatment as suggested previously (Sakaki & Kondo, 1984). This possibility, however, is purely speculative and we have no experimental bases to support the possibility at present.

The rate of photosynthetic CO_2 fixation increased with C_{inorg} influx rate (Fig. 4). The dependence of the rate of CO_2 fixation on that of C_{inorg} influx was different between the protoplasts treated with 1 mM and 10 mM Na_2SO_3. In mesophyll protoplasts treated with 1 mM Na_2SO_3, the ratio of CO_2 fixation rate to C_{inorg} influx one was similar to the ratio of non-treated protoplasts at low C_{inorg} influx rate, but CO_2 fixation ability was saturated at much lower influx rate than that of non-treated protoplasts. In the case of the protoplasts treated with 10 mM Na_2SO_3, the ratio of photosynthetic CO_2 fixation rate to C_{inorg} influx one was lower than that of non-treated protoplasts not only at high C_{inorg} influx rate but also at low one. It is suggested that the depression of C_{inorg} influx rate limited at least partly the photosynthetic CO_2 fixation in Na_2SO_3-treated protoplasts. Kaplan *et al.* (1980) reported that the rate of photosynthetic CO_2 fixation in high CO_2-grown *Anabaena* cells was limited by the rate of C_{inorg} influx into the cells. They suggested that the number of HCO_3^- transport carriers at cell membranes was decreased by such growth condition. In the case of higher plant cells, however, it has still to be determined whether HCO_3^- could cross the cell membranes or not (Volokita *et al.*, 1981; Espie & Colman, 1982). The present results show that the influx of C_{inorg} through the cell membranes should also be an important point for studying the photosynthesis in higher plant cells. Probably the complexed manner of CO_2 fixation kinetics as presented in Fig. 1 is the result from combined effects of sulfite both on C_{inorg} influx and CO_2 fixation processes.

Acknowledgement

We wish to thank the members of the Engineering Division of this institute for their adept cultivation and provision of the plant materials.

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ソラマメ葉肉細胞プロトプラストの亜硫酸による無機炭素の取り込み及び固定の阻害

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ソラマメ（*Vicia faba* L. cv. Otafuku）葉から単離した葉肉細胞プロトプラストをpH4.5で1 mM及び10mM Na_2SO_3と培養した結果，光合成によるCO_2固定速度のみならずプロトプラスト内への無機炭素（C_{inorg}）の蓄積速度も大きく減少した。測定液中のC_{inorg}濃度を変え，プロトプラスト内へのC_{inorg}のインフラックス速度を測定すると，Na_2SO_3処理によってインフラックス速度が大きく阻害されることが明らかになった。C_{inorg}のインフラックス速度を炭酸固定の初速度と比較したところ，1 mM Na_2SO_3処理プロトプラストにおいて低インフラックス速度領域ではC_{inorg}インフラックスが固定速度の律速となっていることが示唆された。1 mM Na_2SO_3処理プロトプラストの高インフラックス領域及び10mM Na_2SO_3処理プロトプラストでは，C_{inorg}インフラックス阻害の他に，光合成経路の阻害が発現していることが示唆された。

ソラマメ葉のカーボニックアンヒドラーゼは1 mM Na_2SO_3によって55%まで阻害されたが4 mM Na_2SO_3によっては阻害されなかった。

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Res. Rep. Natl. Inst. Environ. Stud., Jpn., No. 65, 1984.

Effects of Mixed Gas on Transpiration Rate of Several Woody Plants

1. Interspecific Difference in the Effects of Mixed Gas on Transpiration Rate

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Interspecific difference of transpiration rate in seven woody plants with different tolerances to air pollutants was studied during the simultaneous exposure to two species of NO_2, O_3 and SO_2 whose concentrations were 0.1 ppm. Tested plants were *Nerium indicum* Mill, *Euonymus japonica* Thumb, *Aucuba japonica* Thumb, *Acer buergerianum* Mig. *Viburnum awabuki* K. Koch, *Quercus myrsinaefolia* Blume and *Zelkova serrata* Makino. The obtained results showed that the characterisitc feature of tolerant species to air pollutants was a high stomatal sensitivity to the air pollutants (*N. indicum* and *E. japonica*) or a small value of initial transpiration rate (*A. japonica*). *Z. serrata* had an extreamly unique response of transpiration rate during the exposure to mixed gases with relatively high concentrations.

Key words: mixed gas, woody plants, transpiration rate, SO_2, NO_2, O_3.

In the daytime, plants can absorb CO_2 in air through stomata by a photosynthetic process, and consume H_2O by a transpiration process. Along with these processes, plants unavoidably absorb air pollutants in the smoke polluted area. According to the review by Omasa (1979a), the main factors in plants controlling the absorption of air pollutants by leaves are 1) the concentration of pollutants in the substomatal cavity and 2) the opening of stomata determining principally the gas diffusion resistance. The concentration of pollutants in the substomatatal cavity was reported by several workers (Omasa & Abo, 1978; Omasa *et al.*, 1979b; Black & Unsworth, 1979; Natori & Totsuka, 1980; Unsworth & Black, 1981). Those results showed that the concentration of SO_2, O_3 and NO_2 in the substomatal cavity could be assumed to be 0 ppm. This means that the gas absorption of plants can be limited mainly by the opening of stomata.

It has been well known that the stomatal opening is different among plant species. However, there are only few reports about inter- or intraspecific difference of stomatal opening during the exposure to mixed gases with relatively low concentrations (Beckerson & Hofstra, 1979; Elkiey & Ormrod, 1979; Fujinuma *et al.*, 1981). Furthermore, no report could be found so far concerning the interspecific difference in stomatal opening of woody plants during the exposure to mixed gases.

The responses of stomata during the exposure to gas mixture are different among the gas composition and the environmental conditions. In the present study, the transpiration rate of whole plants, which was known to be an easily measurable indicator of stomatal opening, was examined in seven woody plants with different tolerance to air pollutants during the simultaneous exposure composed of two species of NO_2, SO_2 and O_3 under the same environmental conditions. Based on the obtained data, the relationships between plants resistance and the change of transpiation rate during the gas exposure were discussed.

Materials and Methods

Seven species of woody plants were selected on the basis of the difference of resistance to gaseous pollutants (Noria Suisan Gijutsukaigi Jimukyoku, 1973): *Nerium indicum* Mill and *Euonymus japonica* Thumb as an extremely resistant species, *Aucuba japonica* Thumb and *Acer buergerianum* Mig. as a moderately resistant species, *Viburnum awabuki* K. Koch as a rather sensitive species, and *Quercus myrsinaefolia* Blume and *Zelkova serrata* Makino as a very sensitive species. Young plants of these species were grown for three to six months in plastic pots (1/5000 or 1/10000 are) containing peat moss, vermiculite, perlite, and fine gravel (2:2:1:1 v/v) in a greenhouse. The plants were transferred to a phytotron greenhouse, and they were maintained for further one week before gas treatments under the conditions of 25°C in air temperature and 75% in relative humidity in the phytotron.

The potted plants were placed in a controlled-environmental gas fumigation chamber for 12 hours in order to be preconditioned under the same condition as the gas exposure experiments where the environmental conditions were air temperature 25°C, relative humidity 75% and light intensity about 35 klx at plant height. The light source was composed of twenty-four 400W halide lumps. (Toshiba, Yoko Lump). The light was filterd through heat absorbing glass to exclude the radiation above 800 nm.

The measurements of transpiration rate were conducted during 9:00 – 17:00 to exclude the effects of diurnal rhythm of stomata. Before starting the measurements, potted plants were watered excessively, and they were left to drain for 15 minutes. And then each pot was wrapped in a polyethylene bag and were made air-tight seal around the base of the plant. Several pots were set on an electric top-loading balance (Mettler, Model PE 11), and their weight losses were recorded at the time interval of one min with a thermal data acquisition instrument (Eto Denki, Model Thermodac II). The gas exposure was started after the weight losses of the potted plants attained stable value. After the exposure treatments, leaf areas were measured with an automatic area meter (Hayashi Denko Co., Ltd., Model AAM-7). Transpiration rates were calculated by the recorded weight loss of potted plants for 10 min. The gas concentrations in the fumigation chamber were continuously monitered and regulated using a controlling system based on a chemiluminescent NO-NO_2-NO_x analyzer (Thermo Electron Co., Model 14 D) for NO_2, on a pulse fluorescent SO_2 analyzer (Thermo Electron Co., Model 43) for SO_2 and on a chemiluminescent O_3 meter (Kimoto Electric Co., Model 806) for O_3.

When the initial value of transpiration rate was too small, the data were recollected after exchanging plant materials.

Results

Fig. 1 and 2 show the time course of transpiration rate of the tested plants during the exposure to 0.1 ppmSO_2 + 0.1 ppmNO_2 and 0.1 ppmO_3 + 0.1 ppmNO_2. The decrease in transpiration rate of *E. japonica* was initially rapid, and then became gradual in both mixed gases. That of *N. indicum*, *A. buergerianum* and *Q. myrsiaefolia* was gradual.

Table 1 shows summarized data of the initial and exposed transpiration rate of seven species during the exposure to 0.1 ppmSO_2 + 0.1 ppmNO_2 or 0.1 ppmNO_2 + 0.1 ppmO_3. The initial transpiration rate showed the mean value for one hour before starting the exposure, and the exposed transpiration rate was obtained as the mean value for 2 to 3 h after starting the exposure. Transpiration rate of *N. indicum* and *E. japonica* decreased at the exposure to 0.1 ppmSO_2 + 0.1 ppmNO_2 or 0.1 ppmNO_2 + 0.1 ppmO_3. On the other hand, the initial and exposed transpiration rate of *A. japonica* were very small, and the transpiration rate of the plant was scarcely affected by the exposure to the both mixed gases. Transpiration rate of *V. awabuki*, *Q. myrsinaefolia* and *Z. serrata* showed a tendency not to be affected by the exposure to mixed gases. However, when the initial transpiration rate of *Q. myrsinaefolia* was relatively large, the transpiration rate decreased remarkably during the mixed gas exposure. The

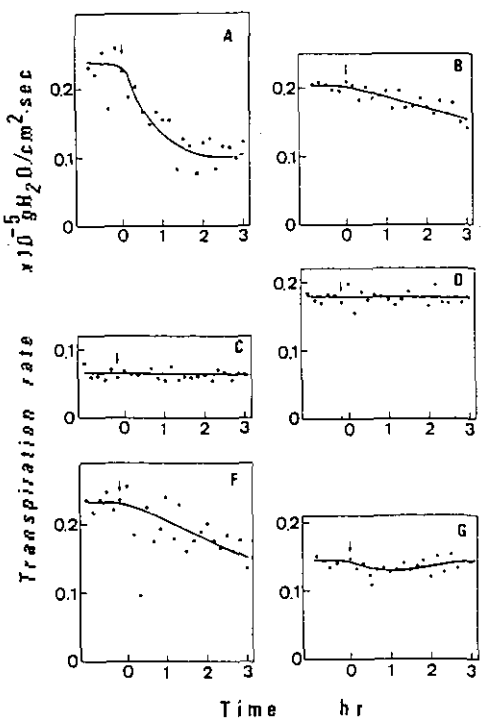

Fig. 1 The time course of transpiration rate of woody plants exposed to 0.1 ppmSO_2 + 0.1 ppmNO_2

Capital letters in the figure indicate plant species: A; *Eunonymus japonica* B; *Nerium indicum* C; *Aucuba japonica* D; *Acer buergerianum* F; *Quercus myrsiaefolia* G; *Zelkova serrata*. Arrows in the figure indicate the time of starting the exposure.

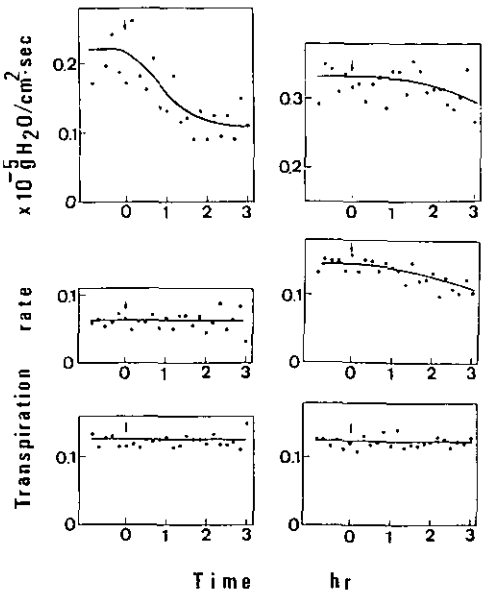

Fig. 2 The time course of transpiration rate of woody plants exposed to 0.1 ppmO_3 + 0.1 ppmNO_2

Capital letters in the figure indicate plant species: A – D and F were the same species as in Fig. 1. E; *Viburunum awabuki*. Arrows in the figure were the same as in Fig. 1.

Table 1 Transpiration rate of woody plants during the exposure to mixed gases

The former values show the initial value which was obtained as a mean value for 1 h immediately before the gas exposure, and the later values show the transpiration rate which was obtained as a mean value from 2 to 3 h after starting the exposure.

Plant species	NO_2 0.1 ppm + SO_2 0.1 ppm		NO_2 0.1 ppm + O_3 0.1 ppm	
Nerium indicum Mill	°0.203–0.163	°0.195–0.160	°0.132–0.116	°0.325–0.299
Euonymus japonica Thumb.	°0.228–0.113	°0.133–0.093	°0.198–0.117	°0.181–0.138
Aucuba japonica Thumb.	0.063–0.064	0.078–0.073	0.063–0.063	
Acer buergerianum Mig.	0.179–0.177	0.159–0.148	0.124–0.112	°0.147–0.111
Viburnum awabuki K. Koch			0.095–0.099	0.121–0.124
Quercus myrsinaefolia Blume	°0.232–0.166	0.106–0.101	0.123–0.125	0.112–0.118
Zelkova serrata Makino	0.143–0.144	0.248–0.239		

° Levels of significance: $P<0.05$ ($\times 10^{-5}$ $gH_2O/cm^2 \cdot s$)

difference in effects of the mixed gases between 0.1 ppmNO_2 + 0.1 ppmSO_2 and 0.1 ppmNO_2 + 0.1 ppmO_3 was not recognized in every plant tested.

Fig. 3 shows the time course of transpiration rate of four species, *N. indicum, E. japonica, Q. myrsinaefolia* and *Z. serrata*, during the exposure to 0.1ppmSO_2 + 1.0ppmNO_2 where NO_2 concentration was 10 times higher than that in Fig. 1. The decrease in transpiration rate of *N. indicum* and *E. japonica* was initially rapid, and then became gradual to attain a given value. On the other hand, *Q. myrsinaefolia* did not show a clear pattern in the change with time. The

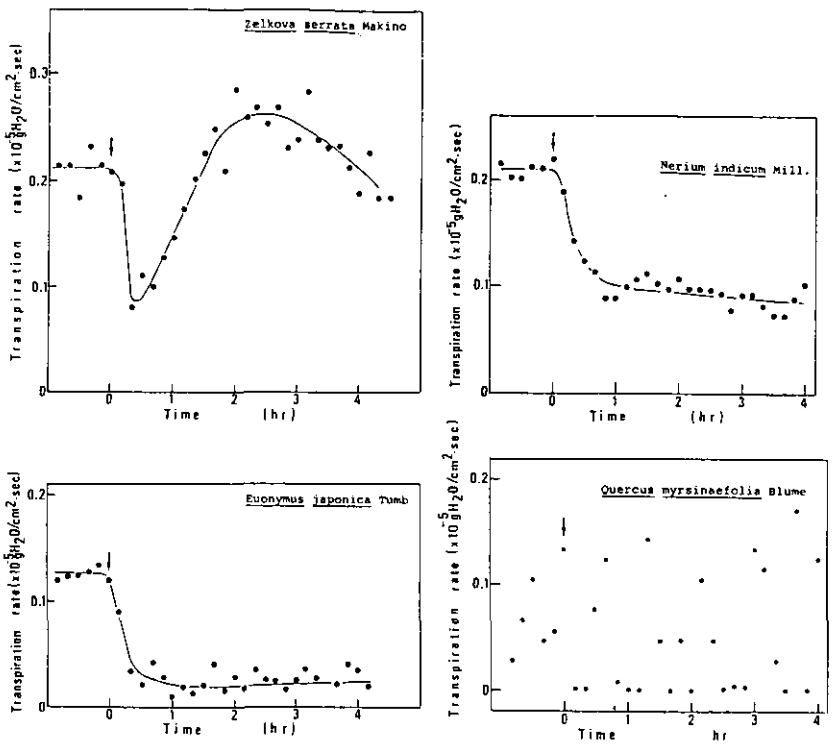

Fig. 3 The time course of transpiration rate of four woody plants during the exposure to 0.1 ppmSO_2 + 1.0 ppmNO_2

Arrows in the figure were the same signs as in Fig. 1.

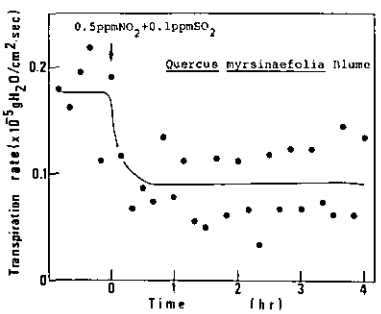

Fig. 4 The same as in Fig. 3, but in *Q. myrsiaefolia* exposed to 0.1 ppmSO_2 + 0.5 ppmNO_2

The arrow in figure was the same signs as in Fig. 1.

reason of the phenomenon might be in the instability of stomatal movement and/or the increase in an error of weighing because of the small weight loss of the potted plants due to the smallness of total leaf area. However, *Q. myrsinaefolia* showed a similar tendency of the decrease in transpiration rate by the exposure to 0.1 ppmSO_2 + 0.5 ppmNO_2 to that of *N.*

indicum and *E. japonica* exposed to 0.1 ppmSO_2 + 1.0 ppmNO_2. As shown in Fig. 4, the transpiration rate of *Z. serrata* decreased rapidly immediately after starting the exposure, but at 30 min after starting the gas exposure, the transpiration rate began to increase. And after 2 hours, the rate became greater than the initial value.

Fig. 5 shows the effects of a change in NO_2 concentrations mixed with 0.1 ppmSO_2 on the time course of transpiration rate of *N. indicum*. The transpiration rate decreased gradually by the exposure to 0.1 ppmSO_2 mixed with 0.1 ppmNO_2 or 0.2 ppmNO_2. On the other hand, the

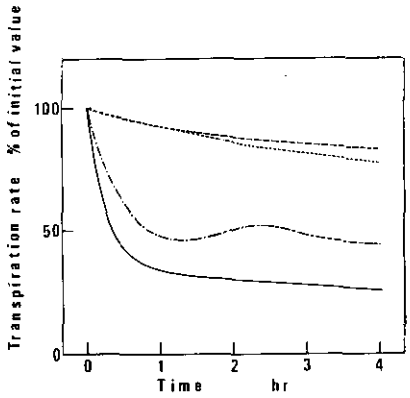

Fig. 5 The time coures of transpiration rate of *Nerium indicum* during the exposure to 0.1 ppmSO_2 mixed with NO_2 of 0.1 – 1.0 ppm

·····; 0.1 ppmSO_2 + 0.1 ppmNO_2 (0.203), - - - - - - ; 0.1 ppmSO_2 + 0.2 ppmNO_2 (0.143), — · — · — ; 0.1 ppmSO_2 + 0.5 ppmNO_2 (0.194), ——— ; 0.1 ppmSO_2 + 1.0 ppmNO_2 (0.196). Values in parentheses following each gas composition show the initial value of transpiration rate (x10^{-5} gH_2O/cm^2·s) at each experiment.

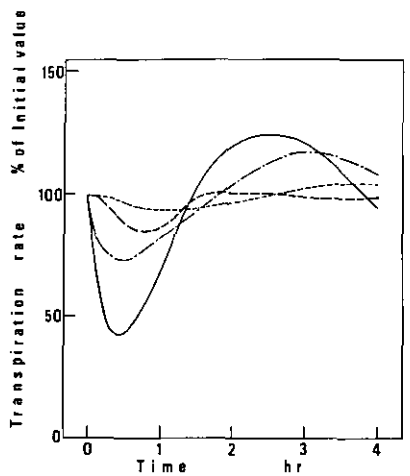

Fig. 6 The same as in Fig. 5, but in *Zelkova serrata*

·····; 0.1 ppmSO_2 + 0.1 ppmNO_2 (0.143) - - - - - - ; 0.1 ppmSO_2 + 0.2 ppmNO_2 (0.219) — · — · — ; 0.1 ppmSO_2 + 0.5 ppmNO_2 (0.157), ——— ; 0.1 ppmSO_2 + 1.0 ppmNO_2 (0.212). Values in parentheses following each gas composition show the initial value of transpiration rate.

transpiration rate decreased remarkably after starting the exposure to 0.1 ppmSO_2 mixed with 0.5 ppmNO_2 or 1.0 ppmNO_2.

Fig. 6 shows the time course of transpiration rate of *Z. serrata* under the same exposure conditions as in Fig. 4. The comparison of the patterns in Fig. 4 with those in Fig. 5 indicates a clear difference between two species. The transpiration rate of *Z. serrata* exposed to 0.1 ppmSO_2 + 0.1 ppmNO_2 was scarcely affected. However, the transpiration rate of the plant exposed to 0.1 ppmSO_2 + 0.2 ppmNO_2 was slightly decreased immediately after the start of the exposure. But at 2 h exposure, it recovered mostly to the initial value. The transpiration rate decreased by the exposure to 0.1 ppmSO_2 + 0.5 ppmNO_2 immediately after the start of the exposure, and at 2 h exposure, it became larger than the initial value. Futhermore, the mentioned time trend became remarkable at 0.1 ppmSO_2 + 1.0 ppmNO_2.

Discussion

In this report, interspecific difference of transpiration rate among seven species of woody plants was examined during the exposure to mixed gases of two species of SO_2, NO_2 and O_3 with relatively low and high concentrations. In the case of the mixed gas exposure with relatively low concentrations such as 0.1 ppmSO_2 + 0.1 ppmNO_2 or 0.1 ppmNO_2 + 0.1 ppmO_3, relatively tolerant species to air pollutants had a high stomatal sensitivity as seen in *N. indicum* and *E. japonica* or the small value of initial transpiration rate as seen in *A. japonica.* On the other hand, relatively sensitive species had a tendency not to be affected for the transpiration rate as shown in *V. awabuki, Q. myrsinaefolia* and *Z. serrata* (Table 1).

Furthermore, the Table 1 indicates that the interspecific difference of stomatal sensitivity of tested plants during the exposure to 0.1 ppmSO_2 + 0.1 ppmNO_2 was similar to that during the exposure to 0.1 ppmNO_2 + 0.1 ppmO_3.

In the case of the mixed gas exposure with relatively high concentrations such as 0.1 ppmSO_2 + 1.0 ppmNO_2 or 0.1 ppmSO_2 + 0.5 ppmNO_2, *N. indicum, E. japonica* and *Q. myrsinaefolia* had a similar pattern of decrease in transpiration rate. The decrease in transpiration rate of these plants was initially rapid and became more gradual thereafter (Fig. 3 and Fig. 4). Omasa *et al.* (1979b) reported that SO_2, NO_2 and O_3 concentrations in the substomatal cavity of sunflower can be assumed to be 0 ppm during the exposure to SO_2, O_3 and NO_2 alone and in combination. If gas concentrations in substomatal cavity are assumed to be 0 ppm in the tested plants, the gas sorption rate will be in parallel with their stomatal opening. Therefore, the data shown in Table 1 and Fig. 3 suggest that the amount of gas sorption of the species tolerant to air pollutants becomes smaller by the closing of stomata than that of sensitive plants during the simultaneous exposure.

Similarly to our results during the exposure to mixed gases, Thomas (1951) has reported that the interspecific difference in resistance to SO_2 is ascribable to the difference in gas absorption into leaves. Kondo and Sugahara (1978) has reported that the rapidity of stomatal closure during SO_2 fumigation may determine the resistance of plant to SO_2. Furukawa *et al.* (1979) have reported that interspecific difference in resistance to SO_2 may be primarily determined by the amount of SO_2 absorbed for a certain period.

On the other hand, *Z. serrata* had an extremely unique response of transpiaration rate during the exposure to SO_2 + NO_2, as shown Fig. 5. The degree of the change in transpiration rate became remarkable with increase in the concentration of NO_2 mixed with 0.1 ppmSO_2. One of reasons why *Z. serrata* is sensitive to gaseous pollutants may be in this unique response of transpiration rate during the exposure to relatively high concentrations of gas mixture. Therefore, to ascertain the resistance of woody plants to the mixed gas exposure, the change of

transpiration rate with time during the exposure to mixed gases is needed to examine.

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数種木本植物の蒸散速度に及ぼす混合ガスの影響
1．混合ガスの蒸散速度への影響についての種間差異

名取俊樹[1]・戸塚　績[1]

0.1ppm SO_2，0.1ppm NO_2，0.1ppm O_3 による2種混合ガスを汚染ガスに対する抵抗性の異なる7種の木本植物に暴露して蒸散速度の変化を測定し，混合ガスの蒸散速度に及ぼす影響の種間差異を検討した。実験植物として，キョウチクトウ，マサキ，アオキ，トウカエデ，サンゴジュ，シラカシ，ケヤキを選定した。実験の結果，汚染ガスに抵抗性のある種の特性として，例えばキョウチクトウとマサキでは混合ガスに対して気孔が敏感に反応して閉じたが，アオキは本来の蒸散速度が低く，ガス暴露しても蒸散速度がほとんど変化しないことが明らかとなった。一方，ケヤキの蒸散速度は，比較的高濃度の混合ガス暴露時に極めて特異的な変化を示した。

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Res. Rep. Natl. Inst. Environ. Stud., Jpn., No. 65, 1984.

Effects of Mixed Gas on Transpiration Rate of Several Woody Plants

2. Synergistic Effects of Mixed Gas on Transpiration Rate of *Euonymus japonica*

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Changes in transpiration rate of *Euonymous japonica* were investigated by the exposure to SO_2, NO_2 and O_3 alone and in combinations under the same experimental conditions. The decrease in transpiration rate by the single exposure to O_3 was most remarkable among that by the exposure to SO_2, NO_2 or O_3 with 0.1 ppm. The simultaneous exposure to 0.1 ppmSO_2 and 0.1 ppmNO_2 resulted in larger decrease in transpiration rate than that at the exposure to SO_2 or NO_2 alone. The mixture of 0.1 ppmO_3 with SO_2 of which concentration varied from 0.05 to 0.5 ppm caused the larger decrease in transpiration rate with increase in SO_2 concentrations. However, effects of the mixture of 0.1 ppmNO_2 with O_3 in different concentrations were approximately the same as those of O_3 alone. The decrease in transpiration rate by the mixed gas exposure to two kinds gases of SO_2, NO_2 and O_3 at the same concentration of 0.1 ppm was approximately similar to each other.

Key words: *E. japonica*, synergistic effect, transpiration rate, SO_2, NO_2, O_3.

In the previous report shown in page 45-53 of the text, the effects of mixed gases on transpiration rate of several woody plants were investigated. There are several papers showing the synergistic effects of mixed gases on transpiration of herbaceous plants (Ashenden, 1979; Beckerson & Hofstra, 1979a,b; Elkiey & Ormrod, 1979, 1980; Fujinuma & Aiga, 1980; Omasa *et al.*, 1980). Ashenden (1979) investigated that the pollutant in combination (10 pphmNO_2 + 10 pphmSO_2) caused a remarkably decrease in transpiration rate. Beckerson and Hofstra (1979a,b) reported that the mixture of SO_2 and O_3 markedly increased the stomatal resistance in white bean, radish, cucumber and soybean. Elkiey and Ormrod (1979, 1980) reported that the exposure of petunia plants to 40 pphmO_3 + 80 pphmSO_2 caused marked increase in leaf resistance. The stomatal response of plants to a given mixed gas can also be changed by the experimental conditions during the gas exposure (Elkiey & Ormrod, 1979; Unsworth & Black, 1981). However, no report could be found so far concerning the effects of the change in constituent of gas mixture on transpiration rate of woody plant under the same experimental conditions.

In the previous report, it has been reported that the stomata of *Euonymous japonica* closed remarkably by the exposure to the mixed gases of 0.1 ppmSO_2 + 0.1 ppmNO_2 or 0.1 ppmNO_2

+ 0.1 ppmO_3 under the constant environmental conditions. In the present report, the synergistic effects of gas mixtures on transpiration rate in *E. japonica* were investigated by the exposure to SO_2, NO_2 and O_3 alone and to the mixture of two species of their gases with different concentrations under the same environmental conditions.

Materials and Methods

Young plants of *E. japonica* were grown for three to six months in plastic pots (25 cm in diameter) containing peat moss, vermiculite, perlite, and fine gravel (2:2:1:1 v/v) in a greenhouse.

Then, the plants were transferred to a phytotron greenhouse, and they were grown there for further one week before the gas treatments. Environmental conditions in the greenhouse were controlled to 25°C in air temperature and 75% in relative humidity. The plants were placed in an air conditioned gas fumigation chamber for 12 hours before the gas treatments under the conditions of air temperature 25°C, relative humidity 75%, and light intensity 35 – 40 klx at plant height.

The measurements of transpiration rate were performed by weighing method during 9:00 – 17:00 to minimize the effects of diurnal rhythme of stomatal movement. Before starting the measurement, the potted plants were watered, and were left to drain for 15 minutes. Each pot was wrapped in a polyethylene bag so as to exclude the water consumption from the surface of the pot. Two or three pots were set on electric top-loading balances (Mettler, Model PE 11), and their weight was recorded every one minite with a thermal data acquisition instrument (Eto Denki, Model Thermodac II). After the fumigation treatments, leaf areas of tested plants were measured with an automatic area meter (Hayashi Denkoh Co. LTD., Model AAM-7). Transpiration rates were calculated in the weight loss in each 10 min for 2–3 hours.

Table 1 shows an experimental design of the combinations of gases used in the present experiments. The gas concentrations in the chamber were continuously monitored and regulated using a controlling system based on a chemiluminescent NO-NO_2-NO_x analyzer (Thermo Electron Co., Model 14 D) for NO_2, on a pulsed fluorescent SO_2 analyzer (Thermo Electron Co., Model 43) for SO_2 and on a chemiluminescent O_3 meter (Kimoto Electric Co., Model 806) for O_3.

Results

Fig. 1 shows a typical time course of the transpiration rate in *E. japonica* during the exposure to 0.1 ppmNO_2 + 0.1 ppmSO_2 for 4 h in the fumigation chamber. After 20 min from the start of gas exposure, the transpiration rate showed a gradual decrease to attain a nearly constant level. The time trend of transpiration rate in Fig. 1 was mostly similar to that in the case of treatment with other combinations of gas fumigations in this experiment.

Fig. 2 shows the decrease in transpiration rates (the initial transpiration rates minus the exposed transpiration rates) at the exposure to NO_2 alone in different concentrations in the range from 0.1 to 1.0 ppm or to the mixture of 0.1 ppmSO_2 with NO_2 of which concentrations varied from 0.1 to 1.0 ppm. As an initial transpiration rate, the mean value for 1 hr before the start of fumigation was applied, and as an exposed transpiration rate, the mean value from 2 to 3 h after the start of fumigation. As seen in the figure, the exposure to 1.0 ppmNO_2 alone decreased the transpiration rate by 0.50×10^{-6} gH_2O/cm^2·s (26% of the initial value).

Table 1 The experimental design of gas exposure which was carried out
+ symbols in the table represent the tested experiments, and symbols represent no testing.

Exp. 1

SO_2/NO_2	0	0.1	0.2	0.5	1.0
0	–	+	+	–	+
0.1	+	+	+	+	+

Exp. 2

NO_2/O_3	0	0.05	0.1	0.2	0.5
0	–	+	+	+	+
0.1	–	+	+	+	+

Exp. 3

O_3/SO_2	0	0.05	0.1	0.2	0.5
0	–	+	+	+	+
0.1	+	+	+	+	+

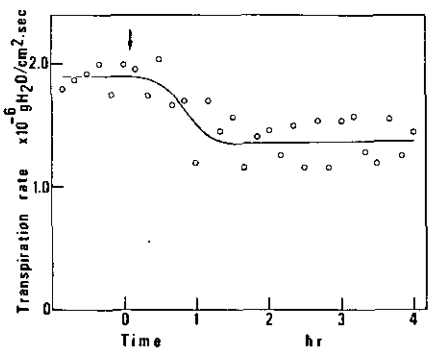

Fig. 1 Typical time course of the transpiration rate in *Euonymus japonica* during the exposure to 0.1 ppmNO_2 + 0.1 ppmSO_2 for 4 h

The arrow in the figure indicates at the time of starting the exposure.

Fig. 3 shows the decrease in transpiration rates during the exposure to O_3 alone in different concentrations in the range from 0.05 to 0.5 ppm or to the mixture of 0.1 ppmNO_2 with O_3 of which concentration varied from 0.05 to 0.5 ppm. The exposure to 0.05 ppmO_3 alone for 2 to 3 h did not affect on the transpiration rate, while transpiration rate decreased by 0.48×10^{-6} $gH_2O/cm^2 \cdot s$ (26% of the initial value) at the exposure to 0.1 ppmO_3 alone. The extent of the decrease in transpiration rate increased with increase in O_3 concentration at the exposure to O_3 alone. However, the exposure to O_3 + NO_2 showed similar effects on transpiration rate to those in the case of exposure to O_3 alone.

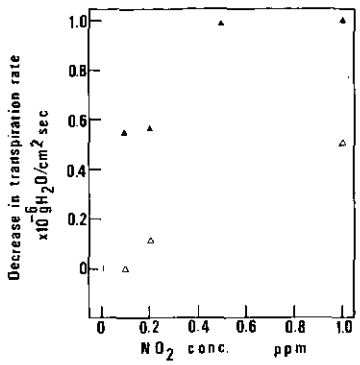

Fig. 2 The decrease in transpiration rate at the exposure to NO_2 alone and to the mixture of 0.1 ppmSO_2 with NO_2 in different concentrations for 2–3 h

Closed triangles represent data at the mixed gas exposure, and open triangles represent data at the exposure to NO_2 alone. Open square represents the result at 0.1 ppmSO_2 alone.

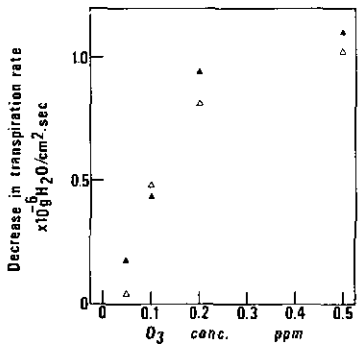

Fig. 3 The same figure as in Fig. 2, but in the case of the exposure to O_3 alone and to the mixture of 0.1 ppmNO_2 with O_3 in different concentrations

Closed triangles were the same as in Fig. 2, and open triangles were the same as in Fig. 2, but at the exposure to O_3 alone.

Fig. 4 shows the same figure as in Fig. 3, but in the case of the exposure to SO_2 alone in different concentrations or to the mixture of 0.1 ppmO_3 with SO_2 in different concentrations in the range from 0.05 to 0.5 ppm. The exposure to SO_2 alone did not decrease the transpiration rate at all. The difference between the decrease in transpiration rate at the exposure to SO_2 alone and that to mixed gas of SO_2 and O_3 increased with increase in SO_2 concentration.

Table 2 shows relative values of the exposed transpiration rate to initial one, which were calculated based on the data in Fig. 2 to 4. The evaluation of synergistic effects of the mixed gases can be read from the data in Table 2 as well as those in Fig. 2–4.

Fig. 5 indicates the comparison of the effects of the exposure to 0.1 ppmSO_2, 0.1 ppmO_3 and 0.1 ppmNO_2, alone and in combination on the decrease in transpiration rate. Transpiration

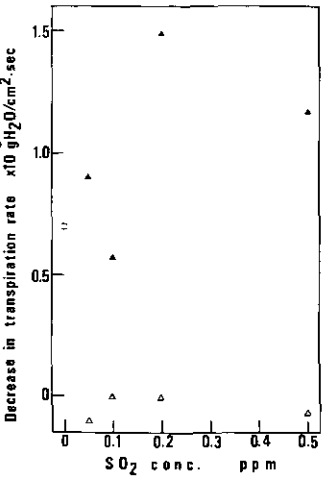

Fig. 4 The same figure as in Fig. 2, but in the case of the exposure to SO_2 alone and 0.1 ppmO_3 with SO_2 in different concentrations

Closed triangles were the same as in Fig. 2, and open triangles were the same as in Fig. 2, but at the exposure to SO_2 alone. Open square represents the result at the exposure to 0.1 ppmO_3 alone.

Table 2 Relative values of the exposed transpiration rate to the initial one, which were calculated from the data in Fig. 2 to 4
The initial value in $\times 10^{-6}$ $gH_2O/cm^2 \cdot s$ was 1.51 ± 0.37, 1.89 ± 0.46 and 2.02 ± 0.23 at the NO_2 i SO_2, SO_2 + O_3 and O_3 + NO_2, respectively.

SO_2 \ NO_2	0	0.1	0.2	0.5	1.0
0		92	93		74
0.1	100	71	51	35	37

O_3 \ SO_2	0	0.05	0.1	0.2	0.5
0		107	100	101	103
0.1	70	44	63	32	26

NO_2 \ O_3	0.05	0.1	0.2	0.5
0	98	74	58	44
0.1	91	76	58	42

rate decreased scarcely during the single exposure to 0.1 ppmNO_2, or 0.1 ppmSO_2. But during the exposure to 0.1 ppmNO_2 + 0.1 ppmSO_2 in combination, it decreased remarkably by 0.54 x 10^{-6} $gH_2O/cm^2 \cdot s$. The so-called synergistic effect of SO_2 + NO_2 mixture on transpiration was recognized clearly. Transpiration rate in relative value at the exposure to 0.1 ppm O_3 + 0.1

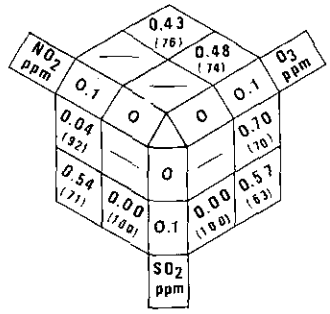

Fig. 5 The decrease in transpiration rate of the plant exposed to SO_2, NO_2 and O_3 alone and in combination for 2–3 h

The values in the figure (10^{-6} $gH_2O/cm^2 \cdot s$) show the difference between the initial and exposed value. *The values in parentheses indicate percentage of the latter value* to the former.

ppmSO_2 showed a similar value to that at the exposure to 0.1 ppmO_3 alone. Transpiration rate at 0.1 ppmO_3 decreased by 0.48×10^{-6} $gH_2O/cm^2 \cdot s$, and that at 0.1 ppmO_3 + 0.1 ppmNO_2 decreased by 0.43×10^{-6} $gH_2O/cm^2 \cdot s$. Therefore, this means that there were no combined effects of 0.1 ppmO_3 + 0.1 ppmSO_2 and 0.1 ppmNO_2 + 0.1 ppmO_3 on transpiration rate in *E. japonica*. The difference in relative values of 70 and 74% at the exposure to 0.1 ppmO_3 at both measurements may be ascribed to the difference in initial value and/or experimental errors.

Discussion

Effects of air pollutant alone

The exposure to 0.05 ppmO_3 alone for 2 to 3 h had no effects on transpiration rate in *E. japonica*, whereas the exposure to above 0.1 ppmO_3 had the remarkable effects (Fig. 3 and Fig. 4). Therefore, the threshold concentration of O_3 to induce the inhibitory effects on transpiration could be in the range from 0.05 to 0.1 ppm. On the other hand, the exposure to SO_2 alone in concentrations varying in the range from 0.05 to 0.5 ppm did not show any effects. The exposure to 0.1 and 0.2 ppmNO_2 had also no effects, while that to 1.0 ppmNO_2 inhibited slightly the transpiration rate. Therefore, it can be said that the toxicity of O_3 on transpiration rate in *E. japonica* was most remarkable among the single exposure to NO_2, SO_2 or O_3 at 0.1 ppm in each gas. On the other hand, Omasa *et al.* (1979) reported that the degree of the stomatal closure in sunflower by the single exposure to NO_2, SO_2 or O_3 at the same concentrations was $NO_2 < SO_2 < O_3$.

Effects of mixed gases

Two methods were applied to evaluate the effects of $SO_2 + O_3$, $NO_2 + O_3$ and $NO_2 + SO_2$ on transpiration. One method is based on the evaluation of the decrease in transpiration rate during the exposure to mixed gas exposure. As shown in Table 2, the transpiration rate by the exposure to $SO_2 + O_3$, $SO_2 + NO_2$ and $NO_2 + O_3$ for 2 to 3 h, where the concentration of each gas was 0.1 ppm, decreased to 63, 71 and 76% of the initial value, respectively. This means that the extent of inhibitory effects on transpiration rate during the exposure to mixed gases was approximately similar among the exposure to $SO_2 + O_3$, $NO_2 + SO_2$ and $NO_2 + O_3$.

Another method for evaluating the combined effects of mixed gases is based on the difference (relative value to the initial value) between the decrease in transpiration rates during the exposure to each gas alone and the decrease during the exposure to their mixed gases. The mentioned difference during the exposure to 0.1 ppmSO_2 alone and during the exposure to 0.1 ppmSO_2 with NO_2 at concentation of 0.1 ppm, 0.2 ppm and 1.0 ppm was 21% (0.54), 42% (0.45) and 37% (0.45), respectively. The difference in the transpiration decrease, as shown by the numerals in parentheses (dimension: 10^{-6} $gH_2O/cm^2 \cdot s$), scarcely changed with increase in NO_2 concentrations, while each value of the numerals in parentheses showed relatively large values. During the mixed exposure to 0.1 ppmNO_2 with O_3 at concentrations of 0.05, 0.1, 0.2 and 0.5 ppm, the difference in the transpiration decrease was 7% (0.13), −2% (−0.5), 0% (0.07) and 2% (0.08), respectively. These values indicated no clear difference among them. In the case of SO_2 + O_3, the exposure to 0.1 ppmO_3 alone decreased to 70% of the initial value, which corresponded to 0.7×10^{-6} $gH_2O/cm^2 \cdot s$. Then, the value at the exposure to 0.1 ppmO_3 was subtracted from the difference between the decrease in transpiration rates during the exposure to SO_2 alone and that during the exposure to mixed exposure. The obtained difference during the mixed exposure to 0.1 ppmO_3 with SO_2 at concentration of 0.05, 0.1, 0.2 and 0.5 ppm, was −7% (0.2), −23% (−0.13), −1% (0.8) and 7% (0.47), respectively. This indicates that the difference increased with increase in SO_2 concentrations.

From the above mentioned results, it can be stressed that the exposure to NO_2 + SO_2 had clear synergistic effects on transpiration rate of *E. japonica*, and the extent of synergistic effects was $SO_2 + NO_2 > SO_2 + O_3 > O_3 + NO_2$. Furthermore, these results suggest that each gas of NO_2, SO_2 and O_3 might have different mechanisms on the inhibition of transpiration rate.

As a conclusion, as mentioned above, the extent of inhibitory effects on the transpiration rate in *E. japonica* was approximately similar among SO_2 + O_3, NO_2 + SO_2 and NO_2 + O_3. This main reason could be ascribed to the strongest toxicity of O_3 and synergistic effects of SO_2 + NO_2. On the other hand, the decrease in transpiration rate was partly depended on initial value. The large initial transpiration can induce greater absorption of air pollutants, if the inhibitory effects of air pollutants on closing of stomata are approximately the same. Therefore, the mentioned fact suggests that the decrease in transpiration rate might be related to the amount of absorbed gases in leaves. For clarifying effects of mixed gases, synergistic effects should be discussed on the basis of the amount of absorbed gases in leaves.

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数種木本植物の蒸散速度に及ぼす混合ガスの影響
2．混合ガスによるマサキの蒸散速度の相乗効果

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同一条件下で，SO_2，NO_2とO_3の単一及び混合ガス暴露を行い，マサキの蒸散速度の変化を調べた。それぞれのガス濃度が0.1ppmのSO_2，NO_2とO_3の単一暴露による蒸散速度の減少はO_3が最も顕著であった。0.1ppm SO_2と0.1ppm NO_2の混合暴露は，それぞれの単一暴露より，蒸散速度の減少は大きかった。0.1ppm O_3と0.05ppmから0.5ppmの範囲で濃度変化させたSO_2との混合暴露は，SO_2濃度の増加に伴い，蒸散速度の減少は大きくなった。しかし，0.1ppm NO_2と種々の濃度のO_3との混合暴露の影響は，それぞれの濃度での単一暴露のそれとほぼ同程度であった。それぞれのガス濃度が0.1ppmのSO_2，NO_2とO_3の2種混合暴露による蒸散速度の減少は，3者の間でほぼ同程度であった。

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Res. Rep. Natl. Inst. Environ. Stud., Jpn., No. 65, 1984.

Differential Effects of Ozone and Sulphur Dioxide on the Fine Structure of Spinach Leaf Cells*

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Spinach (*Spinacia oleracea* L. cv. New Asia) plants were continuously fumigated with 0.5 p.p.m. O_3 and 1.0 p.p.m. SO_2 singly or in combination. The leaf tissues were examined by electron microscopy at various intervals until they were necrosed. The first indication of O_3 injury was swelling of thylakoids in the chloroplasts, which was followed by swelling of Golgi bodies, endoplasmic reticulum and nuclear envelopes. The internal space of the mitochondrial cristae was reduced. Later the chloroplasts were deformed. Sulphur dioxide injury first appeared as swelling of the stroma and deformation of the chloroplasts. Swelling of thylakoids appeared later. After both treatments the cells ultimately collapsed and their contents were aggregated. When O_3 and SO_2 were supplied simultaneously, the appearance and development of injury were markedly accelerated compared with either of the single fumigations. The cells mainly showed the features of SO_2 injury following simultaneous fumigation.
Key words: Air pollution, Fine structure, Ozone, *Spinacia oleracea*, Sulphur dioxide

Ozone and sulphur dioxide are the major components of air pollution. They are usually more injurious to plants than all other pollutants (Kozlowski, 1980). Although many have studied on the effects of O_3 and SO_2 on plants in various ways (see review by Heath, 1980), there are conflicting accounts of the effects of these pollutants on leaf ultrastructure.

Ozone causes thylakoid swelling in chloroplasts of *Zelkova* (Matsushima *et al.*, 1977) or distortion of the thylakoids in *Raphanus* (Athanassious, 1980). It causes granulation of the stroma in *Phaseolus* (Thomson, Dugger & Palmer, 1966) and *Raphanus* (Athanassious, 1980). Chloroplasts shrink without drastic structural distortion in *Nicotiana* (Swanson, Thomson & Mudd, 1973) or degenerate to spherical bodies containing many large globules in *Pharbitis*

* A part of this study was published in; New Phytologist 96, (1984).

(Tōyama, 1975, Nouchi *et al.*, 1977).

Sulphur dioxide causes swelling of the thylakoids in *Pinus* (Malhotra, 1976), *Pisum* (Wong, Klein & Jäger, 1977), *Vicia* (Wellburn, Majernik & Wellburn, 1972) and *Zelkova* (Matsushima *et al.*, 1977). It also causes swelling of stroma in *Larix* (Mlodzianowski & Bialobok, 1977) and *Vicia* (Fischer *et al.*, 1973). In *Spinacia* grana increase in number and plastoglobules increase in both number and size. Swelling of the thylakoids was not observed (Masuch, Weinert & Guderian, 1973). Peculiar rod-like bundles appear in the stroma of *Phaseolus*' chloroplasts (Godzik & Sassen, 1974).

Such variations in ultrastructural observations may be due to differences in plant materials, fumigation conditions or the preparation procedures for electron microscopy: it is still impossible to characterize the effects of O_3 and SO_2 on leaf subcellular structure. Thus this investigation compares the effects of O_3 and SO_2 on subcellular structure of spinach leaves, grown and fumigated under identical conditions.

Since with single fumigations the primary symptoms in subcellular structure differed between O_3 and SO_2, corresponding plants were then fumigated with these pollutants in combination. This second experiment provides some insight into the differential effects of O_3 and SO_2 within the cell.

Material and Methods

We used spinach plants because of low sensitivity of their stomata to air pollutants (Kondo & Sugahara, 1978). Spinach (*Spinacia oleracea* L. cv. New Asia) plants were grown in pots in a phytotron green house (Kondo & Sugahara, 1978). Plants used for the experiments were 4 to 5 weeks old.

Fumigation

Ozone was more injurious to spinach leaves than SO_2 at the same concentration. Therefore, we used 0.5 ppm (v/v) O_3 and 1.0 ppm SO_2 since the gross symptoms developed at almost the same rate under these conditions.

Plants were treated with the pollutants singly or in combination in a controlled environment room (1.7 × 2.3 × 2.0 m) at 25°C and 75% r.h. Illumination was provided by 24 of 400 W stannous halide lamps equipped with a heat absorbing glass filter, which removed radiation above 800 nm. Light intensity was about 33 klx (1000 W m^{-2}) at plant height. Fresh air was passed through charcoal and catalyst bearing (containing MnOx and CuO) filters to remove ambient pollutants and was led into the fumigation room. The air velocity in the room was 0.2–0.4 m s^{-1}, and the ventilation rate was 30 times h^{-1}. Ozone was generated from O_2 with an O_3 generator by way of silent electric discharge. Sulphur dioxide was supplied from a compressed cylinder containing 4000 p.p.m. SO_2 in N_2. The pollutants were injected through thermal mass-flow controllers into the air stream to give the desired concentrations. The concentrations of O_3 and SO_2 within the room were monitored with a chemiluminescent O_3 analyzer and a pulsed fluorescent SO_2 analyzer, respectively. Before the treatment, plants in pots were placed in a similar room for 3 h, then transferred into the fumigation room in which the desired concentrations of pollutants had been established.

Electron microscopy

Leaf samples were taken from fully expanded young leaves at regular intervals up to 12 h after the start of fumigation. Leaf samples before treatment and after 12 h of incubation in a room without pollutants were used as controls. Small pieces of leaf tissues were fixed in 5% glutaraldehyde for 12 h or in a mixture containing 5% glutaraldehyde and 4% paraformaldehyde for 4 h at 4°C. Both fixatives were prepared in 0.05M sodium phosphate buffer, pH 7.2. The samples were washed with the buffer several times and post-fixed in 2% OsO_4 in the buffer for 15 h at 4°C. They were dehydrated in an acetone series and embedded in Quetol 812 after treatment with propylene oxide. Thin sections were stained with uranyl acetate and lead citrate and examined under a JEOL JEM-100C electron microscope at 80 kV. Two μm thick sections were stained with basic fuchsin and methylene blue for light microscopy.

Experiments were repeated at least twice for each treatment of O_3 and/or SO_2.

Results

Fig. 1(a) shows a normal chloroplast with the normal arrangement of grana- and stroma-thylakoids in a spinach leaf before fumigation.

Effects of ozone

The first visible symptom of O_3 injury was water-logged flecks on the adaxial leaf surface, which became apparent 1 h after the start of fumigation. Amorphous yellow green flecks appeared within the water-logged regions of the leaves fumigated for 3 h. After 7 h fumigation, the leaves were slightly wilted and partly browned.

The first indication of ultrastructural injury caused by O_3 treatment was swelling of grana- and stroma-thylakoids of chloroplasts [Fig. 1(b) arrow]. Thylakoid swelling was first detected in the chloroplasts of spongy parenchyma cells after 1 h of fumigation but soon became apparent in the chloroplasts of palisade parenchyma cells. In yellow green regions of the leaves fumigated for 3 h, the cisternae of the Golgi bodies were swollen [Fig. 1(c) g]. Swelling was also observed in the endoplasmic reticulum and nuclear envelopes [Fig. 1(d) er and ne]. The mitochondrial cristae were no longer swollen [Fig. 1(c) cr] compared with those in the normal mitochondria [Fig. 1(a) m]. The swelling of the thylakoids and other membranous structures and the shrinkage of the cristae were observed in materials fixed in both glutaraldehyde and in a mixture of glutaraldehyde and paraformaldehyde. At later stages of O_3 injury, the entire chloroplasts were deformed [Fig. 1(e)] and showed a tendency to aggregate [Fig. 2(a)]. The aggregation of the chloroplasts was associated with the breakdown of compartmentalization following the rupture of the tonoplast and plasma membrane. Thereafter, chloroplast envelopes were disrupted [Fig. 2(b)]. When the leaves began to wilt and necrose, broken organelles and other cellular contents were aggregated [Fig. 2(c)]. Finally, the cells collapsed [Fig. 2(c) upper].

Effects of O_3 were less severe in vascular tissues than in interveinal tissues. Some of the vascular parenchyma cells, especially of larger veins, were intact even after 12 h of fumigation except for slight swelling of the thylakoids [Fig. 2(d)]. The interveinal tissues were almost necrosed at this time.

In spinach leaves palisade and spongy parenchyma cells seemed to be equally sensitive to O_3 although thylakoid swelling appeared somewhat earlier in the chloroplasts of spongy cells. Palisade and spongy cells of *Raphanus* are also equally sensitive to O_3 (Athanassious, 1980), while in some other species palisade cells are more sensitive than spongy cells (Heath, 1980).

Sensitivity difference between the two tissues seems to be dependent on species.

Effects of sulphur dioxide

The first visible symptom of SO_2 injury appeared 3 h after the start of fumigation as water-logged flecks on the adaxial leaf surface. Yellow green spots developed within the water-logged regions after 5.5 h of fumigation. The yellow green spots gradually enlarged to become amorphous patches. Finally the leaves were slightly wilted and partly browned after 8 h of fumigation.

Subcellular changes became apparent before visible symptoms appeared. When the leaf tissues were examined by light microscopy after 2 h of fumigation, stainability of chloroplasts with methylene blue was markedly reduced. The reduction of stainability first appeared in the chloroplasts of spongy parenchyma cells [Fig. 3(a) s] but soon proceeded to the chloroplasts of palisade parenchyma cells. Electron microscopic observation revealed that low stainability of chloroplasts corresponded to swelling of the stroma [Fig. 3(b)] indicating that the low stainability may be due to dilution of stromal component. The swollen chloroplasts were further deformed and the arrangement of internal membrane systems was distorted after 3 h of fumigation [Fig. 3(c)]. The internal space of the thylakoids or loculus was reduced, and electron-dense deposits were often observed in the stroma [Fig. 3(c) d]. These deposits seemed to contain metalic components as they appeared electron-dense without heavy metal staining. They resemble an amorphous type of phytoferritin (Sprey, Gliem and Jánossy, 1976). The mitochondria were apparently normal [Fig. 3(c) m]. In yellow green regions of the leaves fumigated for 5.5 h, the chloroplasts were aggregated and sometimes fused with each other [Fig. 4(b) arrow]. At this stage chloroplasts showed swelling of thylakoids [Fig. 4(c) arrow]. In severely damaged cells the chloroplast envelopes had almost completely disintegrated [Fig. 4(d)]. The mitochondria showed fewer signs of damage than the chloroplasts [Fig. 4(d) m]. Electron-dense materials were observed in contact with the broken chloroplasts, mitochondria and in the cytoplasm [Fig. 4(d) arrow]. In final stages when the leaves began to wilt and necrose, cellular contents were aggregated, and cells collapsed as in the case of O_3 injury.

In contrast to O_3, SO_2 damage appeared simultaneously in the vascular and mesophyll tissues. Fig. 4(a) shows a part of a vascular bundle in a leaf fumigated with SO_2 for 3 h. Chloroplasts of the companion cells and other vascular parenchyma cells are swollen, and their internal membrane systems are distorted as in the mesophyll chloroplasts.

Effects of ozone plus sulphur dioxide

When fumigated with O_3 and SO_2 simultaneously, the appearance and development of symptoms were markedly accelerated. The treated leaves showed water-logging on the adaxial surface within 30 min and began to necrose 2 h after the start of fumigation.

In the water-logged regions of the leaves fumigated with the mixture for 30 min, chloroplasts were swollen and the arrangement of internal membrane systems was distorted [Fig. 5(a)]. Electron-dense particles were observed in the stroma [Fig. 5(a) d]. Chloroplast swelling appeared somewhat earlier in spongy parenchyma cells than in palisade parenchyma cells. The chloroplasts showed a tendency to aggregate and sometimes fused with each other [Fig. 5(b) arrows]. All of these changes were characteristic of SO_2 damage alone. In some of the mesophyll cells, however, features of O_3 injury were also observed. These were swelling of the endoplasmic reticulum and nuclear envelopes and shrinkage of the mitochondiral cristae [Fig. 5(a) cr, (b) er, ne and cr]. In yellow green regions of the plant fumigated for 1 h, swollen chloroplasts and other cellular contents were further aggregated in the central region of cells [Fig. 5(c)]. The cells were collapse after 2 h of fumiation [Fig. 5(d)]. No swelling of thylakoids

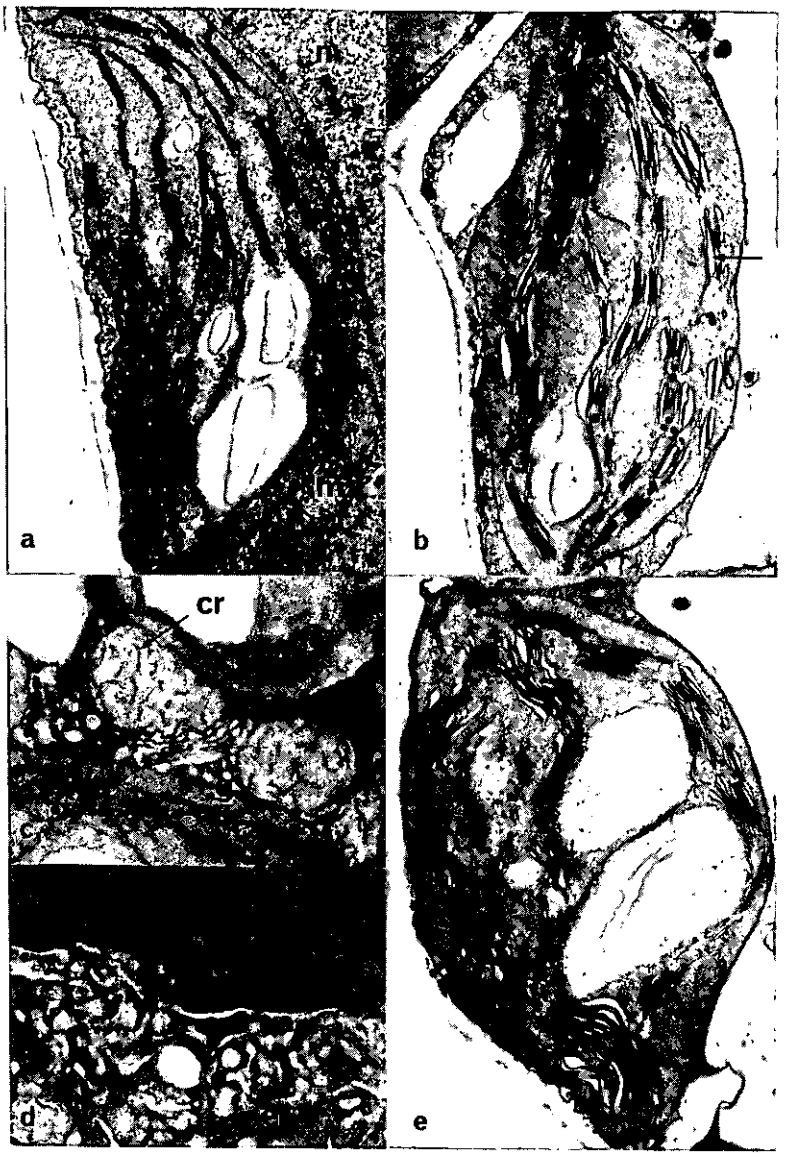

Fig. 1 Electron micrographs of spinach leaves before and after fumigation with 0.5 ppm O_3

(a) Normal chloroplast in a leaf before fumigation. m, mitochondrion; n, nucleus. × 13,000.

(b) Chlorplast in a leaf fumigated with O_3 for 3 h. Arrow indicates swelling of the thylokoids. × 9,200..

(c) Swelling of the Golgi bodies (g) and shrinkage of the mitochondrial cristae (cr) in a leaf fumigated with O_3 for 3 h. × 15,000.

(d) Swelling of the endoplasmic reticulum (er) and nuclear envelope (ne) in a leaf fumigated with O_3 for 3 h. × 17,000.

(e) A deformed chloroplast in a leaf fumigated with O_3 for 5 h. × 12,000.

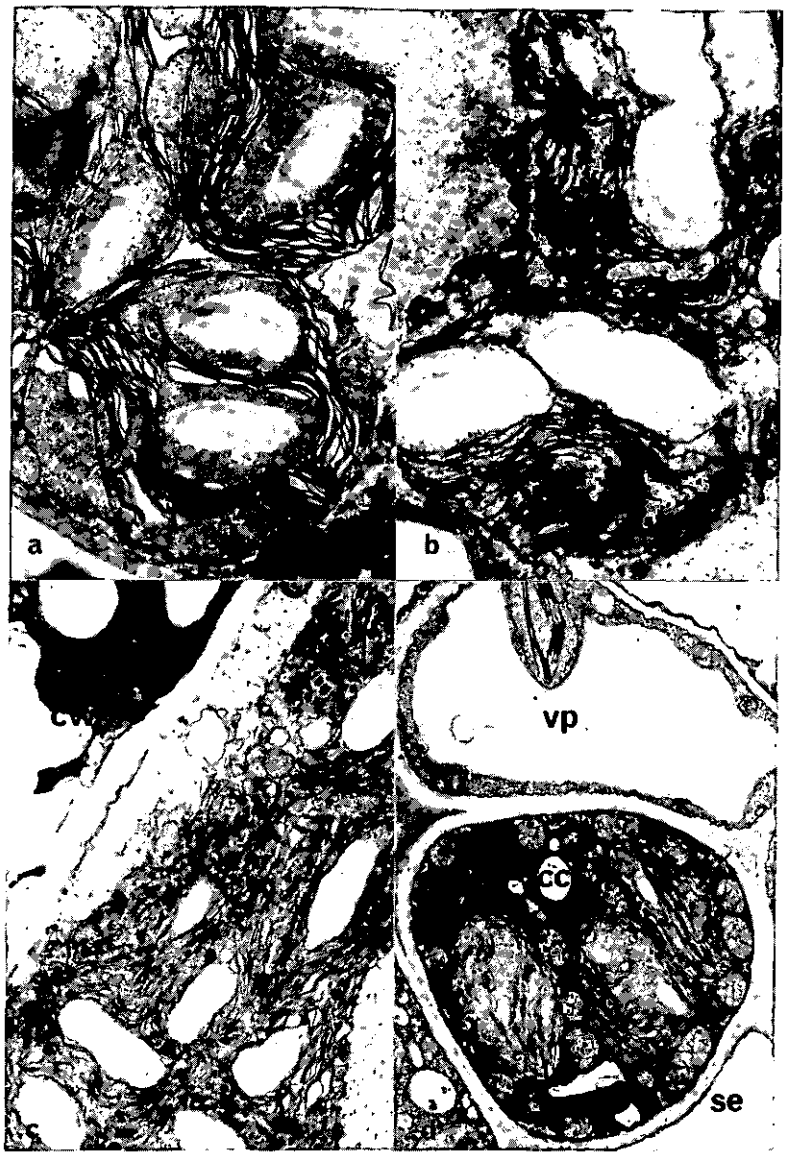

Fig. 2 Electron micrographs of spinach leaves fumigated with 0.5 ppm O_3

(a) Aggregation of deformed chloroplasts in a leaf fumigated with O_3 for 7 h. The tonoplast is distrupted (arrow). × 7,700.

(b) Disrupted chloroplasts in a leaf fumigated with O_3 for 7 h. × 7,700.

(c) Parts of palisade parenchyma cells in a leaf fumigated with O_3 for 7 h. The contents of the lower cell are aggregated. The upper cell has collapsed. Note the undulations in the cell walls (cw). × 5,700.

(d) Part of a vascular bundle in a leaf fumigated with O_3 for 12 h. The companion cell (cc) and other vascular parenchyma cell (vp) are apparently normal except for slight swelling of the thylakoids in the companion cell (arrow). se, sieve element. × 5,700.

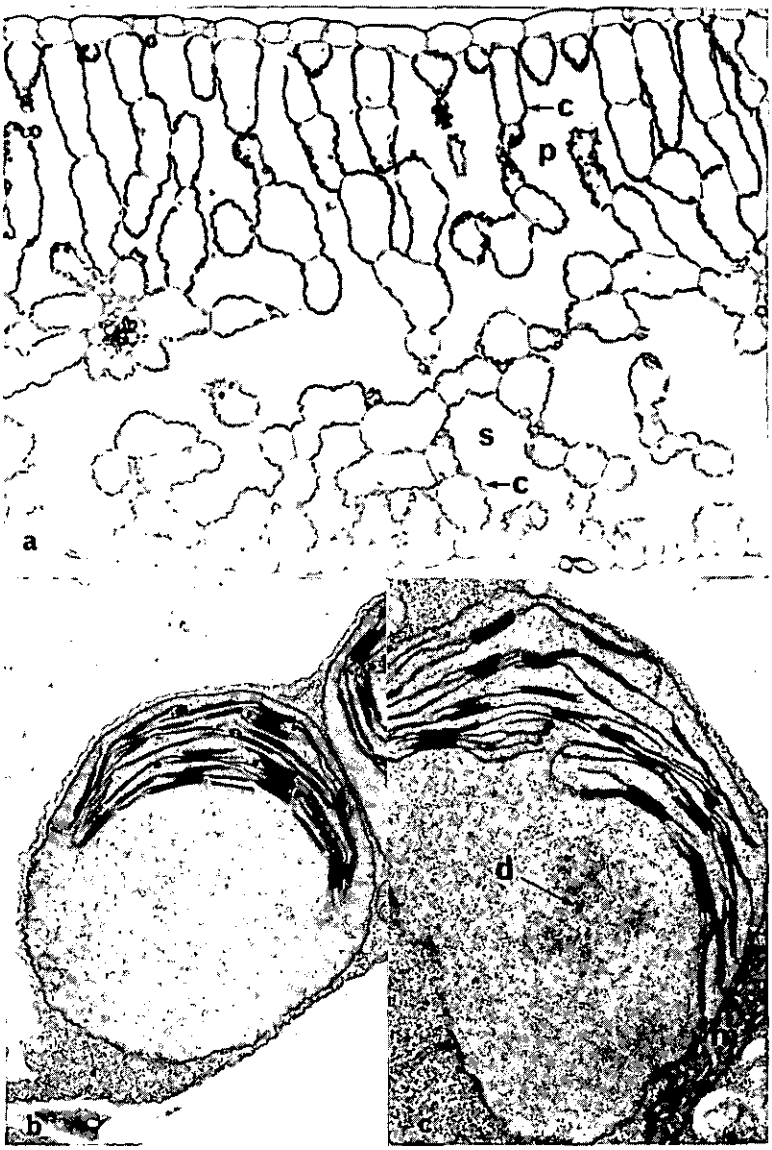

Fig. 3 Light and electron micrographs of spinach leaves fumigated with 1.0 ppm SO_2

(a) Transverse view of a leaf fumigated with SO_2 for 2 h. The section was stained with methylene blue and basic fuchsin. Chloroplasts (c) of the palisade parenchyma (p) are more densely stained than those of the spongy parenchyma (s). x 190.

(b) Chloroplast of a spongy parenchyma cell in a leaf fumigated with SO_2 for 2 h. Note the swelling of the stroma. x 11,000.

(c) A deformed chloroplast in a leaf fumigated with SO_2 for 3 h. Electron-dense deposits (d) are seen in the dilated stroma. A mitochondrion (m) is apparently normal. x 13,000.

Fig. 4 Electron micrographs of spinach leaves fumigated with 1.0 ppm SO_2

(a) Part of a vascular bundle in a leaf fumigated with SO_2 for 3 h. Note swollen and deformed chloroplasts (c) in the companion cells (cc) and vascular parenchyma cells (vp). se, sieve element. × 3,900.

(b) Aggregation and fusion (arrow) of chloroplasts in a leaf fumigated with SO_2 for 5.5 h. × 7,700.

(c) Chloroplast in a leaf fumigated with SO_2 for 5.5 h. Arrow indicates swelling of the thylakoids. d, electron-dense deposits. × 12,000.

(d) Broken chloroplasts in a leaf fumigated with SO_2 for 5.5 h. Arrow indicates accumulation of electron-dense materials. A mitochondrion (m) is not so damaged as the chloroplasts. × 7,400.

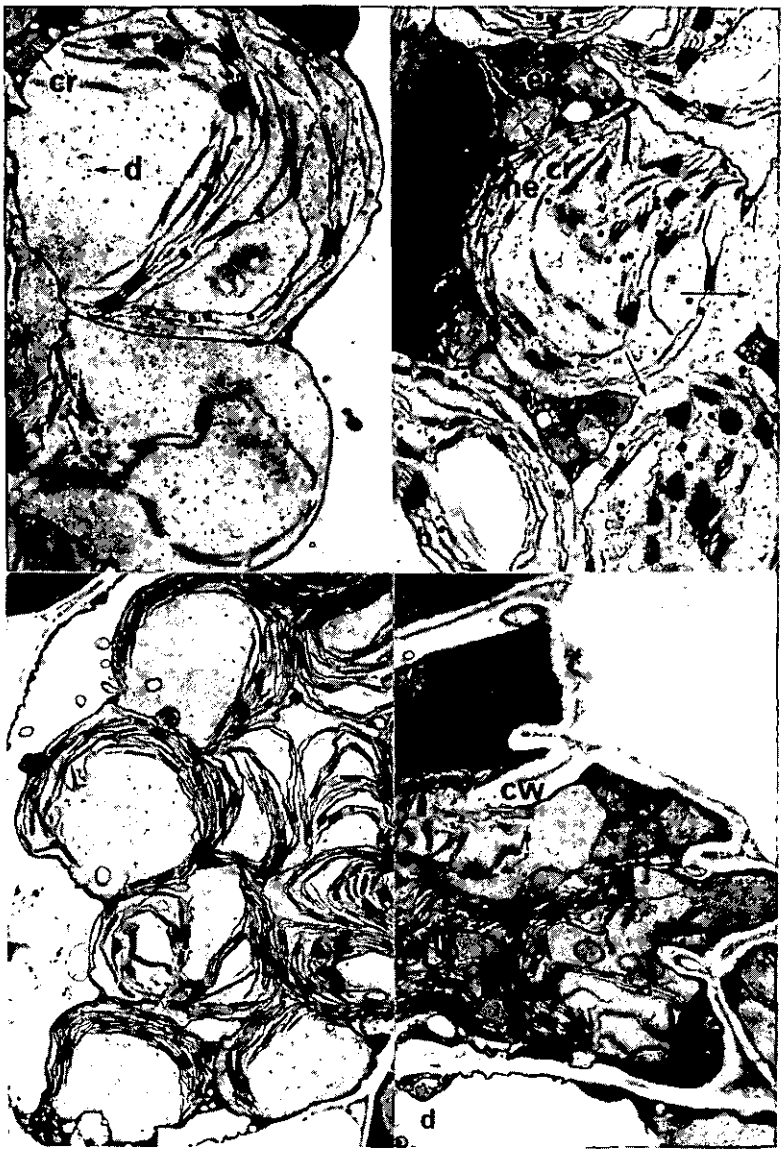

Fig. 5 Electron micrographs of spinach leaves fumigated with 0.5 ppm O_3 plus 1.0 ppm SO_2

(a) Swollen chloroplasts in a leaf fumigated with O_3 plus SO_2 for 30 min. Shrinkage of the mitochondrial cristae (cr) is visible. d, electron-dense deposits. × 7,400.

(b) Chloroplasts in a leaf fumigated with O_3 plus SO_2 for 30 min. Arrows indicate fused regions between adjacent chloroplasts. Swellings of the endoplasmic reticulum (er) and nuclear envelope (ne) and shrinkage of the mitochondrial cristae (cr) are also visible. × 7,500.

(c) Aggregation of swollen chloroplasts in a leaf fumigated with O_3 plus SO_2 for 1 h. × 3,900.

(d) Partially collapsed cell in a leaf fumigated with O_3 plus SO_2 for 2 h. Note folding of the cell walls (cw). × 6,400.

Table 1 Gross and ultrastructural symptoms in spinach leaves fumigated with 0.5 ppm O_3 and 1.0 ppm SO_2 singly or in combination

		Fumigation time (h)									
		0	1	2	3	4	5	6	7	8	9
O_3	Gross		· water-logging		· yellow-green flecks				· brown flecks		
	Ultra-structural*		· swelling of thylakoids		· *swelling of Golgi bodies* · swelling of endoplasmic reticulum · swelling of nuclear envelopes · shrinkage of cristae		· deformation of chloroplasts		· *aggregation of* cullular contents · collapse of cells		
SO_2	Gross				· water-logging			· yellow-green flecks		· brown flecks	
	Ultra-structural			· swelling of stroma	· deformation of chloroplasts			· swelling of thylakoids		· aggregation of cellular contents · collapse of cells	
O_3 + SO_2	Gross		· water-logging · yellow-green flecks · brown flecks								
	Ultra-structural		· swelling of stroma · deformation of chloroplasts · aggregation of cellular contents · collapse of cells								

* The ultrastructural symptoms were observed in the regions of gross symptoms, when the latter were apparent.

was observed throughout the experiment.

Observations on gross and ultrastructural symptoms are summarized in Table 1.

Discussion

In this investigation we used 0.5 ppm O_3 and 1.0 ppm SO_2 to investigate the acute injuries to subcellular structure. Although the gross and ultrastructural symptoms developed at the same rate with both pollutants, it should be noted in comparing their effects that the *concentration of SO_2 was twice that of O_3*.

The gross symptom of damage by both pollutants were very similar, namely water-logged flecking on the adaxial leaf surfaces followed by yellowing within the flecks. Ultimately the leaves wilted and turned brown. However, the ultrastructural changes associated with the flecking differed considerably between O_3 and SO_2, with the former producing swelling of the thylakoids, ER and Golgi bodies and shrinkage of the mitochondrial cristae and the latter swelling of the chloroplast stroma. It is not clear what, if any, ultrastructural changes might be associated with the water-logging but the subsequent yellowing probably reflects loss of chlorophyll attendant on serious damage to the thylakoids. The aggregation of the cellular contents appearing in the final stages of all treatments is indicative of the total breakdown of subcellular compartmentalisation. This aggregation is also observed in the final stages of other pollutant injuries (Thomson, 1975), herbicide treatments (Ashton, Gifford & Bisalputra, 1963; Harvey & Fraser, 1980; Pallett & Dodge, 1980) and plant diseases (Cooper, 1981). This may be a general feature of necrosing tissues.

Similarity between the effects of O_3 and the herbicide paraquat is of interest. Paraquat also induces swelling of the thylakoids, shrinkage of the cristae (Harvey & Fraser, 1980) and breakdown of the tonoplast (Harris & Dodge, 1972) and the plasma membrane (Baur *et al.*, 1969). Both O_3 and paraquat produce the hydroxyl radical, superoxide and other radicals in the plant cell (Dodge, 1975; Hoigne & Bader, 1975). These active oxygens are known to cause lipid peroxidation, an increase in membrane permeability (Pauls & Thompson, 1980) and membrane disruption (Dodge, 1975). Therefore swelling and shrinkage of membranous structures observed in the early stages of O_3 injury seem to be associated with permeability

changes in the membranes. Total breakdown of the organelles is caused by the upset of the osmotic balance or the release of hydrolytic enzymes due to the disruption of the tonoplast and plasma membrane (Harris & Dodge, 1972). Thus O_3 seems to generally attack the membranes in the mesophyll cells of the spinach.

Matsushima *et al.* (1977) report that thylakoid swelling is not a common injury caused by O_3 and appears only after a prolonged fumigation. Coulson and Heath (1974) suggested that O_3 does not penetrate beyond the plasma membrane in the early stages of injury. Some of the previous illustrations of O_3 injury such as granulation of the stroma (Thomson *et al.*, 1966; Athanassious, 1980) and shrinkage of the chloroplasts (Swanson *et al.*, 1973) can be interpreted as a result of cellular dehydration due to a permeability change in the plasma membrane. In the present study however, O_3 apparently penetrated very rapidly beyond the plasma membrane and affected the thylakoids and other membranous structures.

Early effects of SO_2 injury were restricted to the chloroplasts, with swelling of the entire organelles being the first detectable change. Sulphur dioxide inhibits photosystem II activity in the chloroplasts before the gross symptoms appear (Shimazaki & Sugahara, 1979). A photosystem II inhibitor herbicide, monuron, also causes swelling of the chloroplasts (Pallett & Dodge, 1980). However, the chloroplast swelling is not directly associated with the inhibition of photosystem II since monuron induces the chloroplast swelling in the dark as well (Pallett & Dodge, 1980). Whatley (1971) obsered swollen chloroplasts in plasmolyzed cells and associated with hydrostatic imbalance in the cell. In our materials the cells were not plasmolyzed in the early stages of injury [Fig. 3(a)], but the frequent appearance of electron-dense deposits in the stroma suggests a hypertonic status within the chloroplasts (Whatley, 1971). Hypertonicity of the chloroplasts may be induced by a permeability change in the chloroplast envelope (Anderson & Schaelling, 1970).

In the subsequent stages of SO_2 injury, swelling of the thylakoids was observed as in the case of O_3 injury. Sulphur dioxide produces super oxide, the hydroxyl radical and other active oxygens as a consequence of interactions with the photosynthetic electron transport (Asada & Kiso, 1973). These active species of oxygen may destroy the thylakoid components (Shimazaki *et al.*, 1980). Alternatively, photoxidation of chlorophylls and lipid peroxidation may occur in the thylakoids following the inhibition of electron transport and an overloading of the energy trapping system (Dodge, 1975). In either case it is reasonable to suppose that SO_2 injury occurred mainly within the chloroplasts. It is noteworthy that swelling of the thylakoids appears to be a common feature of SO_2 damage in a variety of species and that there are also some reports of swelling of the entire chloroplasts (Fischer *et al.*, 1973; Mlodzianowski & Bialobok, 1977).

Damage caused by SO_2 appeared simultaneously in the vascular and mesophyll tissues, while effects of O_3 were less severe in the vascular tissues than in the mesophyll. Similar observations were made on pine needles by Evans and Miller (1975). Sulphur dioxide seems to be transported in a toxic form(s) for longer distances than O_3.

When O_3 and SO_2 were supplied in combination, the cells mainly showed the features of SO_2 injury. This fact is of interest since O_3 injury appeared somewhat earlier than SO_2 injury when supplied singly (Table 1). Ozone is known to affect the plasma membrane as a primary site of action and to increase permeability of the cell to solutes (Heath, 1980). Therefore, it is suggested that incorporation of SO_2 into the cell was accelerated by O_3 when supplied in combination, and the ultrastructural changes were mainly induced by SO_2.

The sensitivity of individual species seems to be one of the most important factors that lead to confliction accounts of the ultrastructural effects of air pollutants. The fact that the present study has revealed far more severe damage than previously reported in other species

most likely reflects the extreme sensitivity of spinach to O_3 and SO_2.

Acknowledgments

We are indebted to Dr. S. Takenaka and Miss K. Miyasaka of the National Institute for Environmental Studies for their courtesy in the use of electron microscope. We also thank the late Prof. T. Ushijima of Tokyo University of Agriculture and Technology for his interest and encouragement.

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ホウレンソウ葉の細胞微細構造に対するオゾンと二酸化硫黄の作用の相違

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ポット植えにしたホウレンソウ（*Spinacia oleracea* L. cv. New Asia）に，0.5ppmのオゾン（O_3）及び1.0ppmの二酸化硫黄（SO_2）を単独または同時に暴露した。暴露開始後時間を追って葉組織を採集し，組織・細胞構造の変化を光学顕微鏡及び透過型電子顕微鏡で観察した。

O_3単独暴露では最初に葉緑体のシラコイドの膨潤が観察された。次にゴルジ体，小胞体，核膜に膨潤が認められ，ミトコンドリアにはクリステの収縮が観察された。その後葉緑体の変形が観察された。これに対しSO_2単独暴露では最初に葉緑体のストロマの膨潤，これに伴う葉緑体の変形が観察された。シラコイドの膨潤が現れたのは暴露開始後かなり時間が経ってからであった。葉にしおれが現れ，一部に褐変が認められる頃には，O_3，SO_2いずれの暴露処理においても，破壊された細胞内構造体が凝集し細胞がつぶれてゆくのが観察された。O_3とSO_2を同時に暴露すると，障害の進行は著しく促進された。このとき細胞内には主にSO_2障害の特徴が現れていた。

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Res. Rep. Natl. Inst. Environ. Stud., Jpn., No. 65, 1984.

Inhibition of Photosynthesis of Poplar Species and Sunflower by O_3 *

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Three poplar species (I-214: *Populus euramericana* cv. I-214, FS-51: *P. maximowiczii* × *plantierensis*, and Peace: *P. koreana* × *trichoarpa*) and sunflower (*Helianthus annuus* L. cv. Russian Mammoth) differing widely in their foliar susceptibility to ozone were exposed to various concentration of ozone for 2 h in a cylindrical assimilation chamber. The rates of net photosynthesis and transpiration were measured simultaneously during and after the exposure to ozone and the diffusion process of CO_2 was estimated. The foliar susceptibilities of these three poplar species did not reflect the photosynthetic susceptibilities to ozone. Although I-214 shows a very resistant foliar response to ozone as compared with the sensitive Peace, there was no detectable differences in responses of photosynthesis of these two poplar species to ozone. However, the diffusion resistances of CO_2 through the mesophyll cell layer of I-214 and Peace responded differently to ozone. The inhibition of net photosynthetic rates of Peace was attributed solely to the increase in the mesophyll diffusion resistance, while for FS-51 and I-214, stomatal closure was also a factor inducing the reduction of net photosynthetic rates.

Ozone is a major phytotoxicant present in photochemical smog. About a decade ago, a major air pollutant in the urban district in Japan was sulfur dioxide and many investigators concentrated their effort to clarify the effect of sulfur dioxide on plant. In these days, the concentration of sulfur dioxide decreased to the level at which no foliar injury could be

* Some of these results were published in Jpn. J. For. Soc.,65,321–326 (1983).

detected by a single application of sulfur dioxide. In contrast to sulfur dioxide, the concentration of O_3 is high enough to produce the acute foliar necrosis on many susceptible plant species (Furukawa, 1984).

Photosynthesis is a very sensitive physiological process to O_3 and the reduction of photosynthesis occur well before the visible injury on leaves becomes detectable. Heritable differenses of photosynthesis to O_3 is also evident (Hill & Littlefield, 1969). Although the general feature of photosynthesis inhibition caused by O_3 is fairly understood (Heath, 1981), little information is available concerning sensitivity among plant species or cultivars.

Several investigators have paid their attention to the leaf stomata for O_3 injury because the major pathway of gas flux, including O_3, into leaf tissue occurs through stomata. However, stomatal aperture or gas diffusive resistance is not always correlated with the sensitivity to O_3. Ting and Dugger (1968) reported that the age-dependent sensitivity of cotton leaves to O_3 was not correlated with the diffusive resistance of stomata. A further evidence was reported by Harris and Heath (1981) that the resistant and sensitive cultivars of *Zea mays* had similar diffusive resistances of stomata. The present work is therefore concerned with the heritable differences in photosynthetic responses to O_3 and the role of stomatal aperture in susceptibility to O_3. In general, as herbaceous species are considered to be more sensitive to air pollutants than woody species, we selected sunflower as a typical herbaceous species to compare the photosynthetic sensitivity to O_3 with poplar species.

Materials and Methods

Plant materials

Cuttings of three poplar species (I-214: *Populus euramericana* cv. I-214, FS-51: *P. maximowiczii* x *plantierensis*, and Peace: *P. koreana* x *trichocarpa*) and seedlings of sunflower (*Helianthus annuus* L. cv. Russian mammoth) were grown at 25°C and a relative humidity of 70% in a phytotron greenhouse. Plants were cultivated in plastic pots (11 cm diameter, 15 cm deep) filled with a mixture of vermiculite, perlite, and gravel (2:2:1:1, v/v). Each pot contained 5 g Magamp-K and 15 g of magnesia lime. Cuttings of poplar species and seeds of sunflower were propagated at the nursery of National Institute for Environmental Studies. Cuttings were harvested from field-grown poplar trees after the dormant buds were formed. Before planting in pots, cuttings were stored in a refrigerator for at least a month to break the dormancy. Potted plants were watered dialy and with Hyponex solution ($1 g l^{-1}$) once a week.

Fumigation system

The fumigation was performed in an assimilation chamber (50 cm diameter, 20 cm high) which was set in a controlled environment room. The assimilation chamber was made of black vinyl chloride cylinder. The inside wall of the chamber was covered with 7.5 mil semitransparent Teflon (FEP) film. The top of the chamber was covered with 3 mil transparent Teflon (FEP) film. The transmittance of light through the transparent Teflon film was ca. 95%. Illumination system for the controlled environment room was applied for the light source of the assimilation chamber. Illumination system was consisted of twenty four 400 W stannous halide lamps (Yoko Lamp, Toshiba). The light was filtered through heat absorbing glass filter, which removed radiation above 800 nm. The quantum flux density inside the assimilation chamber was 500 μEinstein m^{-2} s^{-1} as measured by a LI-COR LI-190SB quantum flux sensor. Prior to the fumigation, plant was illuminated for more than one hour to open stomata. Ozone

was generated by a silent electrical discharge in dry oxygen. Ozone concentrations within the controlled environment room were monitored continuously by a Kimoto Model 806 chemiluminescent detector of O_3. The outputs from the analyzer were fed to an analogous recorder equipped with a PID controller regulating the pollutant flow through mass flow controllers.

Gas exchange measurement

Fully expanded leaves were accomodated into the assimilation chamber. The stem was led through a port in the bottom of the chamber so that the leaves were inside and the roots and pot were outside. A small impeller (10 cm diameter) which was placed on the bottom stirred the chamber air. Air was continuously sucked by a pump through the suction pipe on the upper side of the chamber. Air flow rate was monitored by a rotameter and was adjusted to 30 l min^{-1}. Wind speed inside the chamber was 0.4 m s^{-1} as measured with a multi-directional hot wire anemometer. Wind speed of this magnitude minimized the boundary layer diffusive resistance to water vapor and CO_2 transfer. Using wet filter paper of similar size and shape as the leaves, the boundary layer resistance to water vapor transfer was determined and was 0.06 to 0.1 s cm^{-1}.

Transpiration rate was determined by measuring water vapor concentrations of the air entering and leaving chamber using thermocouple psychrometer. The rate of O_3 uptake of leaves was determined by measuring O_3 concentrations at the inlet and outlet of chamber. Surfaces of chamber, tubing, tube fitting, solenoid valves, and pumps which came in contact with O_3 were all composed of Teflon to minimize the adsorption or decay of O_3. Without plant materials, no apparent uptake of O_3 by measuring circuit system could be detected. Net photosynthetic rate was determined in an open circuit system by measuring CO_2 concentrations at the inlet and outlet of chamber with an infra-red CO_2 analyzer (Fuji, Model ZAP).

Estimation of diffusive resistance

Because photosynthesis and transpiration were measured simultaneously in the assimilation chamber, diffusive resistances to CO_2 transfer from the bulk air to the site of CO_2 fixation could be determined. Resistances to CO_2 diffusion through boundary layer and internal gas phase of the leaf, and from the surface of mesophyll cells to the site of CO_2 fixation were calculated from the rates of net photosynthesis and transpiration according to the method of Gaastra (1959):

$$J_{cd} = (c_{cd}^{air} - c_{cd}^{ic})/(r_{cd}^{gas} + r_{cd}^{liq})$$
$$J_{wv} = (c_{wv}^{ic} - c_{wv}^{air})/r_{wv}^{gas}$$

where J_{cd} and J_{wv} are net CO_2 uptake and transpiration rates, respectively, c is the concentration of CO_2 (c_{cd}^{air}) and water vapor (c_{wv}^{air}) of the bulk air, or at the site of CO_2 fixation (c_{cd}^{ic}: assumed to be the CO_2 compensation point) and the concentration of water vapor at the transpiring site (c_{wv}^{ic}: assumed to be the saturation water vapor concentration at leaf temperature). Diffusion coefficient of CO_2 can be related to that of water vapor (Jarvis, 1971), so that the conversion factor of 1.65 was applied for the calculation of liquid phase diffusive resistance (r_{cd}^{liq}) using gaseous phase diffusive resistances of CO_2 (r_{cd}^{gas}) and water vapor (r_{wv}^{gas}).

Results

Photosynthetic characteristics in popplar species and sunflower

Differences in gas exchange characteristics found among non-treated poplar species and

sunflower are presented in Table 1. The gas exchange characteristics of sunflower were determined to compare the characteristics of three poplar species, since both sunflower and poplar are C_3 species (Furukawa, 1972) and sunflower shows a relatively higher rate of net photosynthesis among C_3 species (Furukawa, 1981).

Table 1 CO_2 exchange characteristics of poplar species and sunflower

	Poplar Species			Sunflower
	Peace	I-214	FS-51	
J_{CO_2}, $mgCO_2 \cdot m^{-2} \cdot s^{-1}$	0.48 ± 0.02	0.43 ± 0.01	0.52 ± 0.01	0.71 ± 0.02
J_{WV}, $mgH_2O \cdot m^{-2} \cdot s^{-1}$	32 ± 2	31 ± 2	35 ± 2	46 ± 3
$r^{gas}_{CO_2}$, $s \cdot cm^{-1}$	3.0 ± 0.3	3.1 ± 0.4	2.8 ± 0.2	1.3 ± 0.1
$r^{liq}_{CO_2}$, $s \cdot cm^{-1}$	9.2 ± 0.6	10.3 ± 0.7	8.4 ± 0.4	6.9 ± 0.3

Sunflower plant had a higher rate of light saturated photosynthesis than any poplar species tested in the present work. Resistance analysis for the three poplar species and sunflower were made. In the present report, we divided the resistances to CO_2 diffusion from the bulk air to the site of CO_2 fixation into two components: one is gaseous phase diffusive resistance (r^{gas}_{cd}) and the other is liquid phase resistance (r^{liq}_{cd}). The boundary layer resistance (r^{bl}_{cd}) and stomatal resistance (r^{stm}_{cd}) are the components of r^{gas}_{cd}, but r^{bl}_{cd} was very small and fairly constant among species listed in Table 1. As a result, the differences in r^{gas}_{cd} was primarily due to the differences in r^{stm}_{cd}.

Higher rate of photosynthesis in sunflower plant was in part due to the lower r^{gas}_{cd}. However, even when r^{gas}_{cd} of sunflower increased from 1.3 s cm^{-1} (the real value) to 3.1 s cm^{-1} (the value for 1-214), the calculated rate of net photosynthesis in sunflower (0.59 $mgCO_2$ m^{-2} s^{-1}) was still higher than that of any poplar species. Thus another diffusive resistance, r^{liq}_{cd} should also be related to the efficiency of CO_2 fixation in these species. In the liquid phase, the resistance to CO_2 diffusion is determined by barriers of cell walls, plasmalemmas, cytoplasm, chloroplast membranes, and the resistance associated with the carboxylation reaction. If r^{liq}_{cd} of sunflower is assumed to be that of I-214, the calculated rate of net photosynthesis in sunflower was 0.50 $mgCO_2$ m^{-2} s^{-1}, comparable to the photosynthetic rates of poplar species. These results suggest that the higher rate of net photosynthesis in sunflower could be resulted from lower r^{liq}_{cd}.

Effect of O_3 on net photosynthesis and transpiration

Figure 1 A–D represent the time course of the effects of O_3 on the rates of net photosynthesis and transpiration of three poplar species and sunflower. The rate of O_3 uptake is also plotted in each subfigure. The pattern of changes in photosynthesis and transpiration rates during the course of O_3 treatment varied among plant species. For two polar species, I-214 and FS-51, the decline of net photosynthetic rates occurred nearly simultaneously with the decline of transpiration and O_3 uptake rates (Fig. 1A & 1B). For sunflower plants, the oscillations of net photosynthesis and transpiration were induced by the exposure to O_3 (Fig. 1D). In contrast to these simultaneous inhibition of net photosynthesis and transpiration by O_3, Peace species showed quite different responses to O_3 (Fig. 1C). The decrease in

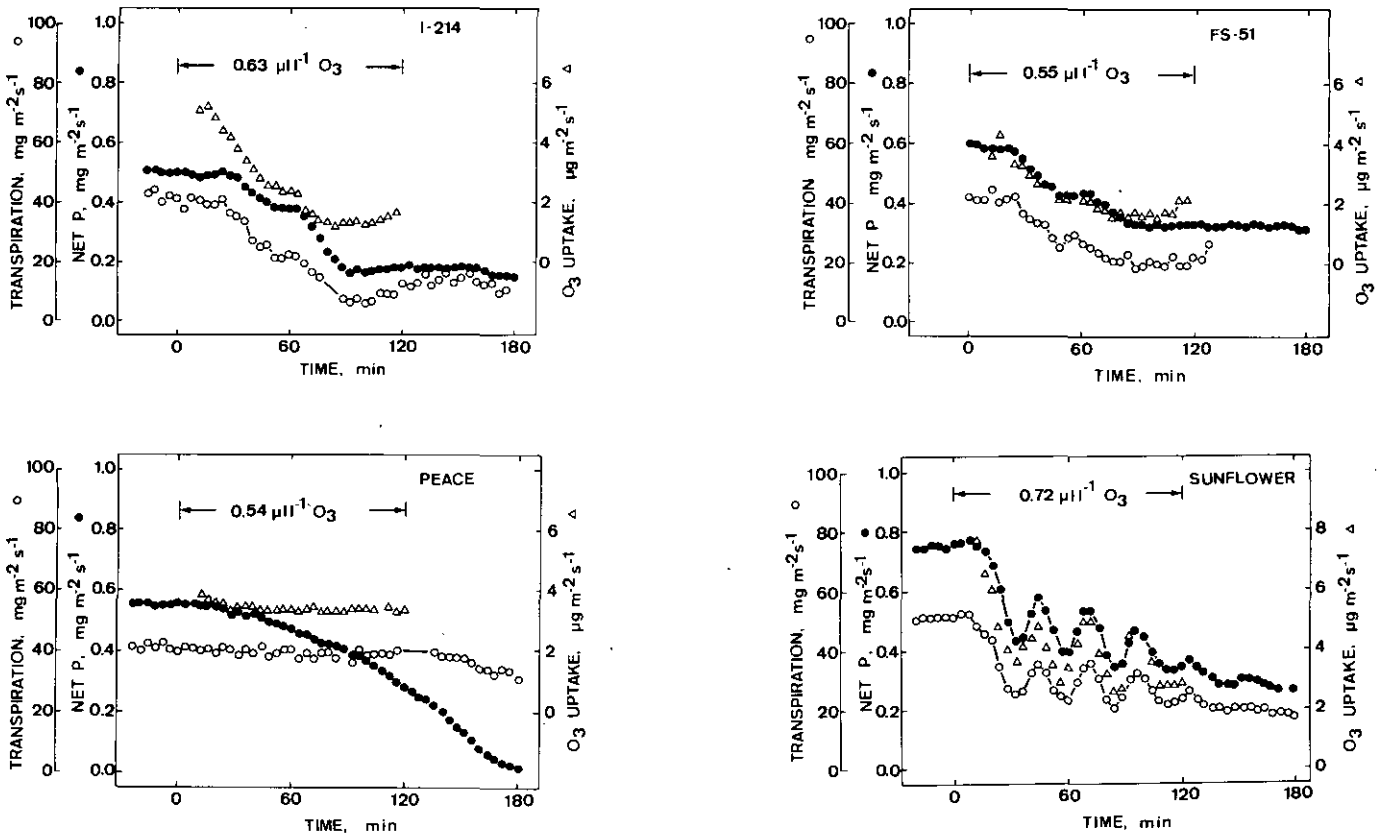

Fig. 1 Changes of rates of net photosynthesis, transpiration, and O_3 uptake of I-214 (A), FS-51 (B), Peace (C) poplars and sunflower (D) during and after the exposure to O_3

transpiration rate in this poplar species could not be observed during the course of O_3 treatment. Even after the 2-h treatment period, the rate of transpiration of Peace species was not inhibited by 0.54 μl/l O_3, while the rate of net photosynthesis declined to 50% of the pre-treatment rate.

The recovery of net photosynthesis and transpiration from the inhibition caused by O_3 could not be detected for 1 h after the termination of O_3 treatment in any poplar species tested in the present experiment. After the termination of O_3 treatment, no further decrease in either photosynthesis or transpiration in I-214 or FS-51 was detected. For sunflower plants, oscillations of net photosynthesis and transpiration were diminished and could not be detected during the post-treatment period. On the contrary, the decline of net photosynthetic rate in Peace species was not stopped by the termination of O_3 treatment. After the termination of ozone treatment, a continuous decline in net photosynthetic rate was noted, and the rate decreased to 0% of the pre-treatment rate within 1-h of post-treatment period.

The extent that net photosynthesis was inhibited by O_3 is shown in Fig. 2. Changes in net photosynthetic rates following exposures to different concentrations of O_3 for 2 h, are calculated as percentages of the respective pre-established rates. Two-hour plant exposures to about 0.2 μl l^{-1} O_3 were required before detecting the clearly measurable inhibition of net photosynthetic rates in Peace, I-214 and sunflower. Furthermore, photosynthesis inhibitions of Peace at various O_3 concentrations followed patterns similar to those observed in I-214. For FS-51 plants, data were scattered from plant to plant. The threshold O_3 concentration in resistive FS-51 plants (identified from data) was 0.6 μl l^{-1}. The threshold concentration was obtained from the hand fitted line drawn in the figure. Then, FS-51 plants are presumably photosynthetically resistive to ozone as compared with Peace plants.

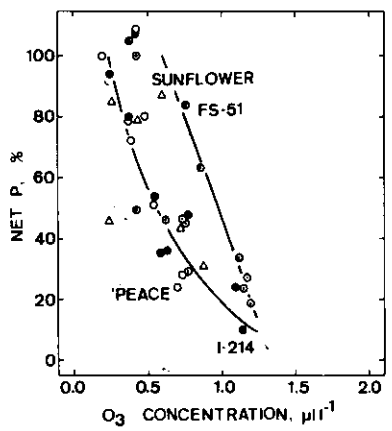

Fig. 2 Relative rates of net photosynthesis in I-214, FS-51, Peace poplars and sunflower as a function of O_3 concentrations

Concentrations of O_3 were the concentration outgoing from the assimilation chamber. Data are obtained from the 2-h exposures to various concentrations of O_3.

Effects of O_3 on CO_2 diffusion process

According to Hill & Littlefield (1969), the decline of net photosynthesis induced by O_3 exposure is attributable to stomatal closure for oat and barley. Simultaneous decline of net

photosynthesis, transpiration and O_3 uptake (an indirect indicator of stomatal behavior [Omasa *et al.*, 1979]) in I-214, FS-51 and sunflower (Fig. 1) may support their idea that the suppression of net photosynthesis is resulted from stomatal closure induced by O_3. To confirm this idea, the percent inhibition of net photosynthesis was plotted against the percent inhibition for three poplar species and sunflower (Fig. 3). The inhibition of net photosynthesis induced by 2-h exposures to various concentrations of O_3 was comparable with the transpiration depression in I-214, FS-51 and sunflower. However, for Peace, the inhibition of net photosynthesis was caused by O_3 with a slight decline in transpiration rate. Only about 10% reduction of transpiration rate in Peace was observed even when plants were exposed to 0.73 $\mu l\ l^{-1}$ O_3 for 2 h.

In order to determine the degree to which net photosynthesis was affected by stomatal behavior during O_3 treatment, diffusion of CO_2 through stomata was calculated and compared with the CO_2 diffusion through mesophyll cell layer (Fig. 4). The effect of O_3 on r_{cd}^{gas} was quite different among poplar species. For Peace, r_{cd}^{gas} remained low and fairly constant over O_3 concentrations applied in the present experiment, while for I-214 and FS-51, r_{cd}^{gas} increased correspondingly with the concentration of O_3. Contrary to the behavior of r_{cd}^{gas}, O_3 can affect r_{cd}^{liq} in all poplar species. The pre-exposure values of r_{cd}^{liq} for three poplar species were similar and were below 10 s cm^{-1}, but r_{cd}^{liq} reached a value of 38 for Peace, 22 (estimated from the curve) for I-214, and 13 s cm^{-1} for FS-51 by 0.75 $\mu l\ l^{-1}$ O_3. The threshold concentration of O_3 for the inhibition of net photosynthesis was apparently identical for Peace and I-214 and was ca. 0.2 $\mu l\ l^{-1}$ (Fig. 2). However, the effect of O_3 on r_{cd}^{liq}, which may reflect the alterations in availability of metabolic intermediates or in enzyme, differed between species. In Peace, r_{cd}^{liq} rose whenever O_3 concentration increased. In I-214, r_{cd}^{liq} remained constant until O_3 concentration increased to 0.4 $\mu l\ l^{-1}$. In FS-51, although data were scattered, r_{cd}^{liq} remained constant upto 0.5 to 0.6 $\mu l\ l^{-1}$ and then increased.

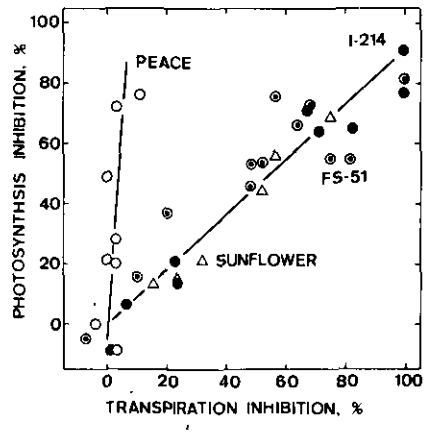

Fig. 3 Relationship between percent inhibition of net photosynthesis and that of transpiration for I-214, FS-51, Peace poplars, and sunflower

Data are obtained from the 2-h exposures to various concentrations of O_3.

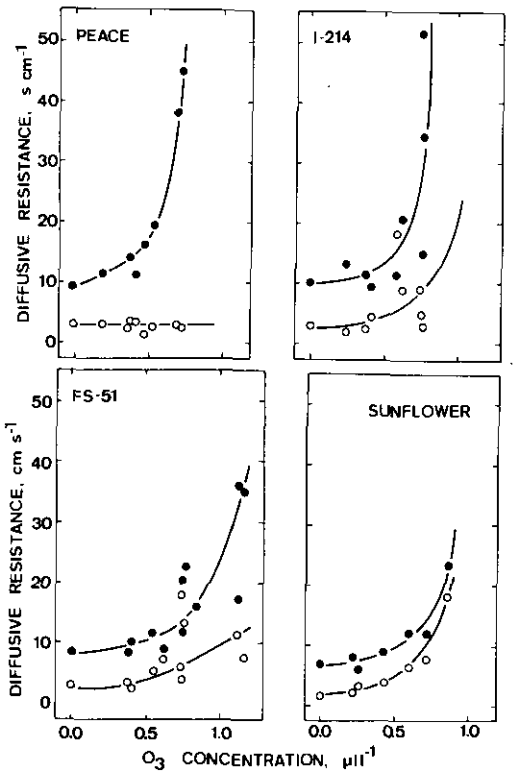

Fig. 4 Gaseous and liquid phase diffusive resistances for CO_2 transfer of I-214, FS-51, Peace poplars and sunflower as a function of O_3 concentration

Concentrations of O_3 were the concentration outgoing from the assimilation chamber. Data are obtained from the 2-h exposures to various concentrations of O_3.

Discussion

Three poplar species, Peace, I-214, and FS-51, and sunflower were treated with various concentrations of O_3 and rates of net photosynthesis and transpiration were determined to elucidate the heritable differences in physiological responses of plants to O_3. Our previous results (Furukawa *et al.*, 1981) have shown that three species differ markedly in susceptibility to O_3. Judging from the degree of folia injury induced by O_3, the susceptibility of three poplar species are ranked in the following order: Peace > FS-51 > I-214. On the basis of this ranking, we selected these species and expected the marked differences in photosynthetic susceptibility among these three poplar species. Although, herbaceous plants are considered to be more sensitive to O_3 as compared with tree species, sunflower responded photosynthetically similar to poplar species. However, the present results show that photosynthesis of different species did not responded to O_3 differently. Although the difference in the degree of foliar injury between Peace and I-214 is remarkable, the relationship between the concentration of O_3 and the

photosynthetic inhibition was similar between these two species. These results may suggest that the foliar sensitivity to O_3 is quite different from the photosynthetic sensitivity. The response of net photosynthesis of FS-51 to O_3 varied from plant to plant and the degree of inhibition of net photosynthesis was scattered. Despite this plant-to-plant variability in FS-51, the percent inhibition of net photosynthesis had a significant correlation with the percent inhibition of transpiration. This result may suggest that the scatter of photosynthetic inhibition for FS-51 is mainly due to the plant-to-plant variability of the response of stomata to O_3. Although the foliar susceptribility of plants to O_3 is not a reflection of stomatal aperture as suggested by our previous results (Furukawa *et al.*, 1981) and the work of Dugger *et al.*, (1962), photosynthetic susceptibility may depend upon stomatal resistance because it must be assumed that both O_3 and CO_2 leaving and entering leaves passes through stomata.

The mechanism which determines the effect of O_3 bringing a reduction in photosynthetic rates is undoubtedly complex. By the exposure to O_3, stomatal closure occurs (Hill & Littlefield, 1969), the CO_2 compensation point is enhanced (Furukawa & Kadota, 1875), photosynthetic electron transport is inhibited (Coulson & Heath, 1974), and rates of respiration may increase or decrease (Todd, 1958; Furukawa & Kadota, 1975). The parallel decline in net photosynthesis and transpiration rates shown in Fig. 3 strongly indicates stomatal closure as the principal causal factor in O_3 effect mediated reduction in net photosynthesis. However, the fact that r_{cd}^{liq} increased with increasing concentration of O_3 for any poplar species and sunflower indicates the possibility that a non-stomatal factor was also responsible for the reduction in net photosynthesis. Furthermore, the result of Peace shows an increase in r_{cd}^{liq} in the O_3 concentration range of 0.2 to 0.4 μl/l which is considered to be a relatively low O_3 concentration. In Peace, r_{cd}^{gas} was not altered by O_3, presumably because stomatal apertures of Peace were not sensitive to O_3. In Peace, therefore, the diffusive resistance of mesophyll (r_{cd}^{liq}) appeared to be the primary factor limiting net photosynthesis during O_3 exposure.

Peace, which showed an insensitive stomatal response to O_3, had a similar photosynthetic tolerance to O_3 to I-214, which showed a very resistive behavior to O_3. Nevertheless, since mesophyll diffusive resistance of Peace began to increase at lower concentration than that of I-214, and since the photosynthetic rates of Peace continued to decrease even after the termination of O_3 exposure, it should be concluded that Peace is the most sensitive species to O_3 in respect of not only foliar injury but also photosynthetic inhibition. These characteristics of Peace suggest the ability of an indicator plant for assessing the impact of photochemical oxidants on vegetation.

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オゾン処理によるポプラ品種とヒマワリの光合成阻害

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可視害によって評価したオゾン感受性が著しく異なるポプラ3種（I-214： *Populus euramericana* cv. I-214, FS-51： *P. maximowiczii* × *plantierensis*, Peace： *P. koreana* × *trichocarpa*）とヒマワリ（*Helianthus annuus* L. cv. Russian Mammoth）の光合成に対するオゾンの影響を調べた。植物葉を種々のオゾン濃度に2時間処理し，純光合成速度，蒸散速度をオゾン処理前，処理中，処理後に同時測定した。CO_2拡散抵抗は，光合成，蒸散速度から計算して求めた。可視害の程度から判別したポプラのオゾン感受性はオゾンによる光合成阻害の程度を反映するものではなかった。ヒマワリの光合成のオゾンに対する感受性もポプラのそれとは大差なかった。可視害によって評価するならば，I-214は最も抵抗性が高く，Peaceが最も低かった。しかし，I-214とPeaceのオゾン感受性は，光合成阻害によって評価するならば，ほとんど差はなかった。しかし，オゾン処理によるPeaceの光合成速度の低下は，第一義的に葉肉組織拡散抵抗の増加によっているのに対して，I-214，FS-51，ヒマワリでは気孔拡散抵抗の増加と葉肉組織拡散抵抗の増加が同時に起こり，光合成速度の低下をもたらすものであった。

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Res. Rep. Natl. Inst. Environ. Stud., Jpn., No. 65, 1984.

The effects of NO_2 and/or O_3 on photosynthesis of sumflower leaves

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Photosynthesis and transpiration rates were simultaneously measured in attached leaves of sunflower (*Helianthus annuus* L. cv. Russian Mammoth) during the exposure to NO_2 and/or O_3 to determine the mixed gas effect on CO_2 diffusion processes. CO_2 diffusive resistances were divided into two components; gaseous phase diffusive resistances (r_{cd}^{gas}), including stomatal and boundary layer diffusive resistances, and liquid phase, or in other word, mesophyll diffusive resistance (r_{cd}^{liq}).

The application of NO_2 alone caused a marked reduction of net photosynthesis with no significant reduction of transpiration, indicating that NO_2 affected CO_2 fixation process with no influence on stomatal aperture. Contradictory, the application of O_3 alone reduced both net photosynthesis and transpiration rates, however, from the estimation of CO_2 diffusive resistances, a main cause affecting photosynthesis reduction during O_3 exposure was not r_{cd}^{gas} but r_{cd}^{liq}.

When the concentration of each gas was below the threshold to inhibit transpiration by either gas alone, the greater than additive reduction of transpiration rate was observed by mixing each gas. In contrast, photosynthetic response to the mixture of NO_2 and O_3 was changed from the additive to the less effect than additive with increasing concentrations of NO_2. In any combination of the mixture, r_{cd}^{gas} and r_{cd}^{liq} increased concurrently.

Key words: Antagonistic effect, Diffusive resistance, Nitrite accumulation, NO_2 and/or O_3, Photosynthesis, Sunflower

The responses of plants to air pollutant mixture are frequently categorized for the convenience into three types: additive, greater than additive (*synergistic*) and less than additive (*antagonistic*). Menser and Heggestad (1966) first noted that mixtures of SO_2 and O_3 caused the visible injury to tobacco plants at the concentration of each air pollutant below the threshold for foliar injury. This was interpreted as *synergistic* interaction between SO_2 and O_3. Similar results have been reported for other combinations of air pollutants. Tingey *et al.* (1971) and Skelly *et al.* (1972) have also shown the evidence of synergism between SO_2 and

NO_2 in their combined effects on plants and the decline of the threshold concentrations above which the injury caused by either gas became apparent. Tingey *et al.* (1973) reported that foliar injury resulting from mixtures of O_3 and SO_2 was dependent on plant species investigated and the gas concentrations used.

All these studies cited above have only used visible foliar injury as a criterion for estimating the effect of pollutants on plants. Plant growth (the increase in dry weight) is the consequnce of the accumulation of photosynthates. Thus it may be conceivable that the possible effects of air pollutants on growth and development should be reexamined in relation to photosynthesis. However, the bulk of research carried out on the effects of air pollutant mixtures on plants has paid little attention to their effects on photosynthetic CO_2 fixation. White *et al.* (1974) reported that photosynthetic rates of alfalfa plants were *synergistically* inhibited by the exposure to NO_2 and SO_2 mixtures. Furukawa and Totsuka (1979) also demonstrated the *synergistic* inhibition of photosynthesis in sunflower by NO_2 and O_3, NO_2 and SO_2, and SO_2 and O_3 mixtures. The present study was, therefore, initiated primarily to distinguish the effects of NO_2 and O_3 mixtures from the effects of NO_2 or O_3 on photosynthesis and transpiration of sunflower.

Materials and Methods

Plant materials

Seedlings of sunflower (*Helianthus annuus* L. cv. Russian Mammoth) were grown at 25°C and a relative humidity of 70% in a phytotron greengouse. Plants were cultivated for four weeks in plastic pots (11 cm diameter, 15 cm deep) filled with a mixture of vermiculite, perlite, and gravel (2:2:1:1, v/v). Each pot contained 5 g Magamp-K and 15 g of magnesia lime. No additional nutrient was supplied to increase the susceptibility to NO_2 (Srivastava *et al.*, 1975c) and to stimulate the accumulation of nitrite in NO_2-treated leaves (Yoneyama *et al.*, 1979).

Fumigation system

Plants were exposed to NO_2 and/or O_3 using an acrylic assimilation chamber (125 liter, cubic) which was set inside a controlled environment room (1.7 X 2.3 X 2.0 m high). The field air was passed in succession through activated charcoal and catalyst bearing (containing MnOx and CuO) filters to remove ambient air pollutants and led into the controlled environment room. This filtration system could remove O_3 and SO_2 almost perfectly, but a trace amount of NO_2 (below 5 nl l^{-1}) was remained in the room. NO_2 gas from a compressed cylinder containing 2 ml l^{-1} NO_2 (at 25°C) in N_2 was injected through a solenoid valve into the air stream. The concentration of NO_2 in the room was regulated by a thermal mass-flow controller equipped with a controlling system of a chemiluminescent NO-NO_2-NO_x analyzer (Thermo Electron, Model 14). Ozone was generated by a silent electrical discharge in dry oxygen and regulated by a system similar with that described for NO_2 using a controlling system of a chemiluminescent O_3 analyzer (Kimoto, Model 806). Recordings of pollutant concentrations inside the room showed that on starting a fumigation, the concentration reached 90 % of the fixed level within 5 min. The concentrations of pollutants could be regulated within ±1 % of the desired levels.

Gas exchange measurement

Fully expanded leaves were accomodated into the assimilation chamber. The stem was led through a port at the bottom of the chamber, so that the leaves were inside and the roots and pot were outside. Measurement was performed at 28 ± 0.5°C, 75 % R. H. Two small fans (10 cm diameter) which were placed on the inner wall stirred the chamber air. Air was continuously sucked by a pump through the suction pipe on the upper side of the chamber. Air flow rate was measured by a rotameter and was adjusted to 3.3 $m^3\ h^{-1}$. Wind speed inside the chamber was 0.4 $m\ s^{-1}$ as measured with a multi-directional hot wire anemometer. Wind speed of this magnitude minimized the boundary layer diffusive resistance to water vapor and CO_2 transfer. Using wet filter paper of similar size and shape to the leaves, the boundary layer resistance to water vapor transfer was determined and was 0.06 to 0.1 $s\ cm^{-1}$.

Illumination system for the controlled environment room was applied for the light source of the assimilation chamber. Illumination system was consisted of twenty four 400 W metal halide lamps (Yoko Lamp, Toshiba). The light was filtered through heat absorbing glass filter, which removed radiation above 800 nm. The quantum flux density inside the assimilation chamber was 500 μEinstein $m^{-2}\ s^{-1}$ as measured with a quantum flux sensor (LI-COR, Model LI-190 SB). Transpiration rate was determined by the gravimetric method using an electronic top-loading balance (Mettler, Model PL-3000). Transpirational water loss was continuously recorded with a thermal data acquisition system. Pot was enclosed in a plastic bag to prevent evaporation of water from pot surface. Net photosynthetic rate was determined in an open circuit system by measuring CO_2 concentrations at the inlet and outlet of the chamber using an infra-red CO_2 analyzer (Shimazu, Model URA-2S).

Estimation of diffusive resistance

Because photosynthesis and transpiration were measured simultaneously in the assimilation chamber, diffusive resistances to CO_2 transfer from the bulk air to the site of CO_2 fixation could be determined. The resistances to CO_2 diffusion through the boundary layer and internal gas phase of the leaf, and from the surface of the mesophyll cells to the site of CO_2 fixation were calculated from the rates of net photosynthesis (J_{cd}) and transpiration (J_{wv}) according to the method of Gaastra (1959):

$$J_{cd}=(c_{cd}^{air} - c_{cd}^{chl})/(r_{cd}^{gas}+ r_{cd}^{liq}) \quad (1)$$

$$J_{wv}=(c_{wv}^{ic} - c_{wv}^{air})/r_{wv}^{gas} \quad (2)$$

where c_{cd}^{air} and c_{cd}^{chl} are CO_2 concentrations of the bulk air and at the site of CO_2 fixation (assumed to be the CO_2 compensation point), respectively; c_{wv}^{ic} and c_{wv}^{air} are the saturation water vapor concentration at leaf temperature and the water vapor concentration of the bulk air, respectively. The diffusion coefficient of CO_2 can be related to that of water vapor (Jarvis, 1971), so that the conversion factor of 1.65 was applied to convert r_{wv}^{gas} into r_{cd}^{gas}. During the exposure to O_3, the boundary layer resistance was held constant by the constant flowing and circulation of the air. As a result, r_{cd}^{gas} could solely be related with r_{cd}^{stm} (stomatal diffusive resistance.) Although the exposure to 0.9 $\mu l\ l^{-1}$ O_3 for 90 min induced the increase in CO_2 compensation points in poplar leaves from 50 to 70 $\mu l\ l^{-1}$ (Furukawa & Kadota, 1975), the CO_2 compensation point in sunflower leaves was fixed to 50 $\mu l\ l^{-1}$ (adopted from Furukawa, 1975). The error introduced by this calculation is less than 10 % of r_{cd}^{liq}, even when the CO_2 compensation point increases from 50 to 70 $\mu l\ l^{-1}$. In the present study, leaf temperature was not measured simultaneously with transpiration, since the changes in leaf temperature during

the treatment with air pollutants differed markedly among portions of leaf surface (Omasa & Abo, 1978). Using the relationship between the rate of transpiration and leaf-air temperature differences in sunflower leaves (Omasa *et al.*, 1978), we estimated the leaf temperature for the calculation of c_{wv}^{ic} as follows:

$$T^{l} = -6.4 \times 10^{5} + J_{wv}\ T^{air} + 2.2 \qquad (3)$$

where T^{l} and T^{air} are leaf and air temperatures, respectively.

Statistical analysis

To test if there was a significant differences between two combinations of pollutants, the *F* statistics was applied. If the difference was not significant at 95 % confidence interval, the effect was desiganted as the additive effect. On the other hand, if the difference was significant at the same confidence interval, the effect was noted as the greater or less than additive effect.

Results and Discussion

Effect of O_3

The 2-h exposure to 0.2 $\mu l\ l^{-1}$ O_3 had no significant effects on either net photosynthesis or transpiration in sunflower leaves (Fig. 1A & 1B). The photosynthetic rate was reduced to 65 % of the pre-exposure rate by 2-h exposure to 0.4 $\mu l\ l^{-1}$ O_3, while the transpiration rate was reduced to ca. 75 %. Under the present experimental conditions, we could not observe the O_3-induced acute foliar necrosis.

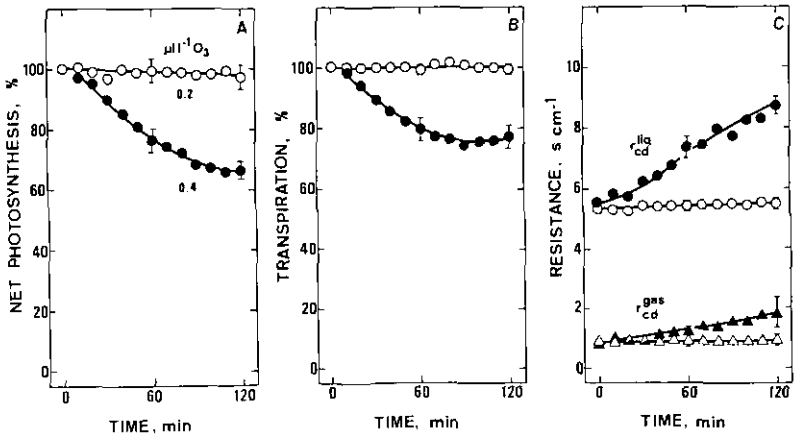

Fig. 1 Effects of 0.2 and 0.4 $\mu l\ l^{-1}$ O_3 on net photosynthesis (A), transpiration (B), and CO_2 diffusive resistances (C) in sunflower leaves

Rates of net photosynthesis and transpiration are expressed as the percentages of the pre-exposure rates. Gas-phase (r_{cd}^{gas}) and liquid-phase (r_{cd}^{liq}) diffusive resistances were estimated using data of net photosynthesis and transpiration rates. Concentrations of O_3 were shown in each subfigure. Each point is the mean of at least three replicates. Error bars indicate representative standard deviations.

Hill & Littlefield (1969) suggested that O_3 may cause photosynthesis inhibition by inducing stomatal closure. Their suggestion seems to be adequate, since the present results also show that the deline of net photosynthesis was nearly comparable with that of transpiration, which is an indirect indicator of stomatal aperture (Gaastra, 1959). However, there is a reported result that the CO_2 compensation point is enhanced by O_3 (Furukawa & Kadota, 1975), suggesting that photosynthetic inhibition is primarily related to nonstomatal factors. Thus, it is necessary to distinguish between the resistance to CO_2 transfer through stomata and mesophyll cell layer, because these two factors limit net photosynthesis at high light intensities used in the present experiment (Zelitch, 1971).

Fig. 1C shows the effect of O_3 on r_{cd}^{gas} and r_{cd}^{liq}. The response of net photosynthesis to O_3 was largely reflected by the changes in r_{cd}^{liq}. If the inhibition of net photosynthetic rates results solely from stomatal closure, r_{cd}^{liq} should remain constant during the exposure to O_3. However, 0.4 ul l^{-1} O_3 caused r_{cd}^{liq} increase gradually from the start of exposure and finally it became 1.5 times of the initial value (Fig. 1C). The increase in r_{cd}^{gas} accounted for only 1 unit of total diffusive resistance at the end of the exposure while r_{cd}^{liq} accounted for 7 units. Therefore, r_{cd}^{gas} appeared to be the secondary cause for the O_3-induced decline of net photosynthesis while r_{cd}^{liq} was the primary one.

Effect of NO_2

Net photosynthesis was more sensitive to NO_2 than transpiration was (Fig. 2A & 2B). Inhibition of transpiration by NO_2 was much smaller than it was for O_3 for a given reduction in net photosynthesis. The exposure to 2 or 4 μl l^{-1} NO_2 for 2 h reduced the rate of net photosynthesis by 20 and 90 %, respectively, but no significant reduction in transpiration could be detected.

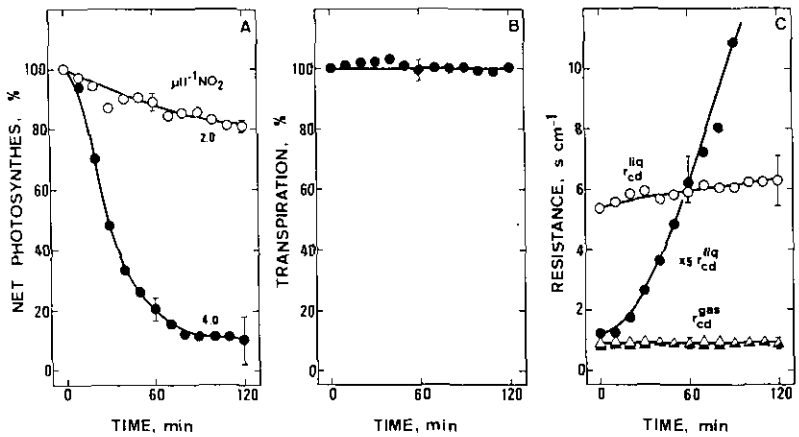

Fig. 2 Effects of 2.0 and 4.0 μl l^{-1} NO_2 on net photosynthesis (A), transpiration (B), and CO_2 diffusive resistances (C) in sunflower leaves

Concentratios of NO_2 were shown in each subfigure. Each point is the mean of at least three replicates. Error bars indicate representative standard deviations.

The reduction of net photosynthesis with no detectable reduction of transpiration is in agreement with the earlier reported results (Hill & Bennett, 1970; Srivastava *et al.*, 1975a, b). In a little difference with our results, Hill and Bennet (1970) reported that after an

appreciable reduction of net photosynthesis, the stomatal closure or the decrease in transpiration rate was observed and they suggested that the stomatal closure induced by NO_2 was caused by an increase in intercellular CO_2 concentration resulting from the inhibition of photosynthesis. However, we could not observe any reduction in transpiration or stomatal closure estimated from r_{cd}^{gas} (Fig. 2C), even when the net photosynthetic rate was reduced to 10 % of the initial rate. Consequently, though there is a little difference between our results and those reported by Hill and Bennett, the increase in r_{cd}^{liq} other than r_{cd}^{gas} had primarily affected photosynthetic response to NO_2, presumably as a result of the inhibition of photosynthetic CO_2 fixation process in chloroplasts (Hill & Bennett, 1970).

Effect of NO_2 and O_3 mixture

The reduction of net photosynthesis induced by the mixture of NO_2 and O_3 were equal to the additive reduction of the individual gases, when the concentration of NO_2 in the mixture was low (Fig. 3A). The treatment with a mixture of 2 μl l^{-1} NO_2 and 0.2 μl l^{-1} O_3 for 2 h reduced the rate of net photosynthesis to 77 % of the initial rate, which was not significantly different from summing the inhibition caused by each gas indicating the additive effect. The exposure to a mixture of 2 μl l^{-1} NO_2 and 0.4 μl l^{-1} O_3 also caused the additive reduction of net photosynthesis. In contrast to these additive effects, when the concentration of NO_2 in the mixture was high enough to inhibit net photosynthesis almost perfectly by NO_2 alone, the less than additive effect was observed. A mixture of 4 μl l^{-1} NO_2 and 0.2 μl l^{-1} O_3 produced less inhibition than the additive from the individual pollutants at the same concentration (Fig. 3A). The behavior of transpiration differed considerably from that of photosynthesis during the exposure to a mixture. A mixture of NO_2 and O_3 at any combination of concentrations of each gas produced a significantly greater effect than would be expected from summing the effects due to each gas alone (Fig. 3B & 4B).

Reinert *et al.* (1975) reported that the plant response to air pollutants mixture was influenced by the concentration ratio of each air pollutant in the mixture. However, the present results suggest that the response of plants to NO_2 and O_3 mixture was affected by the concentrations of each gas in the mixture rather than the concentration ratio of NO_2 to O_3. With increasing concentration of each gas, the greater than additive effect was reduced. When the concentration of each gas was high enough to affect photosynthesis or transpiration separately, the effect turned to be additive. The less than additive effect was observed only when the concentration of NO_2 was significantly high to inhibit net photosynthesis by the treatment with NO_2 alone. These findings were similar with those observed by White *et al.* (1974) in the mixed NO_2 and SO_2 effect on photosynthesis of alfalfa that the effect of NO_2 and SO_2 mixture changed from the greater than additive to the additive effect with increasing concentration of each gas in the mixture.

Because the reductions in transpiration and net photosynthesis occurred nearly simultaneously with the duration of exposure, it is necessary to evaluate the degree to which net photosynthesis is affected by stomatal closure. Fig. 3C and 4C show that both r_{cd}^{gas} and r_{cd}^{liq} increased just after the initiation of the mix treatment. The most significant increase in r_{cd}^{gas} was observed when leaves were treated with the mixture of 4 μl l^{-1} NO_2 and 0.2 or 0.4 μl l^{-1} O_3. For example, r_{cd}^{gas} was 3 s cm^{-1} before the exposure and increased by 2 s cm^{-1} by the treatment with a mixture of 4 μl l^{-1} NO_2 and 0.4 μl l^{-1} O_3 for 2 h, while r_{cd}^{liq} was 8 s cm^{-1} with a representative increase of 3 s cm^{-1}. The increase in r_{cd}^{liq} would reflect the alterations in the availability of metabolic intermediates or in enzyme levels, in as much as r_{cd}^{liq} is influenced by enzymatic activity (Zelitch, 1971). Thus these results indicate that both stomatal closure and photosynthesis inhibition occurred concurrently during the treatment with the mixture.

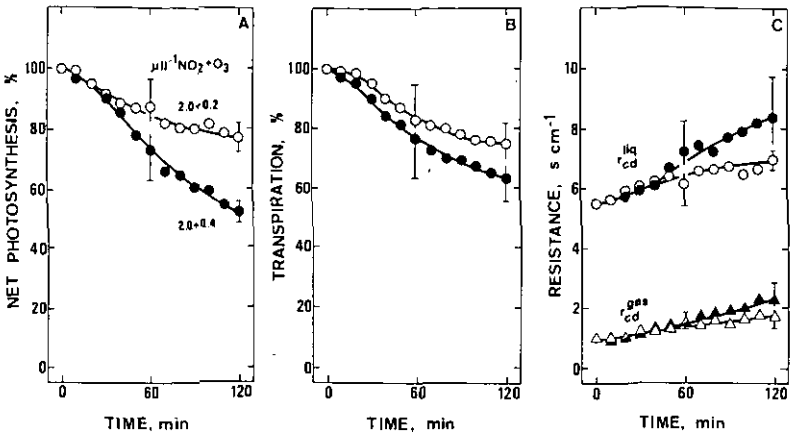

Fig. 3 Effects of 0.2 or 0.4 μl l^{-1} O_3 and 2.0 μl l^{-1} NO_2 mixtures on net photosynthesis (A), transpiration (B), and CO_2 diffusive resistances (C) in sunflower leaves

Concentrations of O_3 and NO_2 were shown in each subfigure. Each point is the mean of at least three replicates. Error bars indicate representative standard deviations.

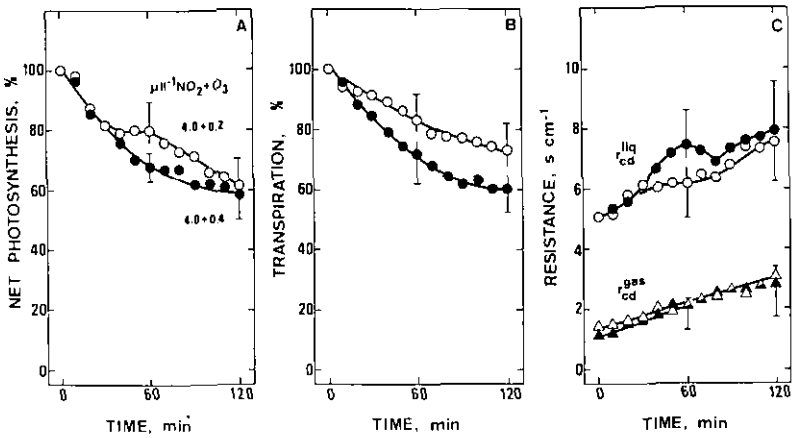

Fig. 4 Effects of 0.2 or 0.4 μl l^{-1} O_3 and 4.0 μl l^{-1} NO_2 mixtures on net photosynthesis (A), transpiration (B), and CO_2 diffusive resistances (C) in sunflower leaves

Concentrations of O_3 and NO_2 were shown in each subfigure. Each point is the mean of at least three replicates. Error bars indicate representative standard deviations.

This is also indicated by the observation that the increase in r_{cd}^{gas} roughly paralleled with the increase in r_{cd}^{liq} over the exposure period applied in the present experiment.

The analysis of CO_2 diffusion process suggests that the photosynthetic response to the individual and mixed gas treatments with NO_2 and O_3 differed markedly. The photosynthetic decline caused by the treatment with NO_2 or O_3 alone was mainly attributed to the increase in

r_{cd}^{liq}, although it could be observed that r_{cd}^{gas} increased slightly during the treatment with O_3 alone. In contrast, the mixed gas treatments affected r_{cd}^{gas} and r_{cd}^{liq} simultaneously. Furthermore, the contribution of the increase in r_{cd}^{gas} to the decrease in net photosynthesis was comparable to that of r_{cd}^{liq}.

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ヒマワリ葉の光合成，蒸散に対する NO_2 と O_3 の混合処理の影響

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ヒマワリ（*Helianthus annuus* L. cv. Russian Mammoth）を同化箱の中に入れて，NO_2，O_3 を処理して，光合成，蒸散速度に対する混合ガスの影響を調べた。光合成速度及び蒸散速度から気孔拡散抵抗，葉肉拡散抵抗を計算して求め，混合ガスの影響が何処に主として作用するかを，拡散抵抗の概念から解析した。

NO_2 単独処理では，光合成はかなり阻害され，濃度が高いとほぼ完全に阻害されたが，蒸散は光合成が初期値の10％以下になってもほとんど変化しなかった。一方，O_3 単独処理の影響は，NO_2 とは異なり，光合成，蒸散がほぼ同じくらい阻害された。しかし，拡散抵抗の解析から，純光合成の低下は，気孔の閉鎖によっているのではなく，葉肉細胞内の生理的変化によっていることが示唆された。

NO_2，O_3 を混合して処理すると，O_3 の影響と類似して，光合成も蒸散も阻害され，低下した。しかし，光合成速度が初期値の10％以下になる濃度の NO_2 と O_3 を同時に処理すると，光合成速度の低下割合は減少し，いわゆるきっこう阻害が観察された。拡散抵抗を用いて，O_3 の場合と同様に解析した結果，混合処理の影響は，気孔拡散抵抗と葉肉拡散抵抗の両方をほぼ同じくらい増加させることが判明した。すなわち，混合処理によって，気孔の閉鎖が純光合成速度を低下させるに十分なほど引き起こされることである。

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Res. Rep. Natl. Inst. Environ. Stud., Jpn., No. 65, 1984.

An Evaluation of High Resistance in *Polygonum Cuspidatum* to Sulfur Dioxide (SO_2)*

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The effects of sulfur dioxide (SO_2) on the photosynthesis of *Polygonum cuspidatum* propagated from shoots sample near at a copper mine at Asio, Tochigi Pref., were compared with those of *Helianthus annuus*, known as a sensitive plant to SO_2. The percentage inhibition of net photosynthesis and leaf conductance was plotted against the calculated SO_2 absorption rate. The threshold value of SO_2 absorption to photosynthetic inhibition in *P. cuspidatum* was larger than that in *H. annuus*. And the photosynthetic inhibition per unit SO_2 absorption rate in *P. cuspidatum* was smaller than in *H. annuus*. Furthermore, we studied the effects of SO_2 on the CO_2 concentration in substomatal cavity. The CO_2 concentration in *P. cuspidatum* did not increase, but that in *H. annuus* did. From these data and the change of the extent of inhibition of photosynthesis and transpiration, the photosynthetic decline in *P. cuspidatum* exposed to SO_2 was primarily due to the stomatal closure of the leaf. It was concluded that photosynthetic activity of *P. cuspidatum* was tolerant to SO_2 firstly because of small SO_2 absorption rate by leaves resulting from the small leaf conductance, and secondly because of high resistace to SO_2 of biochemical process in photosynthetic pathway.

Key words: *Polygonum cuspidatum*, SO_2, photosynthetic rate, SO_2 resistance, smoke polluted area.

In recent years, sulfur dioxide (SO_2) has attracted attention as a gaseous air pollutant which may cause chronic environmental stress for vegetation grown in urban districts. Several workers reported that long-term exposure to SO_2 could inhibit plant growth and alter the species composition of plant communities in SO_2 polluted areas (Archibold, 1978; Asai, 1952; Gordon & Gorham, 1963; Hiroi, 1974; Horsman *et al.*, 1979; Usui *et al.*, 1975; Wagner *et al.*, 1978; Winner & Mooney, 1980c; Wood & Nash, 1976; Yoshioka, 1975).

Usui *et al.* (1975) reported that in smoke polluted area along the leeward of the copper mine at Asio, Tochigi Pref., a large section of deciduous broad-leaved forest was almost completely destroyed by fire in 1891, and that young sprouts which grew afterwards as a secondary succession were probably damaged by the constant attack of SO_2 emitted from the

* A part of this report was published in Jpn. J. Ecol., 34, 153–159 (1984).

smelter. They also suggested that air pollution by SO_2 might induce a primary dominance of *Polygonum cuspidatum* population in this area. Likewise, Hiroi (1974) also reported that *P. cuspidatum* was one of the dominant species of herbaceous communities established in copper mine districts such as Asio, Tochigi Pref. and Besshi, Ehime Pref. On the other hand, Yoshioka (1975) reported that *P. cuspidatum* is one of the dominant species of the natural vegetation found at volcanoes such as Mt. Aso, Kumamoto Pref. and Sakurajima Island, Kagoshima Pref. in Kyushu. It has been reported that fumarolic gases from Mt. Nakadake (Volcano Aso) contained a relatively high SO_2 content of 2.8–12.4% (Iwasaki *et al.*, 1962).

The reason for the establishment of peculiar vegetation in SO_2 polluted areas may be the difference in resistance to SO_2 of the plant species. There are many reports (cf. Japan Society of Air Pollution, 1982 and Katase M., *et al.*, 1983) about the responses of plants to SO_2, but few about the photosynthetic characteristics of native plants surviving in smoke polluted areas. Therefore, it remains unclear why *P. cuspidatum* population can survive dominately in SO_2 polluted areas.

In order to know the response to SO_2 of native plants surviving in smoke polluted areas, the effects of SO_2 were studied on the leaf photosynthesis of *P. cuspidatum* propagated from shoots collected near the copper mine at Asio, Tochigi Pref. The photosynthetic response was compared with that of *Helianthus annuus* cv. Russian Mammoth, which in known as a sensitive plant to SO_2.

Materials and Methods

Shoots of *P. cuspidatum* were collected in autumn in the smoke polluted area on the leeward of the copper mine at Asio, Tochigi Pref. about 110 km north-northwest from Tokyo. The shoots were cut off to a length of 5–10 cm. The base of cut shoots were soaked and rooted in a tray containing water for one month. The rooted plants were transplanted in plastic pots filled with the artificial culture medium composed of peat moss, vermiculite, perlite, fine gravel and Acadamatuchi (granulated loam) (2:2:1:1:2 on a v/v basis). The plants were grown in an air-conditioned greenhouse at 25°C and 75% R.H. for one year. Seeds of *H. annuus* were sown in 1/5000 a plastic pots filled with culture medium composed of peat moss, vermiculite, perlite and fine gravel (2:2:1:1 on a v/v basis), and one plant per pot was grown for 4 to 5 weeks in the greenhouse.

The attached mature leaves of the plants were placed in an acrylic assimilation chamber which was 30 cm long, 17.5 cm wide and 2 cm deep. The photosynthetic and transpiration rates of the plant leaves were measured. The conditions in the chamber were regulated to keep 25–27°C leaf temperature, 40–50% R. H. and 64 klx of light intensisty at the upper surface of the leaf. The CO_2 concentration in the air passing through the chamber was controlled to maintain 341–360 ppm by mixing CO_2-free air with a given volume of 4.88% CO_2 supplied with a cylinder. CO_2-free air was prepared by passing ambient air through tubes filled with sodalime. After the addition of CO_2 to the air stream, the water content of the air entering into the chamber was controlled by passing it through a humidifier and chilling it with a coiled glass tube placed in the water bath. Water temperature in the bath was controlled using a thermoregurator with the accuracy of ±0.5°C. In order to control SO_2 concentration in the air, SO_2 from a cylinder was injected through a thermal mass-flow controller into the air stream and mixed with the air by passing it through a 5 m long teflon tube before it entered the chamber. The rate of air flow entering into the assimilation chamber was maintained at 15 lit·

min^{-1}. The average wind velocity across the transverse section of the chamber was 71 mm s^{-1}. The concentration of SO_2 and CO_2 in the air entering and leaving the chamber was measured alternately for 2 min using solenoid valves. CO_2 concentration was measured by an infrared gas analyzer (Shimazu Seisakusho Co., Model URA-2S) and SO_2 concentration was monitered by a flame photometric detector of SO_2 (Bendix, Model 830). Leaf temperature was measured by three copper-constantan thermocouples (0.1 mm) attached to three different positions on the undersurface of leaves. Light was supplied with four 500W incandescent lamps suspended above the chamber. A water layer about 10 cm in depth was poured between the lamps and the chamber to filter infrared radition, and a semitransparent film made of vinyl chloride was used to obtain uniform distribution of light intensity. After the fumigation treatment, leaf area was measured by an automatic area meter (Hayashi Denkoh Co. Ltd., Model AAM-7). The rates of transpiration and photosynthesis were evaluated from the differences in dew point and CO_2 concentration of the air at the inlet and outlet of the chamber respectively. Dew point of the air was measured by two digital humidity analyzers which were set at the inlet and outlet of the chamber (EG & G, Model 911). Leaf boundary layer resistance to water vapor transfer (r_a) in the chamber was obtained by the measurements on leaf replicas made of wet blotting paper. Leaf conductance to water vapor ($1/r_a + r_s$), r_s: stomatal resistance) was calculated with reference to the methods reported by Koh (1981) and by Furukawa *et al.* (1980).

Results

Leaves of *P. cuspidatum* or *H. annuus* were fumigated for 64 min at 1.70 and 0.74 ppmSO_2, respectively. Fig. 1 shows a typical time course response of net photosynthesis and leaf conductance ($1/r_a + r_s$)) to SO_2 fumigation for both species. Initial rates of net photosynthesis prior to SO_2 treatments were 22.6 mgCO_2 dm^{-2} h^{-1} and 35.5 mgCO_2 dm^{-2} h^{-1}, in *P. cuspidatum* and *H. annuus*, respectively. SO_2 fumigation for 60 min resulted in the decline of photosynthesis for both species. The decrease of leaf conductance was in parallel with that of photosynthesis in *P. cuspidatum* during SO_2 fumigation. But the decrease of leaf condutance in *H. annuus* was slight as compared with that of photosynthesis.

Figure 2 shows the percentage inhibition of net photosynthesis and leaf conductance [(1 − relevant value/initial value) x 100] determined at 60 min after the initiation of fumigation were plotted against the SO_2 concentration. The degree of photosynthetic decline at 60 min after the initiation of fumigation increased with increase of the SO_2 concentration. The threshold concentration of SO_2 to photosynthetic inhibition was 0.56 ppmSO_2 in *P. cuspidatum* and 0.13 ppmSO_2 in *H. annuus* under the experimental conditions. Figure 3 shows the percentage inhibition of net photosynthesis and leaf conductance [(1 − relevent value/initial value) x 100] at 60 min after the fumigation treatment was started, were plotted against the calculated SO_2 absorption rate. The rate of SO_2 absorption in leaves was calculated according to the method reported by Omasa and Abo (1978), on the basis of boundary layer resistance and stomatal resistance under the assumption that SO_2 concentration in substomatal cavity was 0 ppmSO_2. This value was ascertained by Omasa & Abo (1978) for *H. annuus*. The threshold value of SO_2 absorption to photosynthetic inhibition in *P. cuspidatum* was larger than in *H. annuus*. On the other hand, the slope of the regresion line between photosynthetic inhibition and SO_2 absorption rate in the former plant was gentler than in the latter plant. Moreover the decrease of leaf conductance of *P. cuspidatum* was coincident with that of photosynthesis. These results suggested that photosynthetic inhibition of *P. cuspidatum* was mainly due to the stomatal closure and that of *H. annuus* was mainly due to a non-stomatal process, probably the

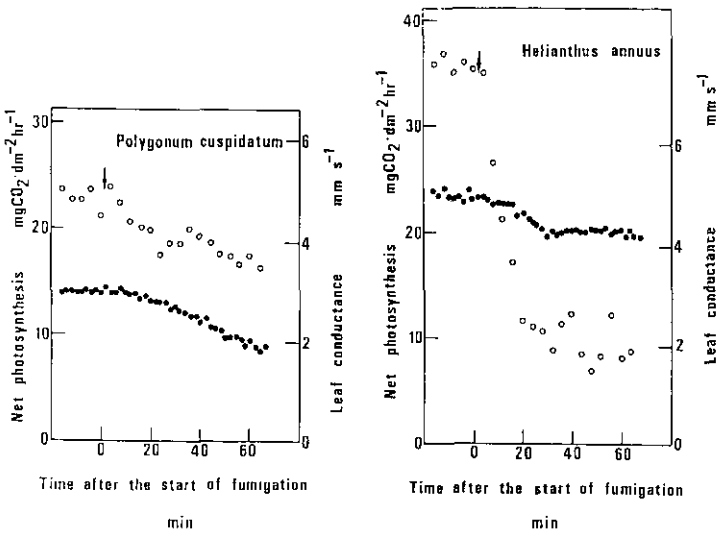

Fig. 1 Effects of SO_2 on net photosynthesis (○) and leaf conductance (●)

SO_2 concentrations fumigated were 0.74 ppm for *H. annuus* and 1.70 ppm for *P. cuspidatum*. Arrows in the figures indicate the start of fumigation.

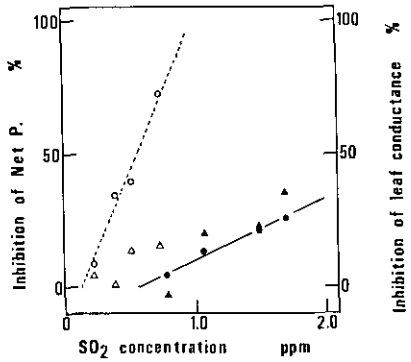

Fig. 2 Relation between inhibition of net photosynthesis (Inhibition of Net P., circles), leaf conductance (triangles) and SO_2 concentrations for *P. cuspidatum* (closed symbols) and *H. annuus* (open symbols)

The lines in the figure indicate the regression line between inhibition of net photosynthesis and SO_2 concentration for *H. annuus* (·····) and *P. cuspidatum* (———). Inhibition of net photosynthesis and inhibition of leaf conductance were expressed as the percentage inhibition [(1 − relevant value/initial value) × 100].

biochemical photosynthetic process.

Table 1 shows the effects of SO_2 on the CO_2 concentration in the substomatal cavity in *P. cuspidatum* and *H. annuus*. The change of CO_2 concentration in the substomatal cavity (Ci) was examined using the following equation: [$Ci = Ca - k\,(1.37\,r_a + 1.54\,r_s)\,P$], where Ca is

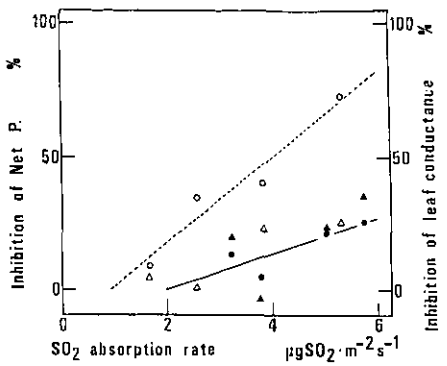

Fig. 3 Relation between inhibition of net photosynthesis (circles), leaf conductance (triangles) and calculated SO_2 absorption rate for *P. cuspidatum* (closed symbols) and *H. annuus* (open symbols)

The lines in the figure indicate regression lines between the calculated SO_2 absorption rate and the inhibition of net photosynthesis for *P. cuspidatum* (———) and *H. annuus* (·····). Inhibition of net photosynthesis and inhibition of leaf conductance were expressed as the percentage inhibition [(1 − relevant value/initial value) × 100].

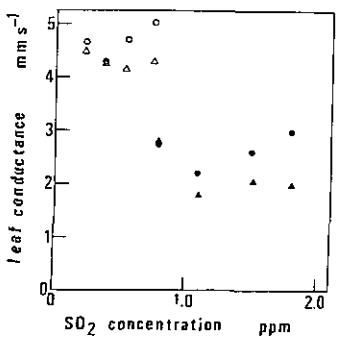

Fig. 4 Effects of SO_2 on leaf conductance for *P. cuspidatum* (closed symbols) and *H. annuus* (open symbols)
Circles show the initial value before fumigation. Triangles show the values at 60 min after initiation of fumigation.

CO_2 concentration in ambient air (ppm), P is net photosynthetic rate ($mgCO_2\ dm^{-2}\ h^{-1}$) and k is a constant (1.544 at 25°C in air at the flow meter). As suggested in Fig. 3 where the inhibition of net photosynthesis in *P. cuspidatum* mainly depended on stomatal closure, the CO_2 concentration in the substomatal cavity in *P. cuspidatum* did not increase by SO_2 fumigation. On the other hand, the value in *H. annuus* increased as suggested in Fig. 3.

Fig. 4 shows the leaf conductance of *P. cuspidatum* and *H. annuus* at the measurements prior to the fumigation and 60 min after starting the fumigation. The leaf conductance of *P. cuspidatum* was smaller than that of *H. annuus* at both measurements.

Table 1 Effects of SO_2 fumigations for 60 min on CO_2 concentration in the substomatal cavity

Values were expressed as the percentage of those before SO_2 fumigation.

H. annuus		*P. cuspidatum*	
Fumigated SO_2 conc. (ppm)	CO_2 conc. in the substomatal cavity (%)	Fumigated SO_2 conc. (ppm)	CO_2 conc. in the substomatal cavity (%)
0.22	104	0.80	106
0.37	129	1.09	96.8
0.54	140	1.50	96.3
0.74	159	1.70	85.5

Table 2 Effects of SO_2 fumigations for 60 min on the ratio of photosynthetic rate and transpiration rate (P/T ratio)

Values were expressed as the percentage of those before SO_2 fumigation.

H. annuus		*P. cuspidatum*	
Fumigated SO_2 conc. (ppm)	P/T Ratio (%)	Fumigated SO_2 conc. (ppm)	P/T Ratio (%)
0.22	97.1	0.80	97.2
0.37	67.3	1.09	101.1
0.54	69.0	1.50	96.9
0.74	29.4	1.70	110.3

Table 2 showes the change of the ratio of photosynthetic rate and transpiration rate (P/T ratio) of *H. annuus* and *P. cuspidatum* during the exposure to SO_2. The P/T ratio of *H. annuus* was decreased with increase of SO_2 concentration. However that of *P. cuspidatum* was unchanged.

Discussion

Several workers (Hiroi, 1974; Usui, 1975; Yoshioka, 1975) reported that *P. cuspidatum* is one of the dominant species in SO_2 polluted area, but causal analysis of the characteristic distribution of *P. cuspidatum* has not been performed.

The photosynthetic inhibition was plotted against SO_2 absorption rate (Fig. 3). The threshold value of SO_2 absorption rate to photosynthetic inhibition rate in *P. cuspidatum* was larger than in *H. annuus*, and the slope of regression line was gentler than that of *H. annuus*. Sij and Swanson (1974) speculated that the stomatal closure could not account for the reduction of photosynthetic rate caused by SO_2 exposure in Pinto bean from the results obtained by the simultaneous measurements of photosynthesis and transpiration. Winner and Mooney (1980a) reported when SO_2 absorption was 5 μgSO_2 cm^{-2} 8 h^{-1} or less (0.174 $ngSO_2$ cm^{-2} s^{-1}), photosynthetic inhibition for *Diplacus aurantiacus* and *Heteromeles arbutifolia* was due entirely to the stomatal closure, and that when SO_2 absorption was as high as 15 μgSO_2 cm^{-2} 8 h^{-1},

photosynthetic inhibition for both species was due to non-stomatal factors. As shown in Table 1 and Fig. 3, the present results showed that the decrease of photosynthesis in *H. annuus* was not in parallel with that of leaf conductance during SO_2 fumigation, and CO_2 concentration in the substomatal cavity increased. Raske (1975) reported that DCMU fed to leaves through the transpiration stream caused inhibition of photosynthesis in the mesophyll, and subsequently, an increase in intercellular CO_2 concentration. Consequently, it may be suggested that the biochemical photosynthetic process (non-stomatal processes) of *H. annuus* was inhibited by SO_2 fumigation, as reported by some workers (Ohshima *et al.*, 1973; Furukawa *et al.*, 1980). But the decline of photosynthetic rate of *P. cuspidatum* was in parallel with that of leaf conductance, and CO_2 concentration in the substomatal cavity did not increase. Therefore, the photosynthetic decline was thought to be due primarily to stomatal closure, and photosynthesis was apparently not limited by the biochemical photosynthetic process. Furthermore, it was easily surmised from the above description that the P/T ratio of *P. cuspidatum* was not affected by SO_2 fumigation except the effect of increase of leaf temperature resulting from stomatal closure on the P/T ratio. This idea was ascertained in Table 2.

Under consideration of the assumption that SO_2 concentration in the substomatal cavity is 0 ppm, as discussed by several workers (Omasa, 1978; Black *et al.*, 1979b; Winner *et al.*, 1980a), it can be said that smaller stomatal conductance resulted in smaller SO_2 absorption by leaves. As shown in Fig. 4, the leaf conductance of *P. cuspidatum* was smaller than that of *H. annuus* before and during the fumigation. Therefore, it was considerable that the absorption rate of SO_2 in *P. cuspidatum* was innately smaller than that in *H. annuus* under the same SO_2 concentration.

In conclusion, we postulated that one of the reasons why *P. cuspidatum* could survive in smoke polluted area was due to the tolerance of its photosynthetic activity to SO_2 fumigation because of the small stomatal conductance and the higher resistance of biochemical processes in photosynthetic pathway, and probably because of its high SO_2 detoxication ability.

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イタドリの二酸化イオウ（SO_2）に対する高い抵抗性について

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足尾煙害地より採取し栄養繁殖させたイタドリとSO_2に対して感受性が高いとされているヒマワリについて，SO_2暴露下での光合成反応を比較検討した。気相コンダクタンスより推定したSO_2吸収速度に対して両種の光合成阻害度を図示した結果，イタドリのほうがヒマワリより光合成阻害に対するSO_2吸収速度のしきい値は大きく，単位SO_2吸収速度当たりの光合成阻害度は小さかった。さらに，SO_2暴露による気孔腔内のCO_2濃度は，イタドリでは増加しないが，ヒマワリでは顕著に増加した。この結果及びSO_2暴露時の気孔閉鎖及び光合成阻害度の変化の結果より，イタドリの光合成阻害は主に気孔閉鎖であることが暗示された。したがって，イタドリが煙害地に生育できる要因の一部は，遺伝的に気相コンダクタンスが小さく，SO_2が葉内に侵入しにくいこと，さらに，生理生化学的な光合成過程のSO_2に対する抵抗性が強いことであると結論される。

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Res. Rep. Natl. Inst. Environ. Stud., Jpn., No. 65, 1984.

Effects of Low Concentrations of O_3 on the Growth of Sunflower Plants*

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Fourteen-day-old sunflower plants (*Helianthus annuus* L. cv. Russian Mammoth) were exposed to 0.1 or 0.2 ppm ozone (O_3) for 12 days in an artificially-lighted growth cabinet. Plants were harvested 0, 6 and 12 days after the start of gas exposure, and the growth analysis was performed. White fleck of injury developed on many leaves after the exposure to 0.1 or 0.2 ppm O_3 for 1–2 days, and subsequently visible injury and withering of old leaves were accelerated. Twelve days after the start of exposure, the dry weight of whole plant was reduced by 11% and 32% of the control by 0.1 and 0.2 ppm O_3, respectively. Root growth was markedly inhibited by O_3, while leaf growth was slightly inhibited. Relative growth rate (RGR) and net assimilation rate (NAR) were reduced by 0.1 ppm O_3 for the first 6 days, but were not affected for the following 6 days. RGR and NAR were reduced by 0.2 ppm O_3 throughout the exposure period. For the last 6 days, RGR was less affected by 0.2 ppm O_3 than NAR, due to the increase in leaf area ratio (LAR). Leaf weight ratio (LWR) was also increased by O_3 exposure, whereas stem weight ratio (SWR) and root weight ratio (RWR) were reduced. These changes in growth parameters suggest that the chronic exposures to low concentrations of O_3 could affect the net photosynthesis and the pattern of partitioning of assimilates in sunflower plants.
Key Words: Air pollution, Dry weight growth, Growth analysis, NAR, Ozone (O_3), Partitioning, RGR, Sunflower plants.

Photochemical oxidants are the most important and widespread types of air pollutants in recent years. Injurious effects of the oxidants on agricultural and native vegetations have been investigated by many workers (see reviews Middleton, 1961; Ting & Heath, 1975). Ozone (O_3), which is a major component of photochemical oxidants, is probably more injurious to plants

*A part of this study was published in; Environ. Control in Biol., 19, 137–147 (1981).

than any other air pollutants (Heggested, 1969; Hill *et al.*, 1970). Acute injuries induced by O_3 have been studied extensively. High concentrations of O_3 usually cause the visible symptoms of injury on leaves (Heggestad & Middleton, 1959; Hill *et al.*, 1970) and affect the rates of photosynthesis, respiration and transpiration within a few hours (Todd, 1958; Hill & Littlefield, 1969; Furukawa & Kadota, 1975).

Chronic effects on plants of long-term exposures to relatively low concentrations of O_3 are considered to be more practical and important subjects at present. There has been an increasing number of studies concerning the chronic effects of O_3 on growth and yield of many plants. Feder (1970) reported that low concentrations of O_3 inhibited the flower formation of carnation and geranium plants. Heagle *et al.* (1972) documented that the exposure to 0.1 ppm O_3 for 67 days significantly reduced the yield of a hybrid sweet corn. The results from Tingey *et al.* (1973) showed that 3-week exposure to 0.1 ppm O_3 could reduce the growth of soybean plants. Similar reduction in growth and/or yield have been also reported on many other plant species (Tingey *et al.*, 1971; Manning *et al.*, 1971; Jensen & Dochinger, 1974; Tingey & Reinert, 1975; Oshima *et al.*, 1975; Heagle *et al.*, 1979). However, Harward and Treshow(1975) reported that some understory plants in the aspen zone significantly increased in the dry weight growth and the seed production with the exposure to 0.06 or 0.15 ppm O_3. From many other investigations, Bennett *et al.* (1973) compiled evidences indicating the increase in growth of plants exposed to low concentrations of O_3 and showed in their own experiments that 12-days exposure to 0.03 ppm O_3 stimulated the growth of bean, barley and smart weed.

These conflicting results indicate that the chronic effects of low concentrations of O_3 on plant growth have not been clarified. Furthermore, almost all of these experiments were conducted to determine the effects of O_3 only with a single harvest procedure. The changes in growth and yield should be the results of many physiological and biochemical changes. In order to examine the chronic effects of O_3 on plant growth precisely, the effects on several growth parameters should be investigated.

In the present study, we exposed sunflower plants to 0.1 or 0.2 ppm O_3 continuously for 12 days, and detected the effects of O_3 on several growth parameters by means of the plant growth analysis. This procedure might provide the information concerning the physiological changes in O_3-exposed plants. Furthermore, we tried to give an explanation of the conflicting results reported by other workers.

Materials and Methods

Plant material and growth conditions

Sunflower seeds from the plants bred in our institute were immersed in water for 12 h and then sterilized by dipping into 1 g l^{-1} Benlate T solution (20% Bis (dimethylthiocarbamyl) Disulfide and 20% Methyl 1- (Butylcarbamyl)- 2-benzimidazolecarbamate, Dupont, Delaware, U.S.A.) for 30 min, followed by rinsing with running tap water for 2 h. Three seeds were directly sown in each plastic pot (diameter: 11 cm, height: 20 cm) containing a 1.8 *l* mixture of vermiculite, peat moss, perlite and fine gravel (4:4 :2:1, v/v). As basal fertilizers, 5 g pot^{-1} of Magamp K (N: P_2O_5 :K = 6:40:5, W.R. Grace Co, Tennessee, U.S.A.) was added, and the pH of wet medium was adjusted to 6.4 with magnesia lime (about 15 g pot^{-1}). Plants were fertilized regularly (1–3 times $week^{-1}$) with 100–200 ml of 0.1% Hyponex solution (N:P_2O_5 :K = 6.5:6:19 w/w Hyponex Co. Inc, Copley, Ohio, U.S.A.) plus Hoagland's No.2 micro elements solution (Hewitt, 1966) and watered regularly or daily as needed.

After sowing the pots were brought into a controlled environment growth cabinet (1.7 x 2.3 x 1.9 m^3 Koito Co. Ltd.) to cultivate plants under the following environmental conditions. Air temperature in the cabinet was 25 ± 0.5°C, and relative humidity was 75 ± 5%. Light source consisted of 24 stannous halide lamps (400 W, Yoko lamp, Toshiba), and long wavelength (>800 nm) of the emitted radiation was eliminated through a heat absorbing glass filter. Light intensity was about 420 $\mu E\ m^{-2}s^{-1}$ (ca. 115 Wm^{-2}, 30 klx) at plant height. Light/dark cycle was 14/10h. Fresh air was led into the cabinet after being passed through activated charcoal and catalist-bearing (containing Mn Ox and CuO)filters to remove ambient air pollutants. Air velocity in the cabinet was 0.2–0.4 m s^{-1}, and ventilation rate was 75 m^3h^{-1} (ca. 10 times h^{-1}). The concentration of carbon dioxide (CO_2) in the cabinet was continuously monitored and regulated at 400 ± 4 ppm throughout the experimental period by a controlling system based on an infrared CO_2 gas analyzer (URA-2S, Shimadzu). In the growth cabinet, plants were rotated (2.5 times h^{-1}) on a turntable to minimize possible position effects.

Seeldings were selected for uniformity and thinned to a single plant per pot 7 days after sowing. Twelve-day-old plants were transferred to another controlled environment growth cabinet for O_3 exposure (Koito Co. Ltd.). The size and the environmental conditions in this cabinet were the same as those in the cabinet where plants had been cultivated previously, except for the high ventilation rate up to 800 m^3h^{-1} (ca. 110 times h^{-1}) to minimize the effects of unknown pollutants which might be produced by photochemical reactions in the cabinet.

Exposure to O_3

Fourteen-day-old plants were exposed to O_3 for 12 days. O_3 was generated by a silent electrical discharge in dry oxygen and mixed with the filtrated fresh air, and the mixed air was led into the cabinet. The concentration of O_3 in the cabinet was monitored continuously and regulated by a controlling system based on a chemiluminescent O_3 analyzer (Model 806, Kimoto). Growth experiments consisted of three separate experiments. The concentration of O_3 in each experiment was 0.0 ppm (control), 0.1 ± 0.002 ppm or 0.2 ± 0.004 ppm continuously. Environmental conditions inside the cabinet were almost identical among the three experiments except for the O_3 concentration and were kept constant during the growth experiments.

Harvests and growth analysis

In each experiment, 10 plants were harvested just before the start of O_3 exposure (0 day) and other 10 plants were also harvested 6 and 12 days thereafter. The extents of visible injury and withering in each leaf were visually assessed in 5% increments of leaf area. The area of each leaf was measured by an automatic planimeter (model 3100, LICOR Co. Ltd.). Total number of leaves more than 10 mm in length and plant height were recorded. Plants were divided into leaf laminae, stem, root, flower bud and withered leaves, and were dried at 80–90°C for 3–5 days for weighing the dry matter. Stem part included leaf petioles.

Relative growth rate (RGR), net assimilation rate (NAR), leaf area ratio (LAR), specific leaf area (SLA). leaf weight ratio (LWR). stem weight ratio (SWR) and root weight ratio (RWR) were calculated according to the following formulae (Evans, 1972):

$$RGR = \frac{1}{W}\cdot\frac{dW}{dt} = \frac{\ln W_2 - \ln W_1}{t_2 - t_1}$$

$$NAR = \frac{1}{\bar{F}}\cdot\frac{dW}{dt} = \frac{(W_2 - W_1)(\ln \bar{F}_2 - \ln \bar{F}_1)}{(t_2 - t_1)(\bar{F}_2 - \bar{F}_1)}$$

$$LAR = \bar{F}/W$$
$$SLA = \bar{F}/F$$
$$LWR = F/W$$
$$SWR = S/W$$
$$\text{and } RWR = R/W,$$

Where W_i and $\bar{F}_i$ are the dry weight of whole plant and the leaf area at time t_i (i: 1 and 2), respectively. F, S and R are the dry weight of leaves, stem and root, respectively.

Results

Visible injury and withering of leaves

The symptom of visible injury was noted as white fleck (Hill *et al.*, 1970) within 2 days the start of exposure to 0.1 or 0.2 ppm O_3, and was most notable on the tip or the edge of upper surface of leaf laminae, especially of matured leaves. As the exposure continued, the symptom spread on the expanded leaves and appeared even on the upper young leaves (Fig. 1). Furthermore, O_3 exposure accelerated the withering of lower old leaves (Fig. 2), which was apparently similar to that of naturally senescent leaves. With increase in concentration of O_3, the extents of visible injury and of withering of leaves increased. Effects of O_3 exposure on the total dry weight of withered leaves are shown in Table 1.

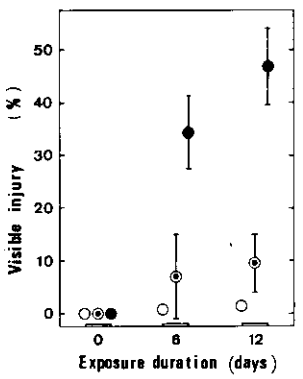

Fig. 1 Effect of O_3 exposure on the visible injury of leaves

Percentage of total leaf dry weight was represented. Natural senescent yellow coloring and withering of leaves were included. O_3 exposure was started 14 days after sowing and continued for 12 days thereafter. Each symbol is the mean of 12 plants and vertical bars indicate ± standard deviation of the mean. ○: 0 ppm (control), ◉: 0.1 ppm, ●: 0.2 ppm O_3 exposure treatments. See Materials and Methods in details.

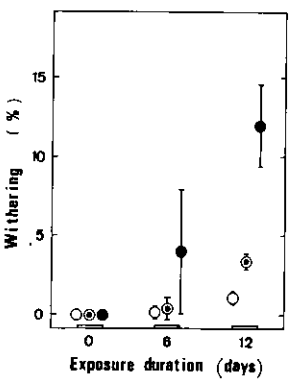

Fig. 2 Effect of O_3 exposure on the withering of leaves

Percentage of total leaf dry weight was represented. O_3 exposure was started 14 days after sowing and continued for 12 days thereafter. See legends for Fig 1.

Table 1 Effects of O_3 exposure on several characteristics of sunflower plants[1)]

	O_3 concentration (ppm)		
	0.0 (control)	0.1	0.2
Withered leaves (mg dry wt)	68.2 ± 23.8	206.5 ± 38.2***[2)]	695.2 ± 139.7***
Flower bud (mg dry wt)	17.3 ± 6.9	16.0 ± 5.8	10.6 ± 3.8**
Plant height (cm)	48.2 ± 3.4	47.1 ± 3.0	42.8 ± 3.8**
Number of leaves	24.4 ± 1.2	23.7 ± 1.8	26.6 ± 2.2**

1) Plants were harvested 12 days after the start of exposure. Mean of 12 plants and standard deviation are indicated.
2) Significance of difference from control (t-test), **p<0.01, ***p<0.001. See Materials and Methods in details.

Effects on plant growth

As shown in Fig. 3, dry weight growth of whole plant was significantly reduced (P<0.001) by the exposure to 0.1 or 0.2 ppm O_3 for 6 days. At the final harvest, the dry weight of plants exposed to 0.1 and 0.2 ppm O_3 was smaller by 11% and 32% than that of control plants, respectively.

The effects of O_3 on the dry weight growth of stem, root and leaf laminae are shown in Fig. 4, 5 and 6, respectively. Six-day exposure to 0.1 or 0.2 ppm O_3 was sufficient to reduce the dry weight growth of each organ. As compared with the control plants, the stem dry weight at the final harvest revealed 13% and 38% reductions for plants exposed to 0.1 and 0.2 ppm O_3, respectively. Drastic reduction in the dry weight growth of root was induced by O_3 exposure. At the final harvest, the root dry weight of 0.1 ppm O_3-exposed plants was smaller by 15% than that of control plants, while a 50% reduction was caused by 0.2 ppm O_3. Leaf growth was

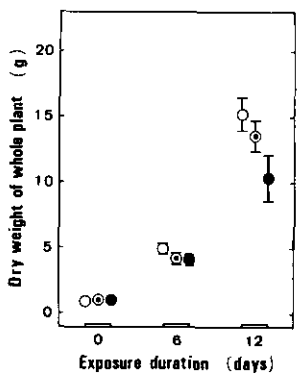

Fig. 3 Effect of O_3 exposure on the increase in dry weight of whole plant

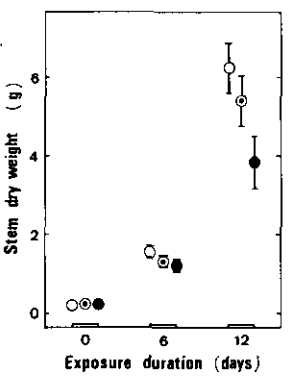

Fig. 4 Effect of O_3 exposure on the increase in dry weight of stem

Dry weight of withered leaves was excluded. O_3 exposure was started 14 days after sowing and continued for 12 days thereafter. Each symbol is the mean of 12 plants and vertical bars indicate ± standard deviation of the mean. o: - ppm (control), ⊙: 0.1 ppm, ●: 0.2 ppm O_3 exposure treatments. See Materials and Methods in details.

also reduced by O_3 exposure. Although the withering of lower old leaves was accelerated, the growth reduction of leaf dry weight caused by O_3 exposure was less than that of stem or root. The exposure to 0.2 ppm O_3 reduced leaf dry weight by 18%, and 0.1 ppm O_3 reduced by only 6% ($P<0.05$) even when the exposure was continued for 12 days. A slight reduction in leaf area growth was also detected in the O_3-exposed plants (Fig. 7). At the final harvest, the leaf area of plants exposed to 0.1 and 0.2 ppm O_3 was smaller by 9% and 14% than that of control plants, respectively.

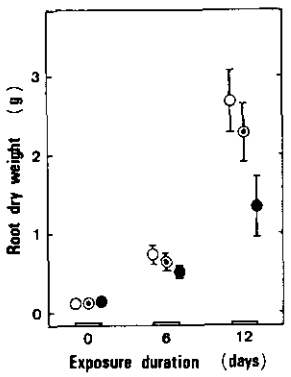

Fig. 5 Effect of O_3 exposure on the increase in dry weight of root

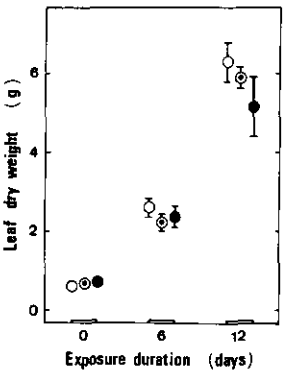

Fig. 6 Effect of O_3 exposure on the increase in dry weight of leaves

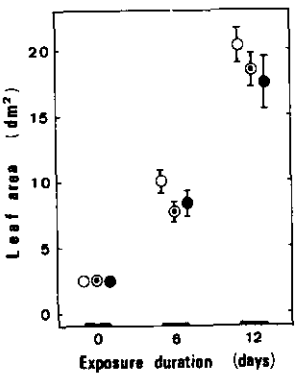

Fig. 7 Effect of O_3 exposure on the increase in leaf area

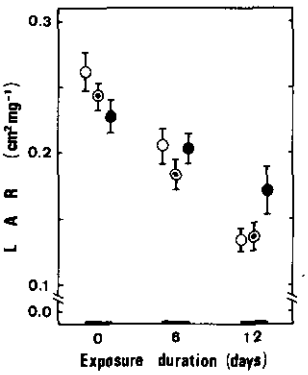

Fig. 8 Effect of O_3 exposure on the leaf area ratio (LAR)

Dry weight of withered leaves was excluded. O_3 exposure was started 14 days after sowing and continued for 12 days thereafter. Each symbol is the mean of 12 plants and vertical bars indicate ± standard deviation of the mean. o: - ppm (control), ⊙: 0.1 ppm, ●: 0.2 ppm O_3 exposure treatments. See Materials and Methods in details.

As shown in Table 1, the exposure to O_3 did not influence the number of leaves emerged, irrespective of the withering of old leaves, except that 12-days exposure to 0.2 ppm O_3 stimulated the development of new leaves. A small flower bud was observed on the top of stem of almost all plants at the final harvest. The exposure to 0.2 ppm O_3 reduced the dry weight of flower bud by 39% of the control value, though 0.1 ppm O_3 had no significant effect. Stem elongation expressed as plant height was depressed by O_3 exposure, but the suppression was not so remarkable as the reduction in dry weight growth of stem.

Effects on growth parameters

The data presented in the previous section (Fig. 3–7) were subjected to the growth analysis. Table 2 shows the changes in RGR and NAR of plants in each treatment. For the first 6 days of exposure, the RGR of sunflower plants was reduced in 0.1 and 0.2 ppm O_3 by 15% and 19% of that in control, respectively. For the following 6 days, the exposure to 0.2 ppm O_3 resulted in the same extent of reduction in RGR as before, whereas 0.1 ppm O_3 caused a slight increase. The change in NAR caused by O_3 resembled with the change in RGR. The NAR of plants exposed to 0.1 ppm O_3 was smaller than that of control plants for the first 6 days but somewhat larger for the following 6 days. The exposure to 0.2 ppm O_3 reduced NAR during the exposure period for 12 days. By the way, the reduction in NAR amounted to 29% of the control value and was larger than that in RGR for the last 6 days.

As RGR is the product of NAR and LAR, effect of O_3 on LAR should be investigated (Fig. 8). The exposure to 0.2 ppm O_3 increased the LAR by 28% of the control valur at the final harvest. LAR was further divided into SLA and LWR. The SLA was only slightly increased by O_3 exposure (Fig. 9), whereas the LWR of plants pronouncedly increased with prolonged duration of exposure and with increased concentration of O_3 (Fig. 10). At the final harvest, the exposure to 0.2 ppm O_3 increased LWR by 21% of the control value, while 0.1 ppm O_3

Table 2 Effects of O_3 exposure on relative growth rate (RGR) and net assimilation rate (NAR) of sunflower plants[1)]

	Exposure duration (days)	O_3 concentration (ppm)		
		0.0 (control)	0.1	0.2
RGR	0–6	0.276	0.235	0.224
($mg\ mg^{-1}\ day^{-1}$)	6–12	0.189	0.196	0.154
NAR	0–6	1.232	1.149	1.058
($mg\ cm^{-2}\ day^{-1}$)	6–12	1.185	1.274	0.844

1) Dry weight of withered leaves was excluded to calculate these values. See Materials and Methods in details.

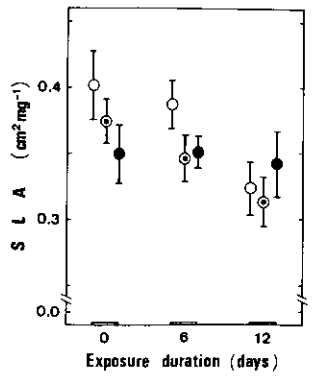

Fig. 9 Effect of O_3 exposure on the specific leaf area (SLA)

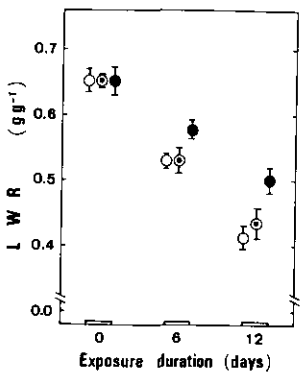

Fig. 10 Effect of O_3 exposure on the leaf weight ratio (LWR)

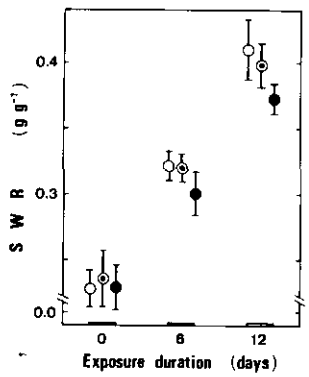

Fig. 11 Effect of O_3 exposure on the stem weight ratio (SWR)

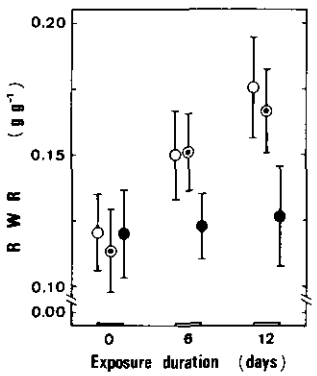

Fig. 12 Effect of O_3 exposure on the root weight ratio (RWR)

Dry weight of withered leaves was excluded. O_3 exposure was started 14 days after sowing and continued for 12 days thereafter. Each symbol is the mean of 12 plants and vertical bars indicate ± standard deviation of the mean. o: - ppm (control), ⊙: 0.1 ppm, ●: 0.2 ppm O_3 exposure treatments. See Materials and Methods in details.

increased LWR by 5% ($P<0.05$). We also calculated the ratio of dry weight of stem or root to that of whole plant (Figs. 11 and 12). The exposure to 0.1 ppm O_3 for 12 days only slightly reduced both SWR and RWR, whereas 0.2 ppm O_3 significantly reduced SWR and RWR by 9% and 28%, respectively.

Discussion

Dry matter production in sunflower plants was significantly reduced by the exposure to both 0.1 and 0.2 ppm O_3 for 6 and 12 days (Fig. 3). These findings support the previously

reported results that the chronic exposures to low concentrations of O_3 inhibited plant growth and yield (Feder, 1970; Manning *et al.*, 1971; Tingey & co-workers, 1971, 1973, 1975; Heagle *et al.*, 1972, 1979; Jensen & Dochinger, 1974; Oshima *et al.*, 1975). In addition, results of growth analysis in the present investigation suggested that several physiological functions participating in the plant growth were altered by O_3 through the course of exposure period. The reduction in RGR caused by O_3 exposure appeared to be derived from the effect of O_3 on NAR (Table 2). It has been well documented that the exposures to high concentrations of O_3 inhibit the net photosynthesis and stimulate the respiration in several plants (Todd, 1958; Hill & Littlefield, 1969; Furukawa & Kadota, 1975). The reduction in NAR represented in the present study suggests that chronic exposures to low concentration of O_3 could also induce the inhibition of net photosynthesis and/or the acceleration of respiration in plants. The reduction in NAR by exposure to 0.09 ppm O_3 has been reported with other herbaceous plant species (Horsman *et al.*, 1980, 1981).

However, for the last-half period of 0.1 ppm O_3 exposure in the present experiments, NAR was recovered or rather accelerated in the similar manner to the change in RGR. A similar result was reported by Oshima *et al.* (1978), who observed the reduction in RGR of the parsley plants caused by the exposure to 0.2 ppm O_3 for the first several weeks, followed by the higher RGR than that of control plants. Such changes in NAR and RGR might be at least partly responsible for the stimulative effects of O_3 on plant growth reported by several workers (Bennett *et al.*, 1973; Harward & Treshow, 1975). During the exposure to O_3, plants might have adapted to the given environmental condition. Although the mechanisms of O_3 phytotoxicity have not yet been defined, there were some studies that O_3 in high concentrations could affect the activities of several enzymes (Dass & Weaver, 1972; Tingey *et al.*, 1976a). Tanaka and Sugahara (1980) reported that poplar plants exposed to 0.1 ppm SO_2 increased in superoxide dismutase activity and thereby became tolerant to acute toxicity of SO_2. In the plants adapted to low concentrations of O_3, the enzymes which participate in the defence of O_3 toxicity might be also induced or activated during the exposure period. Studies on the enzymes relating to O_3 tolerance should be prerequisite to know the mechanisms of adaptation to O_3.

Plants exposed to 0.2 ppm O_3 exhibited the smaller reduction in RGR than that in NAR for the last-half of exposure period (Table 2). The remarkable reduction in NAR induced by O_3 could be compensated by the increase in LAR resulting in smaller reduction in RGR (Fig. 8). The increase in LWR and the reduction in SWR and RWR caused by O_3 indicated that low levels of O_3 had changed the partitioning ratio of photosynthate among leaves, stem and root (Figs. 10, 11 and 12). Bennett and Oshima (1976) observed the reduction in RWR of the carrot plants by a long-term exposure to O_3. Other investigators have demonstrated that O_3 could reduce the dry weight growth of root most severely, resulting in a reduction in RWR in plants (Tingey & co-workers, 1971, 1973, 1975; Oshima *et al.*, 1979). It has been reported that the plants in deficiency of photosynthate showed larger reduction in dry weight growth of root than that of shoot (Curtis & Clark, 1950). The reduction in RWR in O_3-exposed plants might also result from the deficiency of photosynthate caused by O_3. Another report indicated that O_3 reduced the proportion of dry weight of fruits to taht of whole plants (Bennett *et al.*, 1979). Besides, present experiments also showed the reduction in dry weight growth of flower bud caused by O_3 exposure (Table 1). These results suggest the changes in partitioning of assimilates in O_3-exposed plants. The change in the partitioning ratio was probably caused by the inhibition of translocation of photosynthate or by the enhancement of respiration in the dissimilation parts, i.e. root, stem and flower. Recently, Tingey *et al.* (1976c) have suggested the inhibitory effect of O_3 on translocation of photosynthate in ponderosa pine. Accordingly, the increase in total number of leaves and the acceleration of senescence of old leaves in

O_3-exposed plants (Table 1) should be further investigated in view of the changes in partitioning and translocation of photosynthate and other metabolites.

Retardation of stem elongation in O_3-exposed plants might be resulted from the reduction in dry weight growth of stem. On the other hand, ethylene production was reported in plants exposed to high concentration of O_3 (Craker, 1971; Tingey *et al.*, 1976b). It is known that ethylene inhibits stem elongation (Burg & Burg, 1966) and accelerates senescence (Burg, 1968). By exposure to low concentrations of O_3, plants might produce ethylene. Therefore, it is probable that retardation of stem elongation and acceleration of senescence caused by O_3 exposure in the present experiment can be explained by ethylene production.

Shimizu *et al.* (1980) investigated the effects of a long-term exposures to low concentrations of SO_2 on the growth of sunflower plants. They found that SO_2 altered the several growth parameters, which suggested some changes in physiological functions in plants. The method of the growth analysis seems to be usefull to analyze the changes in physiological functions resulting in the changes in growth and yield of plants exposed to an air pollutant and probably to pollutant mixture. However, in order to understand the precise mechanisms of the growth reduction of plants induced by chronic exposures to low concentrations of O_3 and the mechanisms of adaptation of these plants, more direct studies on the effects of O_3 on physiological phenomena, such as photosynthesis, respiration, enzyme activity, translocation of assimilates and hormonal balance must be studied. Studies on some these problems are in progress in our laboratory.

Acknowledgments

We are grateful to the members of the Division of Engineering of our institute, who helped to maintain the environmental conditions of the growth cabinets and advised for cultivation of the plant materials. We are indebted to Dr. N. Kondo for helpful advice and discussion during the course of the present study and the preparation of this manuscript. We are also indebted to Dr. O. Ito for criticizing this paper.

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ヒマワリの生長に及ぼす低濃度オゾン長期暴露の影響

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人工光型の環境制御室で，播種後14日のロシアヒマワリを12日間，0.1及び0.2ppmのオゾンに暴露し，植物の生長に及ぼすO_3の影響について検討した。O_3暴露開始直前と6日め，12日めに植物を選出して，葉面積，器官別乾重などを測定し，生長解析法を用いて，生長の各パラメーターを算出した。0.1及び0.2ppm O_3によって，小白斑状の可視障害が葉面に発現し，下位葉の枯死が促進された。0.1及び0.2ppm O_3に暴露した植物の個体乾重は，対照より各々11%，32%減少した。O_3暴露によって各器官とも乾物生長が抑制されたが，根の生長抑制が著しかったのに比べ，葉の生長はそれほど抑制されなかった。生長解析の結果，0.1ppm O_3は暴露前半の6日間に植物の相対生長率（RGR）や純同化率（NAR）を減少させたが，後半は減少させなかった。これに対して，0.2ppm O_3は暴露期間を通じてRGRやNARを減少させたが，後半におけるRGRの減少率はNARの減少率に比べて小さかった。これは葉面積比（LAR）が0.2ppm O_3によって増加したためであった。また0.2ppm O_3は植物の葉重比（LWR）を増加させたが，茎重比（SWR）や根重比（RWR）を減少させた。これらの結果から，低濃度O_3の長期間暴露は，植物の純光合成速度や光合成産物の分配率に影響することが示唆された。

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Res. Rep. Natl. Inst. Environ. Stud., Jpn., No. 65, 1984.

Effects of Low Concentrations of NO_2 and O_3 Alone and in Mixture on the Growth of Sunflower Plants

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Fourteen-day-old sunflower plants (*Helianthus annuus* L. cv. Russian Mammoth) were exposed to 0.1 ppm nitrogen dioxide (NO_2) continuously and/or 0.1 ppm ozone (O_3) during light period in an artificially-lighted growth cabinet for 12 days. Plants were harvested 0, 6 and 12 days after the start of gas exposure. O_3 alone induced a visible injury as white fleck on matured leaves and the mixture of NO_2 and O_3 increased the extents of the white fleck of injury and of the withering of old leaves, whereas no particular visible injury was observed in NO_2-exposed plants. NO_2 stimulated plant growth, especially in stem dry weight, during the exposure period. O_3 also enhanced dry weight growth, especially in leaves, but only for the last 6 days. However, the mixture treatment reduced the growth of plants. The root dry weight of plants exposed to the mixture of both gases was significantly lower than those of control and of either NO_2- or O_3-exposed plants during the treatment. Growth analysis showed that NO_2 increased relative growth rate (RGR) for the first 6 days of exposure and O_3 increased RGR for the last 6 days, whereas the mixture treatment reduced RGR for the first 6 days. The changes in RGR were largely due to the changes in net assimilation rate (NAR). Although the mixture treatment reduced NAR throughout the exposure period, it resulted in no decrease in RGR for the last 6 days of exposure, because the increase in leaf area ratio (LAR) compensated the decrease in NAR. NO_2 increased stem weight ratio (SWR) and decreased root weight ratio (RWR). O_3 slightly increased leaf weight ratio (LWR) and decreased RWR. The mixture treatment increased LWR and decreased RWR remarkably. These changes in growth parameters indicate that chronic exposures to low concentrations of NO_2 and/or O_3 could affect the net photosynthesis and the pattern of partitioning of assimilates in sunflower plants. The multiple regression analysis represented that the significant interaction effect of $NO_2 \times O_3$ was observed on visible injury, leaf area, dry weight growth of each organ and whole plant, LAR, SWR and RWR.

Key words: Air pollutant mixture, Dry weight growth, Growth analysis, Interaction effect, NAR, NO_2, O_3, Partitioning, RGR, Sunflower plant.

The atmosphere in pollution in industrial regions of the world has been progressed gradually with increase in the human activities, especially after the Industrial Revolution. It has

been reported that high concentrations of such air pollutants as sulfur dioxide (SO_2), ozone (O_3), etc. have induced visible symptoms of injury on plant leaves in the field (Brennan *et al.*, 1967; Hindawi, 1968). During the past decade, however, as the concentration of each pollutant in the atmosphere has become lower, many workers have paid attention to the chronic effects of low concentrations of air pollutants on plant growth (see reviews, Feder, 1973; Unsworth & Ormrod, 1982).

Recently, the concentrations of nitrogen dioxide (NO_2) and O_3 are relatively higher in the urban and industrial areas (Furukawa *et al.*, 1978, 1979). There were several reports that chronic exposures to NO_2 had depressed plant growth, whereas several workers have pointed out that low concentrations of NO_2 have a possibility to increase plant growth, though the plant responses were different depending upon many factors such as NO_2 concentration, species and organs of plants, environmental conditions and the nutrient status of nitrogen (N) (Troiano & Leone, 1977; Totsuka *et al.*, 1978; Yoneyama *et al.*, 1980, Matsumaru *et al.*, 1981). O_3 is one of the most toxic air pollutants. Although many authors reported the growth reduction in plants with chronic exposures to O_3, low concentrations of O_3 could stimulate the plant growth in some cases (Bennett *et al.*, 1973, Harward & Treshow, 1975). Therefore, chronic effects on plant growth of exposures to these pollutants alone have not been clarified.

Furthermore, ambient atmosphere contains many species of pollutants in the field. Many workers have reported the acute and/or chronic effects of low concentrations of pollutants in mixture on visible symptoms of injury, physiological activities, growth and yield of plants. The significant interaction effects of pollutants on plants were also documented (see reviews, Reinert *et al.*, 1975; Ormrod, 1982). However, very few studies were conducted on the chronic effects of exposure to mixed gases of NO_2 and O_3 and the significant interaction effects of $NO_2 \times O_3$ on plant growth were not detected (Sanders & Reinert, 1982), although several authors reproted the interaction effects of $NO_2 \times O_3$ on the acute injury (Nakada *et al.*, 1976; Furukawa & Totsuka, 1979; Furukawa *et al.*, 1981).

In the present study, we investigated the chronic effects of low concentrations of NO_2 and/or O_3 on the dry weight growth of sunflower plants. We exposed sunflower plants to 0.1 ppm NO_2 continuously and/or 0.1 ppm O_3 during light period for 12 days and observed the effects on several growth parameters by means of the plant growth analysis, in order to consider the physiological changes of NO_2- and/or O_3-exposed plants. The interaction effects of $NO_2 \times O_3$ on several attributes to plant growth were analyzed using the multiple regression analysis.

Materials and Methods

Plant material and growth conditions

Sunflower seeds from a single plant bred in our institute were used in all the present experiments. Seeds were immersed in water for 12 h and sterilized by dipping into 1 g l^{-1} Benlate T solution (20% Bis (dimethylthiocarbamyl) Disulfide and 20% Methyl 1-(Butylcarbamoyl)-2-benzimidazolecarbamate, Dupont, Delaware, U.S.A.) for 30 min, followed by rinsing with running tap water for 2 h. Three seeds were sown in each plastic pot (diameter: 11 cm, height: 20 cm) containing a 1.8 *l* mixture of vermiculite, peat moss, perlite and fine gravel (4:4:2:1, v/v). As basal fertilizers, 5 g pot^{-1} of Magamp K ($N:P_2O_5:K = 6:40:5$, w/w, W.R. Grace Co., Tennessee, U.S.A.) was added and the pH of wet medium was adjusted to 6.4 with about 15 g pot^{-1} of magnesia lime. Plants were fertilized regularly (1–3 times $week^{-1}$) with 100–200 ml of 0.1% Hyponex solution ($N:P_2O_5:K$ = 6.5:6:19, w/w, Hyponex Co. Inc.,

Copley, Ohio, U.S.A.) plus Hoagland's No. 2 micro elements solution (Hewitt, 1966), and watered regularly or daily as needed.

After sowing, the pots were brought into a controlled environment growth cabinet (1.7 × 2.3 × 1.9 m^3, Koito Co. Ltd.) to cultivate plants under the following environmental conditions. Air temperature in the cabinet was 25 ± 0.5°C, and relative humidity was 75 ± 5%. Light source consisted of 24 stannous halide lamps (400W, Yoko Lamp, Toshiba), and long wavelength (>800 nm) or emitted radiation was eliminated through a heat absorbing glass filter. Light intensity was about 435 $\mu Em^{-2}s^{-1}$ (ca. 120 Wm^{-2}, 31 klx) at plant height. Light/dark cycle was 14/10 h. Fresh air was led into the cabinet after being passed through activated charcoal and catalist-bearing (containing MnOx and CuO) filters to remove ambient air pollutants. Air velocity in the cabinet was 0.2–0.4 m s^{-1}, and ventilation rate was 75 m^3 h^{-1} (ca. 10 times h^{-1}). The concentration of carbon dioxide (CO_2) in the cabinet was continuously monitored and regulated at 400 ± 4 ppm throughout the experimental period by a controlling system based on an infrared CO_2 gas analyzer (URA-2S, Shimadzu). In the growth cabinet, plants were rotated (2.5 times h^{-1}) on a turntable to minimize possible position effects.

Seedlings were thinned to a single plant per pot for uniformity 7 days after sowing. Twelve-day-old plants were transferred to another controlled environment growth cabinet (Koito Co. Ltd.) for exposure to O_3 and/or NO_2 gases. The size and the environmental conditions in this cabinet were the same as those in the cabinet where plants had been cultivated previously, except for the high ventilation rate up to 800 m^3 h^{-1} (ca. 110 times h^{-1}) to minimize the effects of other pollutants which might be produced by photo- and dark-chemical reactions in the cabinet.

Exposure to NO_2 and/or O_3

Fourteen-day-old plants were exposed to NO_2 and/or O_3 for 12 days. NO_2 induced from a compressed cylinder containing 500 ppm NO_2 in N_2 was injected through a thermal mass-flow controller into the filtrated fresh air stream. The concentration of NO_2 in the cabinet was continuously monitored and regulated by a controlling system based on a chemiluminescent NO-NO_2-NOx analyzer (Model 14, Thermo Electron). O_3 was generated by a silent electrical discharge in dry oxygen and was also injected similarly as NO_2 into the filtrated fresh air stream. The concentration of O_3 in the cabinet was continuously monitored and regulated by a controlling system based on a chemiluminescent O_3 analyzer (Model 806, Kimoto).

The present study consisted of separate growth experiments of 4 treatments: NO_2, O_3, NO_2 plus O_3 (mixture) and control treatments. Each treatment was conducted 2 times except for O_3 treatment which was done only once because of an accidental plant disease during the cultivation at the 2nd time. In NO_2 and mixture treatments, NO_2 concentration was regulated at 0.1 ± 0.005 ppm continuously through the course of treatments, and NO_2 was hardly detected (<0.005 ppm) in other treatments. In O_3 and mixture treatments, the introduction of O_3 into the cabinet was started at the time of light-on and the concentration was linearly raised for 2 h to reach 0.1 ppm. The concentration of O_3 was regulated at 0.1 ± 0.002 ppm for 10 h, and was also linearly lowered for the following 2 h to 0 ppm at the time of light-off. This diurnal change of O_3 concentration was achieved by controlling O_3 flow rate through the thermal mass-flow controller automatically. The average concentration of O_3 during 24 h is about 0.05 ppm. O_3 concentration wsas not above 0.005 ppm in other treatments. Environmental conditions inside the cabinet were almost identical among all growth experiments except for the concentration of each gas, and were kept constant during each experiment.

Harvest

In each experiment, 10 plants were harvested just before the start of gas exposure (0 day) and other 10 plants were also harvested 6 and 12 days thereafter. The extents of visible injury and withering of each leaf were visually assessed in 5% increments of leaf area. The area of each leaf was measured by an automatic planimeter (Model 3100, LI-COR Co. Ltd.). Total number of leaves more than 10 mm in length and plant height were recorded. Plants were divided into leaf laminae, stem, root, flower bud and withered leaves, and were dried at 80–90°C for 3–5 days for weighing the dry matter. Stem part included leaf petioles.

Statistical analysis

The mean values and standard deviations of all growth characteristics of plants in each treatment were calculated at each harvesting time. Significant differences of mean values between every 2 treatments were examined as follows (See Snedecor & Cochran, 1967) At first, Bartlett's test (χ^2-test) was performed to confirm the homogenity of variance. Then the analysis of variance in the one-way classifications (F-test) was done to clarify the effect of the treatments. If a significance was obtained, the difference between 2 treatments was tested one after another using the least significant difference (LSD) and studentized range test. In the present experiments, the extents of visible injury and withering and the dry weight of withered leaves were transformed to a logarithm to satisfy the Bartlett's test.

Because the number of plant in each treatment was not the same in the present study, we used the multiple regression analysis to determine the main and the interaction effects of NO_2 and O_3 instead of the analysis of variance as follows (see Okuno *et al.*, 1971; Hirosaki & Kobayashi, 1979). At first, multiple regression equation was estimated as:

$$Y = b_0 + b_1 x_1 + b_2 x_2 + b_3 x_3$$

where Y is the regression estimate of each growth characteristics (criterion variable), x_1, x_2 and x_3 are the predictor variables (x_1 is ranked as either 1 when NO_2 was exposed or -1 when not, x_2 is also ranked as either 1 when O_3 was exposed or -1 when not, and x_3 is ranked as either 1 or -1 as the result of $x_1 \times x_2$), and b_0, b_1, b_2 and b_3 are the best unbiased estimates of intercept β_0 and partial regression coefficient β_1, β_2 and β_3, respectively. Then the significance of variance due to the multiple regression was examined by F-test, and t-test for the null hypothesis as $\beta_i = 0$, where i = 1, 2 and 3, was performed to clarify the significances of the main effect of NO_2, the main effect of O_3 and the interaction effect of $NO_2 \times O_3$, respectively. We could discuss each main effect when the interaction effect was not significant.

Growth analysis

Relative growth rate (RGR), net assimilation rate (NAR), leaf area ratio (LAR), specific leaf area (SLA), leaf weight ratio (LWR), stem weight ratio (SWR) and root weight ratio (RWR) were calculated in each treatment according to the following formulae (Evans, 1972):

$$RGR = \frac{1}{W} \cdot \frac{dW}{dt} = \frac{\ln W_2 - \ln W_1}{t_2 - t_1}$$

$$NAR = \frac{1}{\bar{F}} \cdot \frac{dW}{dt} = \frac{(W_2 - W_1)(\ln \bar{F}_2 - \ln \bar{F}_1)}{(t_2 - t_1)(\bar{F}_2 - \bar{F}_1)}$$

$$LAR = \bar{F}/W$$

$$SLA = \bar{F}/F$$

$$LWR = F/W$$

$$SWR = S/W$$
and $$RWR = R/W,$$

where W_i and $\bar{F}_i$ are the dry weight of whole plant and the leaf area at time t_i (i: 1 and 2), respectively. F, S and R are the dry weight of leaves, stem and root, respectively. The latter 5 growth parameters were also tested for statistical significance as mentioned before.

Results

Visible injury and withering of leaves

In the plants treated with the mixture of NO_2 and O_3, white fleck which is one of the typical symptoms of visible injury caused by O_3 (Hill *et al.*, 1970; Shimizu *et al.*, 1981) appeared on leaves within 6 days after the start of gas exposure. The symptom was first notable on the tip and the edge of upper surface of leaf laminae, especially of matured leaves, and was spread over the expanded leaves as the exposure was continued. There appeared on leaves neither water-soaked lesions nor necrotic patches which are a typical symptom causedby NO_2 (Taylor & Maclean, 1970). The white fleck was also appeared on leaves in O_3-exposed plants, but the extent of injury was less than those in plants treated with mixture gases (Fig. 1) and in plants subjected to continuous O_3 treatment (Shimizu *et al.*, 1981). In NO_2-exposed and control plants, no particular symptoms were noted, but only the natural senescent yellowing was observed, which was accelerated by NO_2 exposure. The extents of visible injury were significantly different among treatments (Fig. 1), and the interaction effect of $NO_2 \times O_3$ was observed ($P < 0.1$, Table 2).

Lower old leaves became gradually senescent and withered in all treatments, but the dry weight of withered leaves (Table 1) and withering ratio (Fig. 2) were significantly increased by the mixture treatment. However, we could not detect the interaction effect of $NO_2 \times O_3$ on withering, but could observe the main effect of O_3 (Table 2).

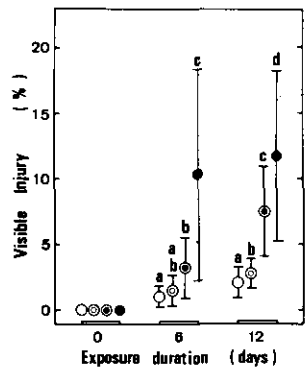

Fig. 1 Effects of exposures to NO_2 and/or O_3 on the visible injury of leaves

Percentage of total leaf dry weight was represented. Natural senescent yellow coloring and withering of leaves were included. Exposures were started 14 days after sowing and continued for 12 days thereafter. Each symbol is the mean of 10 or 20 plants and vertical bars indicate ± standard deviation of the mean. ○: control, ◎: NO_2, ◉: O_3, ●: NO_2 plus O_3 (mixture) treatments. Treatments in each harvest marked by the same letters are not significantly different (LSD, $p < 0.05$). See Materials and Methods in details.

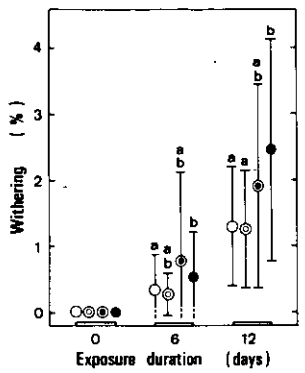

Fig. 2 Effects of exposures to NO_2 and/or O_3 on the withering of leaves

Percentage of total leaf dry weight was represented. Exposures were started 14 days after sowing and continued for 12 days thereafter. See legends for Fig. 1.

Effects on plant growth

As shown in Fig. 3, NO_2 increased the dry weight growth of whole plants for 6 and 12 days of exposure. O_3 also showed the promotive effect on growth for 12 days of exposure. At the final harvest, 9% and 8% increases were detected in NO_2 and O_3 treatments, respectively, as compared with control treatment. However, the dry weight of whole plant treated with mixture gases was slightly smaller than that in control.

Effects of these treatments on the dry weight growth of stem, root and leaf laminae were examined (Fig. 4, 5 and 6). NO_2 increased stem and leaf dry weight significantly for 6 days of exposure, and at the final harvest, 14% and 8% increases were detected, respectively. O_3 also increased stem and leaf dry weight significantly for 12 days of exposure by 9% and 11%, respectively. The root dry weight was not significantly changed by neither NO_2 nor O_3 alone treatment. However, the mixture of NO_2 and O_3 caused the reduction in root dry weight for 6 days of exposure. At the final harvest, root dry weight was reduced by the mixture treatment by 16% relative to control, while stem and leaf dry weight were only slightly reduced and increased, respectively. Each treatment changed the leaf area growth as similar extent as the changes in leaf dry weight growth (Fig. 6 and 7).

As shown in Table 1, the height growth was slightly increased by NO_2 treatment, and slightly decreased by O_3 and mixture treatments. The number of leaves was not so remarkably changed by any treatments, except for the slight increase by NO_2 treatment. A small flower bud appeared on the top of stem of almost every plant at the final harvest, and no significant difference was found among treatments.

The significant interaction effects of $NO_2 \times O_3$ ($P < 0.05$) were obtained in dry weight of leaves, stem, root and whole plant and leaf area (Table 2). On the number of leaves, the significant main effect of NO_2 was obtained, and the main effect of O_3 was significant on height growth.

Effects on growth parameters

Growth analysis was performed to estimate the physiological changes caused by NO_2 and/or O_3. The changes in RGR and NAR in all treatments were presented in Table 3. As compared with control, NO_2 increased RGR by 7% for the first 6 days, while no increase was

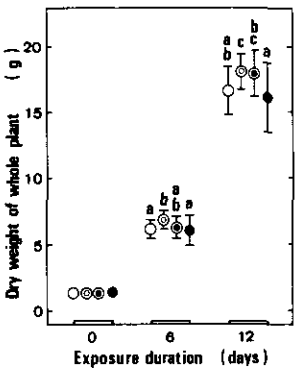

Fig. 3 Effects of exposures to NO_2 and/or O_3 on the increase in dry weight of whole plant

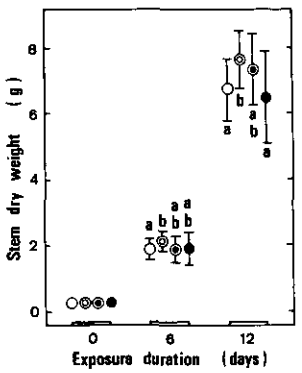

Fig. 4 Effects of exposures to NO_2 and/or O_3 on the increase in dry weight of stem

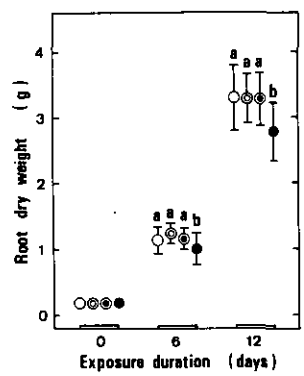

Fig. 5 Effects of exposures to NO_2 and/or O_3 on the increase in dry weight of root

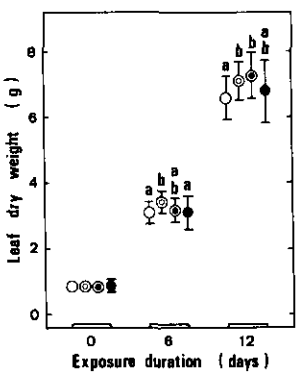

Fig. 6 Effects of exposures to NO_2 and/or O_3 on the increase in dry weight of leaves

Dry weight of withered leaves was excluded. Exposures were started 14 days after sowing and continued for 12 days thereafter. Each symbol is the mean of 10 or 20 plants and vertical bars indicate ± standard deviation of the mean. ○: control, ◎: NO_2, ◉: O_3, ●: NO_2 plus O_3 (mixture) treatments. Treatments in each harvest indicated by the same letter are not significantly different (LSD, $p < 0.05$). See Materials and Methods in details.

detected for the following 6 days. O_3 hardly changed RGR for the first 6 days, but increased RGR by 6% for the following 6 days. In contrast, mixture treatment slightly reduced RGR for the first 6 days, and did not affect for the following 6 days. RGR consists of NAR and LAR. The changes in NAR caused by NO_2 and/or O_3 exposures resembled the changes in RGR. NO_2 first increased NAR by 7% but not for the later period, whereas O_3 increased NAR by 6% for the last 6 days. However, the mixture treatment reduced NAR by 5–6% during the exposure period of 12 days.

LAR did not differ among treatments at each harvesting time, except for the plants grown

Table 1 Effects of exposures to NO_2 and/or O_3 on several characteristics of sunflower plants[1)]

	Treatment			
	Control	NO_2	O_3	$NO_2 + O_3$
Withered leaves (mg dry wt)	$84.0 \pm 54.9^{a2)}$	91.1 ± 66.9^{a}	142.0 ± 115.9^{ab}	162.3 ± 106.7^{b}
Flower bud (mg dry wt)	7.3 ± 4.0^{a}	9.0 ± 11.5^{a}	4.7 ± 3.3^{a}	5.7 ± 4.9^{a}
Plant height (cm)	37.5 ± 3.5^{ab}	39.3 ± 4.6^{a}	35.3 ± 4.9^{b}	36.3 ± 4.0^{b}
Number of leaves	25.6 ± 2.1^{a}	27.5 ± 1.8^{b}	25.4 ± 2.1^{a}	26.3 ± 1.8^{a}

1) Plants were harvested 12 days after the start of exposure. Mean of 10 or 20 plants and standard deviation are indicated.
2) Values in each column followed by the same letters are not significantly different (LSD; $p<0.05$). See Materials and Methods in details.

Table 2 The main and the interaction effects of NO_2 and O_3 on several characteristics of sunflower plants[1)]

effect	Days after the start of exposure					
	6			12		
	NO_2	O_3	$NO_2 \times O_3$	NO_2	O_3	$NO_2 \times O_3$
Visible injury	**2)	**	+	**	**	+
Withering	ns	+	ns	ns	*	ns
Withered dry weight	ns	+	ns	ns	**	ns
Plant height	ns	ns	ns	ns	*	ns
Number of leaves	*	ns	ns	**	+	ns
Leaf area	ns	+	*	ns	ns	*
Leaf dry weight	ns	ns	*	ns	ns	*
Stem dry weight	ns	ns	ns	ns	ns	**
Root dry weight	ns	*	**	*	*	*
Flower dry weight	–	–	–	ns	+	ns
Total dry weight	ns	ns	*	ns	ns	**

1) Results were obtained using the multiple regression analysis. See Materials and Methods in details.
2) Significant levels; +: $p<0.10$, *: $p<0.05$, **: $p<0.01$, ns: not significant, –: not detected.

in the mixture treatment which resulted in a significant increase in LAR by 8% at the final harvest (Fig. 8). LAR can be further divided into SLA and LWR. The SLA of sunflower plants was almost equal among all treatments at each harvest (Fig. 9), while the LWR of O_3-exposed plants became slightly greater and that of mixed gases-exposed plants became significantly (by 7%) greater than that of control plants (Fig. 10). The dry weight ratios of other organs to that of whole plant were also calculated (Fig. 11 and 12). The SWR of NO_2-exposed plants increased by 5% only at the final harvest. In the case of RWR, only the mixture treatment caused a significant reduction of 10% for the first 6 days. At the final harvest, RWR was reduced by exposures to NO_2 and/or O_3 and most strongly by the mixture treatment (by 12%).

The multiple regression analysis represented that the interaction effects of $NO_2 \times O_3$ were

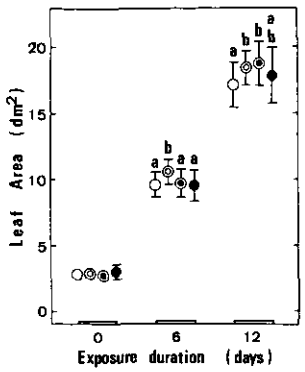

Fig. 7 Effects of exposures to NO_2 and/or O_3 on the increase in leaf area

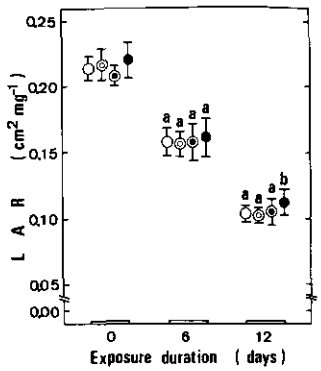

Fig. 8 Effects of exposures to NO_2 and/or O_3 on leaf area ratio (LAR)

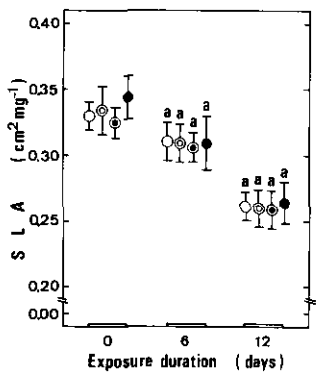

Fig. 9 Effects of exposures to NO_2 and/or O_3 on specific leaf area (SLA)

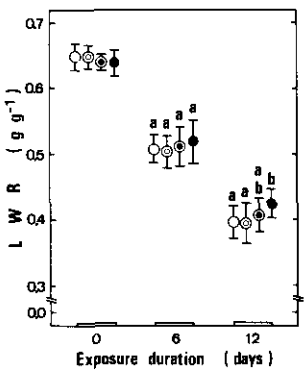

Fig. 10 Effects of exposures to NO_2 and/or O_3 on leaf weight ratio (LWR)

Dry weight of withered leaves was excluded. Exposures were started 14 days after sowing and continued for 12 days thereafter. Each symbol is the mean of 10 or 20 plants and vertical bars indicate ± standard deviation of the mean. ○: control, ◎: NO_2, ◉: O_3, ●: NO_2 plus O_3 (mixture) treatments. Treatments in each harvest indicated by the same letter are not significantly different (LSD, $p < 0.05$). See Materials and Methods in details.

obtained in LAR, SWR and RWR, while the main effects of O_3 were detected in LWR and RWR, and the main effect of NO_2 was also detected in RWR (Table 4).

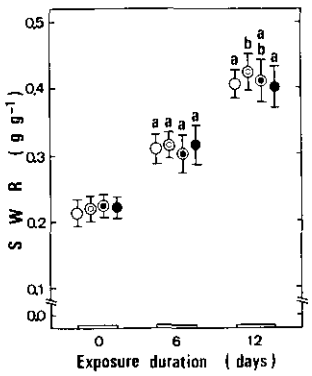

Fig. 11 Effects of exposures to NO_2 and/or O_3 on stem weight ratio (SWR)

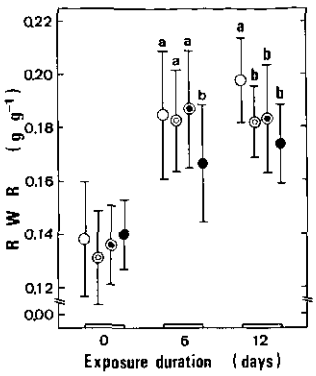

Fig. 12 Effects of exposures to NO_2 and/or O_3 on root weight ratio (RWR)

Dry weight of withered leaves was excluded. Exposures were started 14 days after sowing and continued for 12 days thereafter. Each symbol is the mean of 10 or 20 plants and vertical bars indicate ± standard deviation of the mean. ○: control, ◎: NO_2, ◉: O_3, ●: NO_2 plus O_3 (mixture) treatments. Treatments in each harvest indicated by the same letter are not significantly different (LSD, $p < 0.05$). See Materials and Methods in details.

Table 3 Effects of exposures to NO_2 and/or O_3 on relative growth rate (RGR) and net assimilation rate (NAR) of sunflower plants[1)]

	Exposure duration (days)	Treatment			
		Control	NO_2	O_3	$NO_2 + O_3$
RGR	0–6	0.254	0.272	0.258	0.245
($mg\ mg^{-1}\ day^{-1}$)	6–12	0.166	0.162	0.176	0.164
NAR	0–6	1.437	1.542	1.480	1.365
($mg\ cm^{-2}\ day^{-1}$)	6–12	1.340	1.321	1.414	1.264

1) Dry weight of withered leaves was excluded to calculate these values. See Materials and Methods in details.

Table 4 The main and the interaction effects of NO_2 and O_3 on several growth parameters of sunflower plants[1)]

effect	Days after the start of exposure					
	6			12		
	NO_2	O_3	$NO_2 \times O_3$	NO_2	O_3	$NO_2 \times O_3$
LAR	ns[2)]	ns	ns	ns	**	*
SLA	ns	ns	ns	ns	ns	ns
LWR	ns	ns	ns	ns	**	ns
SWR	ns	ns	ns	ns	ns	*
RWR	*	ns	+	**	**	ns
FWR	–	–	–	ns	ns	ns

1) Results were obtained using the multiple regression analysis. See Material and Methods in details.
2) Significant levels; +: $p<0.10$, *: $p<0.05$, **: $p<0.01$, ns: not significant, –: no data available.

Discussion

Effects of NO_2 alone

In the present experiments, exposure to 0.1 ppm NO_2 for 6 and 12 days increased the dry matter production in sunflower plants (Fig. 3–6). The present results confirmed the earlier works that the low concentrations of NO_2 have a possibility to increase the growth of some plants (Troiano & Leone, 1977; Totsuka *et al.*, 1978; Yoneyama *et al.*, 1980; Matsumaru *et al.*, 1981). The growth analysis indicated that the increase in growth was mainly due to the greater NAR for the earlier period of NO_2 exposure (Table 3). The increase in NAR of sunflower plants exposed to NO_2 was also reported previously (Totsuka *et al.*, 1978). There were several reports that NO_2 depressed the net photosynthetic rate, although exposures were carried out at rather high concentrations (Hill & Bennett, 1970; Srivastava *et al.*, 1975). The increase in NAR observed in the present experiments suggests the possibility that low concentrations of NO_2 increase the net photosynthesis of plants. Yoneyama and Sasakawa (1979) and Kaji *et al.* (1980) clearly demonstrated that NO_2 in atmosphere was absorbed by leaves and was assimilated into amino acids. From these studies, it might be assumed that NO_2 can act as a useful source of N in plants and that NO_2-N was used for constituents of the proteins which participate in the assimilation of CO_2, resulting in the increase in NAR and dry weight growth. It might be also attributable for growth increase that chronic exposure to NO_2 increased the chlorophyll content of leaves (Taylor & Eaton, 1966; Horsman & Wellburn, 1975). Several other characteristics changed by NO_2 exposure such as increases in plant height, leaf area, number of leaves and leaf yellowing might be the secondary effects due to the increase in dry weight growth.

The assimilates increased by NO_2 exposure were mainly accumulated in stem part, as indicated by the increase in SWR (Fig. 11). This suggests that NO_2, as well as SO_2 and O_3 (Shimizu *et al.*, 1980, 1981), also can change the partitioning of the assimilates. Stem might act as a storage organ for the excess assimilates. There were several observations that stem kept assimilates in temporary store at the time of flowering (Wardlaw, 1968). In the present experiments, SWR was significantly increased by NO_2 during the last 6 days, when the flower bud began to form and developed. In sunflower and several other plants, whose dry weight was

increased by NO_2, accerelation of stem growth was most remarkable (Yoneyama *et al.*, 1980).

Effects of O_3 alone

It is unexpected that O_3 accerelated the dry weight growth after 12 days of exposure (Fig. 3–6). There were a few other investigations where low concentrations of O_3 can stimulate the plant growth (Bennett *et al.*, 1973, Harward & Treshow, 1975). According to the growth analysis, the dry weight increase in O_3-exposed plants was also derived from the increase in NAR, which occurred for the later period of exposure (Table 3). Although many authors observed the inhibitory effects of O_3 on net photosynthesis at rather high concentrations (Todd, 1958; Hill & Littlefield, 1969), chronic exposure of sunflower plants to low concentrations of O_3 might accrelate the net photosynthetic rate. The increase in NAR as compared with that of control during the later period of O_3 exposure had been also observed by Walmsley *et al.* (1980) and Shimizu *et al.* (1981). O_3 was thought neither to act as a nutrient, as SO_2 and NO_2 do, nor to increase the chlorophyll content of leaves. The individual processes of carbon assimilation such as membrane permeability of CO_2 and enzyme activities participating in CO_2 fixation should be investigated with regard to O_3 exposure.

In the previous paper, we observed the growth reduction of sunflower plants continuously exposed to 0.1 ppm O_3 for 12 days (Shimizu *et al.*, 1981). The discrepancy between the results of the present study and the previous one might be due to the lower average concentration of O_3 per 24 h, the intermittently exposure to O_3 or the absence of O_3 at night time in the present experiments. Although the average concentration of O_3 was lower in the present experiments, the concentration during day-time was the same as that in the pervious one. The exposure dose of O_3 is not thought to be important but the dose of O_3 absorbed in leaves should be effective. The transpiration rate of sunflower plants during night-time was almost 10–20% of that during day-time. Because the absorption rate of O_3 can be approximately estimated from the transpiration rate (Omasa *et al.*, 1979), the amount of O_3 absorbed in plants would not be so different between the present and the previous studies. The translocation of assimilates might be related to the difference of O_3 effects between both studies. The translocation of assimilates occurred during night-time as well as during day-time (Warblaw, 1968), and could be inhibited by O_3 exposure (Tingey *et al.*, 1976; McCool & Menge, 1983). The accumulation of assimilates in leaves caused by the inhibition of the translocation should induce the endproduct inhibition on net photosynthesis. Other many metabolic activities which might be inhibited by O_3 exposure should be also recovered during the period without O_3 exposure. There is also a possibility that the enzyme which participates in the neutralization of O_3 toxicity is not or less available in the dark.

We observed the representative responses of plants to O_3 in the present experiments as well as in the previous ones. O_3 induced visible symptoms of injury as white fleck, accelerate withering and inhibited height growth (Fig. 1 and 2, Table 1). As compared with control, RGR and NAR were increased in the later period of O_3 exposure (Table 3). The ratio of the increase in dry weight of leaves to that of stem or root became greater in O_3-exposed plants as observed in the slight increase in LWR (Fig. 10). O_3 could also change the partitioning of assimilates, which might be induced by the O_3 inhibition on translocation of assimilates (Tingey *et al.*, 1976; McCool & Menge, 1983). These changes caused by O_3 were clearly different from the changes caused by NO_2. The significant main effects of O_3 detected by the multiple regression analysis also confirmed that depression in height growth, the withering of leaves and the increase in LWR were caused by O_3 exposure, specifically.

Effects of mixture of NO_2 and O_3

The mixture treatment decreased the growth of sunflower plants, significantly as compared with the treatment of NO_2 or O_3 alone and slightly as compared with control (Fig. 3). The growth analysis clarified that the reduction in RGR in the mixture treatment for the first 6 days was due to the depression in NAR (Table 3). The NAR of plants exposed to mixed gases was markedly lower than those of other 3 treatments for the exposure period of 12 days. Furukawa and Totsuka (1979) reported the significant reduction in net photosynthetic rate of sunflower plants caused by simultaneous exposure to NO_2 and O_3 although each gas alone had no depressive effect. The reduction in NAR observed in the present experiments suggests that simultaneous exposure to low concentrations of NO_2 and O_3 also could depress the net photosynthetic rate.

However, for the last 6 days of exposure, the reduction in NAR induced by the mixture treatment was compensated by the increase in LAR (Fig. 8), resulting in the similar value of RGR to that of control plants (Table 3). These changes resembled the response of plants to 0.2 ppm O_3 in the previous study (Shimizu *et al.*, 1981). The elevated LAR was almost due to the increase in LWR (Fig. 10). RWR was significantly reduced (Fig. 12), due to the significant decrease in growth of root dry weight (Fig. 6). These results indicate that the mixture treatment also altered the partitioning of assimilates in the same way as O_3 alone exposure induced, but the extent of changes by the mixture treatment was greater than that by O_3 alone treatment. Similar changes in LWR and RWR have been reported several plants exposed to O_3 (Bennett & Oshima, 1976; Oshima *et al.*, 1978; Shimizu *et al.*, 1981; etc.).

It has been reported that plants deficient in photosynthates showed the larger reduction in dry weight growth of root than that of shoot (Curtis & Clark, 1950; Ryle & Powell, 1976). However, in the present experiments, dry matter production of whole plant became larger in O_3 alone or hardly smaller in the mixture treatment than in control treatment, whereas O_3 alone and the mixture treatments increased LWR and decreased RWR (Fig. 10 and 12). Therefore, it can be stressed that the changes in LWR and RWR should be directly influenced by O_3. In the mixture treatment, we also observed the white fleck symptom, the increase in withering and the decrease in height growth (Fig. 1 and 2, Table 1). These phenomena and the changes in RGR, NAR and partitioning of assimilates were almost similar to those of O_3 alone treatment in the previous study (Shimizu *et al.*, 1981). Therefore, effects of the mixture of NO_2 and O_3 seem to be similar to the effects of rather high concentrations of O_3 alone.

The significant interaction effects of $NO_2 \times O_3$ were observed in many attributes to plant growth (Table 2 and 4). Reinert and Gray (1981) and Sanders and Reinert (1982) reported that the interaction effects of $NO_2 \times O_3$ could not be significant on the growth of radish and azalea plants. However, in their studies, O_3 alone treatment strongly affected the growth of the test plants. In other studies, even when NO_2 or O_3 alone had little or no effects, the significant interaction effects of $NO_2 \times O_3$ was detected on visible injury (Furukawa *et al.*, 1981), photosynthesis (Furukawa & Totsuka, 1979) and pollen-tube elongation (Nakada *et al.*, 1976). In other combination such as SO_2 plus NO_2 or SO_2 plus O_3, there have been similar trends of the significance of the interaction effects of these gases (see reviews Ormrod, 1982).

The multiple regression analysis used here is based on the assumption that there is a linearity between the plant response and the concentration of a pollutant. However, many responses of plants to air pollutants are thought to be changing as sigmoidal curve. Then if each gas affects the plants in such a manner as the other do, the observed interaction effects were only statistically significant and it is not clarified whether biological meaning exists or not. In the present experiments, however, growth alteration by NO_2 alone was remarkably different from that by O_3 alone, and the mixture treatment seems to only increase the O_3-induced

visible injury and growth change. Therefore, it was suggested that NO_2 may act as catalyst only to enhance the action of O_3 on physiological processes and entire growth of plants. In order to clarify the meaningful interaction effects in biology (see Heagle & Johnston, 1979), more precise mechanisms for the effects of each pollutant alone and in mixture on physiological phenomena concerning the plant growth must be investigated.

There seems a serious problem that the mixture of low concentrations of NO_2 and O_3 may depress NAR and alter the partitioning of assimilates. In the field, more susceptible plants than sunflower plants might be remarkably affected by the ambient atmosphere containing low concentrations of NO_2 and O_3. Furthermore, ambient atmosphere also contains low concentrations of SO_2. SO_2 was also known to affect the physiology and growth of plants (Shimizu *et al.*, 1980), and the significant interation effects of $SO_2 \times NO_2$ or $SO_2 \times O_3$ were also reported (Reinert *et al.*, 1975; Ormrod, 1982). Plants grown in the field receive these mixed gases with relatively low concentrations continuously or intermittently. The mixed gases of SO_2, NO_2 and O_3 might affect seriously on plants, as reported by Fujiwara *et al.* (1973), Furukawa and Totsuka (1979), Elkiey and Ormrod (1980), Reinert and Gray (1981), Reinert *et al.* (1982), Sanders and Reinert (1982) and Kress *et al.* (1982). The more intensive investigations on the effects of chronic exposures to mixed gases should be carried out in order to determine the air quality standard of ambient atmosphere.

Acknowledgments

We are grateful to the members of the Division of Engineering of our institute, who helped to maintain the environmental conditions of the growth cabinets and advised for cultivation of the plant materials and Mr. T. Kitagawa for technical assistance in harvest. We are indebted to Dr. N. Kondo, Dr. N. Kachi and Dr. K. Okano for helpful advice and discussion during the course of the present study and the preparation of this manuscript. We are also indebted to Dr. S. Hirosaki for his kind advice on the statistical evaluation. His computer programe of the multiple regression analysis was used in the present study.

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ヒマワリの生長に及ぼす低濃度の二酸化窒素とオゾンの単独及び混合暴露の影響

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人工光型の環境制御室で，播種後14日のロシアヒマワリを12日間，0.1ppm NO_2（連続），0.1ppm O_3（明期），及びこれらの混合ガスに暴露し，植物の生長に及ぼす NO_2 と O_3 の複合汚染の影響について検討した。ガス暴露開始直前と6日め，12日めに植物を選出して，葉面積，器官別乾重などを測定し，生長解析法を用いて，生長の各パラメーターを算出した。O_3 単独暴露区及び混合暴露区の植物では，O_3 に特有な小白斑状の可視障害が葉面に発現し，また下位葉の枯死が促進されたが，NO_2 単独暴露区の植物には，可視障害は認められなかった。NO_2 は暴露前半から植物の生長，特に茎の生長を促進した。O_3 は暴露後半になって特に葉の生長を促進した。しかし混合暴露では，前半から植物の生長が顕著に抑制された。生長解析の結果，NO_2 は暴露前半に植物の相対生長率（RGR）や純同化率（NAR）を増加させた。また O_3 は暴露後半にRGR や NAR を増加させた。混合暴露では，前半に RGR や NAR が抑制されたが，後半は NAR が抑制されていたにもかかわらず，RGR は対照と変わらなかった。これは暴露後半における葉面積比（LAR）が NO_2 と O_3 の混合暴露によって増加したためであった。NO_2 は茎重比（SWR）を増加させ，根重比（RWR）を減少させた。O_3 は葉重比（LWR）を若干増加させ，RWR を減少させた。一方混合暴露は顕著に LWR を増加し，また RWR を減少させた。これらの結果から，低濃度の NO_2 と O_3 の単独及び混合暴露は，植物の純光合成速度や光合成産物の分配率に影響することが示唆された。植物の生長に関する上記の属性について重回帰分析を行った結果，有意な NO_2 と O_3 の複合効果が可視障害や葉面積，各器官及び植物個体の乾重，LAR，SWR，RWR などに認められた。

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Res. Rep. Natl. Inst. Environ. Stud., Jpn., No. 65, 1984.

An Analysis of Height Growth of Japanese Black Pine *(Pinus thunbergii)* in Kashima Industrial Area by Curve-fitting Techniques

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Height growth of Japanese black pine (*Pinus thunbergii*) under different conditions of air pollution in an industrial area in Kashima, Japan was analyzed by fitting the growth curves to the Gompertz equation. Growth of different individuals with different sizes was recorded at three sites for one year. Parameters of the Gompertz equation were estimated from the regression of RGR against the logarithm of the size. The estimated growth curves were different from site to site, but we could not obtain evidence which suggested deteriorating effects of air pollution on the growth of Japanese black pine.
Key words: Air pollution, Field observation, Gompertz curve, Kashima, *Pinus thunbergii*, Plant growth.

There have been reported by several workers that plants grown in Kashima industrial area, Japan were affected by phytotoxic air pollutants such as SO_2, NO_2, oxidants, fluoride, and ethylene (Tominaga, 1974; Tominaga & Miyamoto, 1975 a, b; Miyamoto & Saijo, 1976; Ebara, 1977, 1978; Miyamoto, 1977, 1978; Yokobori, 1978; Yokobori & Taoda, 1980, Yokobori & Ohta, 1983). Yokobori and Ohta (1983) suggested that the width and density of tree rings of Japanese red pine (*Pinus densiflora*) grown in this area were influenced by the intensity of combined air pollution.

To study human impact on environment, it is a useful method to compare the growth of a particular species grown under different conditions and at different places. In most cases, however, it is difficult to apply this method to the plants grown in the field, because their initial conditions are different from site to site. In such a case it is convenient to employ a growth curve specified by a few parameters. Using this process we should be able to compare the growth patterns grown at different sites.

In this report, we measured growth of various sized Japanese black pine (*Pinus thunbergii*) for one year at differently polluted sites in Kashima industrial area. Based on the size-growth rate data, we compared the height growth using Gompertz curve to examined the possibility that the air pollution would affect the growth of *Pinus thunbergii* in this region.

Study Area

Kashima is located in the east of Kanto Plane, facing the Pacific Ocean (latitude 35°55′ N and longitude 140°40′ E). Geological features of this area are characterized by alluvium lowlands and coastal sand dunes. The climax vegetation is a warm-temperate evergreen forest dominated by *Machius thunbergii* and *Castanopsis cuspidata* var. *sieboldii* (Akatsu & Horiuchi, 1971), but prevailing forest vegetation is a secondary forest dominated by *Quercus serrata*, *Pinus densiflora* and *Pinus thunbergii* and a plantation of these pine species. Table 1 shows monthly mean air temperatures and monthly rainfall at Choshi (30 km south-east of Kashima) from 1976 to 1980. The climate around Choshi is relatively mild and oceanic, where an annual mean air temperature is 15.5°C and annual rainfall is about 1550 mm. North-east sea winds prevail during the growing season.

The development of Kashima industrial area (about 2100 ha) depends on Kashima Port, which was commenced from 1962. The operations of factories including steel and petrochemical industries and a electric power plant were started in 1970. Smokestacks of factories about 200 m high are located at the entrance of the port.

Table 1 Mean monthly air temperatures and monthly distributions of rainfall at Chosi, 30 km SE of Kashima

Month	Mean temperature (°C)	rainfall (mm)
January	6.0	79.4
February	6.4	82.9
March	9.2	163.2
April	13.2	120.0
May	17.2	132.2
June	20.3	126.3
July	23.1	100.0
August	24.3	115.0
September	23.3	190.1
October	19.2	219.1
November	14.7	156.1
December	9.4	65.4
Annual	15.5	1550

Methods

Collection of data

Growth of *Pinus thunbergii* was determined at three localities (Site A–C) in and around Kashima industrial area. The selection of the sites were based on the isopleth of air pollution reported by Yokobori (1978) (Fig. 1).

Site A was situated in Mizu-Jinja Shrine at Kamisu-Cho. This site was in the south-east of the port and faced a traffic road. The pine trees had been planted and their density was highest among the three sites surveyed. The mutual shading of the plant was remarkable in individuals which were more than 3 meters high. Soils were composed of sands containing low amounts of organic matter. Although the soil surface was covered with litter of pine, there was no distinct

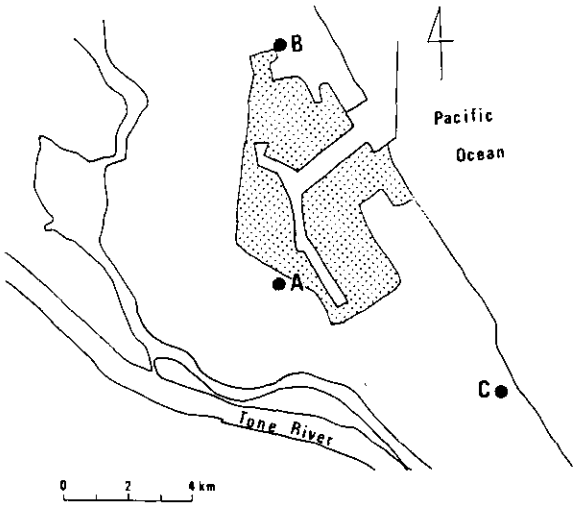

Fig. 1 Map showing sampling sites in and around Kashima industrial area
Dotted area indicates the industrial area. A, Kamisu-Cho; B. Kashima-Cho; C, Hazaki-Cho.

soil layer structure.

Site B was set in Kuriu-Kohen Park at Kashima-Cho. It was situated in the north of the port and was adjacent to a factory of metalworking. There were pine plantations including some naturally occurring individuals. Density of the pine plants was lower than that at site A and mutual shading was insignificant in higher trees more than 3 meters, but some trees lower than 1.5 meters were shaded by larger ones. Soils were sands, whose properties were similar to those in site A.

Site C was near the seashore and was located ca. 5 km south-east of the industrial area. There was natural growth of pine plants on fixed dunes with low densities. Mutual shading was not apparent. The site was topographically uneven, containing wet lowlands and dry dunes. All the observed plants were on the dry dunes. A grass vegetation of *Miscanthus sinensis* and *Imperata cylindrica* var. *koenigii* was dominant and pine trees lower than 1 meters were shaded by these grasses in summer seasons.

In each site, about 50 individuals of various sized *Pinus thunbergii* were selected. They were marked by winding a wire numbered with DYMO-tape around the trunk. On March 24, 1980 and on March 25, 1981, tree heights, girths beneath the first lateral branch, lengths of internodes of a major axis for the previous three years were determined. RGR of tree height was calculated for each individuals by:

$$RGR = (\ln H_1 - \ln H_0)/\Delta t \qquad (1)$$

where H_0 and H_1 are tree height (cm) in 1980 and 1981, respectively and Δt is a time period of 1 year.

Data analysis

Growth data of pine plants were analyzed by HITAC M180 Computer, using a program

package for statistical analyses, BMDP, supplied by Hitachi-Seisakujo.

In general, variables of plant growth have a tendency that the variance increases with increasing mean. Since comparisons of variables with different variances have statistically many limitations, such variables should be transformed into those having similar variances before comparisons. When the standard deviation increases proportionally with increasing mean, a log-transformation of the variable results in a constant variance (Snedecor & Cochran, 1967). Therefore, in this report comparisons of the mean were made after log-transformation of variables. Because the sampling was not made randomly, direct comparisons of plant sizes among the three sites were not suitable. Thus, growth patterns of averaged plants in each site were estimated from one-year growth of individual trees as a function of plant size under the assumption that the environmental conditions during the whole life time of the plants would be the same as those during the period when the growth data were collected. Kaufmann (1981) suggested that the growth estimation as a function of size allows a comparison of growth curves obtained from several different treatments of the test organisms. Size is usually more closely related to the growth rate than age. Furthermore, collecting size data is faster, easier and cheaper than measuring individual growth directly throughout the whole life time.

Estimation of growth patterns from the relationship between the size and the growth rate are based on the method of curve-fitting of individual growth. This method was reviewed by Yamagishi (1977). In this report, a method for curve fitting to Gompertz equation was applied. Gompertz curve is sigmoid in shape and asymmetrical about the inflection point. The equation is specified as:

$$H = KA^{pt} \quad (0 < p,\ A < 1) \tag{2}$$

where K is the maximal tree height (H approaches K with increasing t, because $0 < p < 1$). Hear let $b = -\ln p$, Eq (2) becomes:

$$H = KA^{\exp(-bt)} \tag{3}$$

Let H at $t = 0$ be H_0, and Eq (3) is solved for A

$$A = H_0/K \tag{4}$$

Substituting this relation to Eq (3) results in:

$$H = K\,(H_0/K)^{\exp(-bt)} \tag{5}$$

Let $H_0/K = \exp(-a)$, Eq (5) becomes:

$$H = K \exp\ -a^{\exp(-bt)} \tag{6}$$

Taking logarithms of both sides results in:

$$\ln H = \ln K - a \exp(-bt) \tag{7}$$

Thus RGR of H is described as:

$$1/H\ dH/dt = ab \exp(-bt) \tag{8}$$

Because 'a' and 'b' are constants, RGR of H decreases exponentially with time.
From Eq (7)

$$a \exp(-bt) = \ln K - \ln H \tag{9}$$

Substituting Eq (9) into Eq (8) results in:

$$RGR = b \ln K - b \ln H \tag{10}$$

Because 'K' and 'b' are constants, the relationship between RGR and ln H is linear.

When H_0 is known, 'a' in Eq (9) is directly solved by putting t = 0 and $H = H_0$:

$$a = \ln K - \ln H_0 \tag{11}$$

However, in the present study H_0 was unknown. In this case, let H at time t and t+1 be H_t and H_{t+1}, Eq (7) becomes:

$$\ln H_t = \ln K - a \exp(-bt), \text{ and} \tag{12}$$
$$\ln H_{t+1} = \ln K - a \exp[-b(t+1)] \tag{13}$$

Deleting 'a' from these equations results in:

$$\ln H_{t+1} = \exp(-b) \ln H_t + \ln[K[1-\exp(-b)]] \tag{14}$$

Eq (14) implies that a linear relationship is obtained between logarithms of H_t and H_{t+1}, characterized by Y-intersept $\ln[K[1-\exp(-b)]]$ where X = 0 and a slope $\exp(-b)$.

Results

Table 2 summarizes the data on plant size of *Pinus thunbergii* grown at three sites in Kashima industrial area. The order of the mean tree height was A > B > C, while the values at site B showed the largest variations and included both highest and lowest individuals of all plants observed at the three sites. The size distribution at site B was bimodal with peaks of 5 and 1 meters whereas sites A and C showed monomodal distribution. However, there is no statistical bases of concluding that these patterns of size distribution reflected the patterns of the original populations, because the sampling was not done randomly.

Processes in growth of Pinus thunbergil

(a) Relationships between girths, lengths of internodes, and tree height

Fig. 2 (a)–(c) and Fig. 3 (a)–(c) show the allometric relations of the girth to the tree height at each site in 1980 and 1981, respectively. There were high correlations between these two variables (r = 0.810 – 0.953), and all of the regression coefficients were within the range of 0696 and 0.991. The regression coefficients less than 1.0 imply that the relative growth rates (RGR) of the tree height were lower than those of the girth. No significant differences in the regression lines among the three sites could be detected in either year. Thus, all the data obtained at the three sites were combined. The regression lines were:

$$\log Y = 0.871 \log X + 1.190 \text{ in 1980, and} \tag{15}$$
$$\log Y = 0.779 \log X + 1.349 \text{ in 1981,} \tag{16}$$

where X is the girth (cm) and Y is the tree height (cm).

In general, biomass of an individual tree is estimated by the allometric relation:

$$\log W = p \log(D^2 H) + q$$

where W is the biomass, D is the tree daiameter at breast height, and H is the tree height, Constants 'p' and 'q' are specific for plant species and/or localities. In most cases 'a' is close to 1.0 (Ogawa & Kira, 1977). Constant 'a' is considered to be unchanged even when the parameter D is substituted by the girth. Therefore, $(\text{girth})^2 H$ can be the indicator of biomass. Fig. 4 (a) and (b) shows the relationships between $(\text{girth})^2 H$ and H^3 at the three sites in 1980 and 1981 on a log-log plot. No significant differences were observed between the three sites. In both years

Table 2 Attributes concerning plant size of *Pinus thunbergii* in three sites (A, B and C) in Kashima industrial area

Measurements were made on March 24, 1980 and March 25, 1981.

Attributes	Sites	Year	Mean	S.D.	Minimum	Maximum
Tree height (cm)	A	1980	248.2	85.0	113.0	416.0
		1981	313.5	84.5	163.0	480.0
	B	1980	220.0	174.6	39.0	670.0
		1981	258.9	184.2	68.0	680.0
	C	1980	193.9	65.9	80.0	357.0
		1981	234.8	67.4	102.0	384.0
Girth (cm)	A	1980	25.4	9.2	12.0	46.0
		1981	28.6	9.2	17.0	51.0
	B	1980	17.5	13.6	4.8	43.0
		1981	19.5	14.7	5.0	44.0
	C	1980	19.5	7.4	5.0	37.0
		1981	25.0	8.5	9.5	45.0
Length of internode in 1978 (cm)	A	1980	43.8	16.9	10.0	73.0
		1981	58.7	19.4	21.0	107.0
	B	1980	28.4	15.0	6.0	70.0
		1981	34.4	16.8	14.5	81.0
	C	1980	34.5	14.5	8.0	74.0
		1981	49.7	15.9	24.0	79.0
Length of internode in 1979 (cm)	A	1980	58.0	18.6	19.0	105.0
		1981	56.9	15.0	33.0	97.0
	B	1980	34.6	16.8	15.0	79.0
		1981	25.9	16.5	5.5	70.0
	C	1980	47.5	15.4	22.0	78.0
		1981	34.4	11.0	16.0	65.0
Length of internode in 1980 (cm)	A	1980	56.3	15.6	31.0	97.0
		1981	72.6	23.1	28.0	150.0
	B	1980	26.7	17.4	5.0	74.0
		1981	43.4	19.5	13.0	94.0
	C	1980	34.0	11.6	16.0	70.0
		1981	47.6	16.5	20.0	90.0
Length of internode in 1981 (cm)	A	1981	46.7	17.1	12.0	76.0
	B	1981	29.7	15.0	7.0	70.0
	C	1981	39.3	15.8	18.0	88.0

there were very high correlations ($r = 0963$ in 1980 and 0.951 in 1981) and the regression lines were:

$$\log(G^2 H) = 0.944 \log H^3 + 2.323, \text{ and} \quad (17)$$

$$\log(G^2 H) = 0.880 \log H^3 + 2.717, \text{ respectively,} \quad (18)$$

where G means girth in cm. The above mentioned high correlations demonstrate that tree height is a good indicator for plant biomass. However, RGR of the biomass is expected to be larger than that of the tree height, because the regression coefficients were less than 1.0. The coefficients less than 1.0 result from the advanced hypertrophic growth of the trunk over the height growth of the trees.

Positive allometric relationships were obtained between the length of the internodes and the tree height. The regression coefficients between them fluctuated from 0.131 to 1.160.

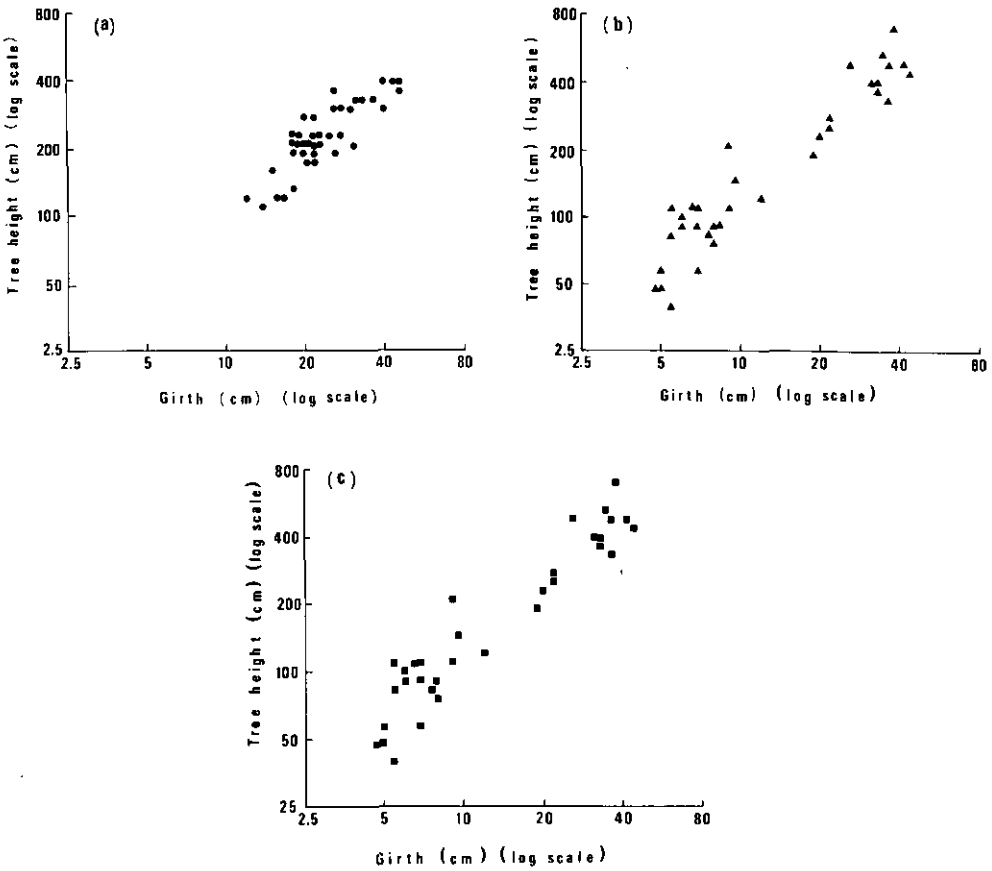

Fig. 2 Allometric relationships between girth (cm) and tree height (cm) of *Pinus thunbergii* grown at three sites: A (a), B (b), and C (c)
Measurements were made on March 24, 1980.

(b) RGR of tree height in relation to size

Fig. 5 (a)–(c) shows the relationships between RGR of the tree height and the tree height in 1980. At all sites negative correlations were obtained, suggesting sigmoidal growth of the tree height. The X-intercept where RGR is zero indicates the estimated maximal tree height. Since height growth is a summation of growth of each internode, it is expected that RGR of internodes shows a negative correlation to the lengths of the internodes. Fig. 6 (a)–(c) show the relationships between lengths of internodes developed in 1979–1981 and their RGRs for the period from March 24, 1980 to March 25, 1981 at three sites. Except for the internodes developed in 1979 at site B, there were clear negative correlations.

Fitting of height growth to the Gompertz curve

Estimations of parameters of growth curves in tree height were made from the relationships between RGR and plant size.

Tree heights in 1981 were plotted against the tree height in 1980 on a log-log plot (Fig. 7) and a linear relationship was obtained;

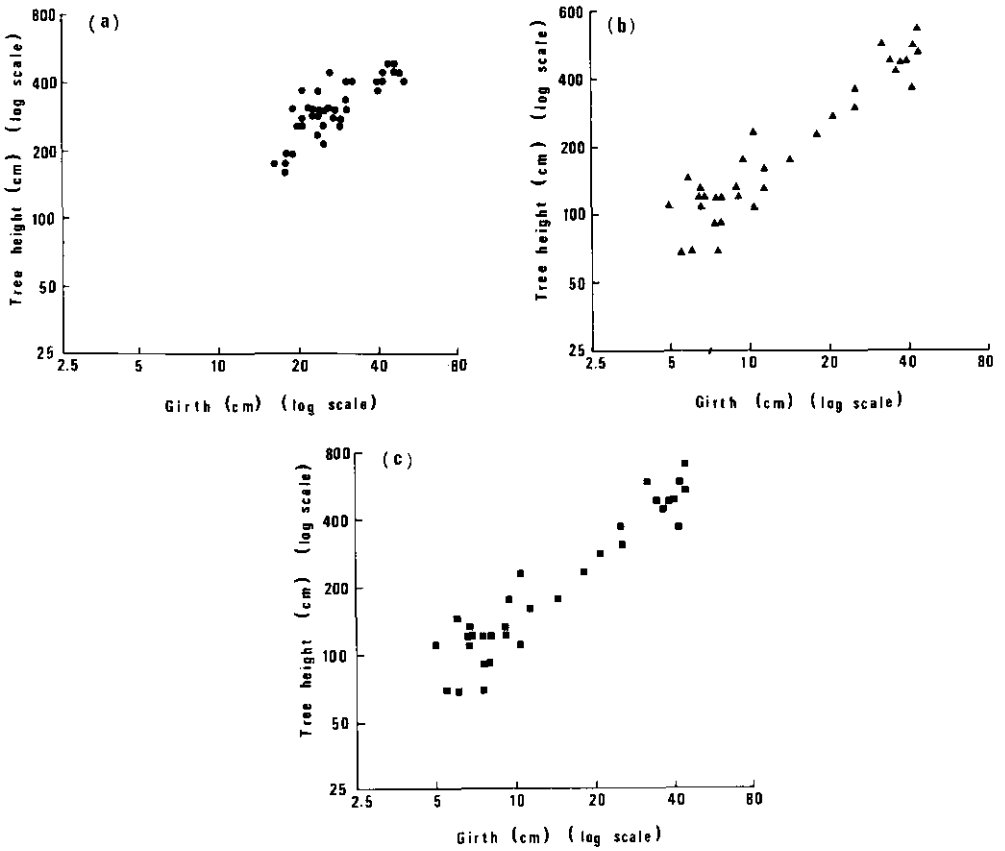

Fig. 3 Allometric relationships between girth (cm) and tree height (cm) of *Pinus thunbergii* grown at three sites: A (a), B (b), and C (c)
Measurements were made on March 25, 1981.

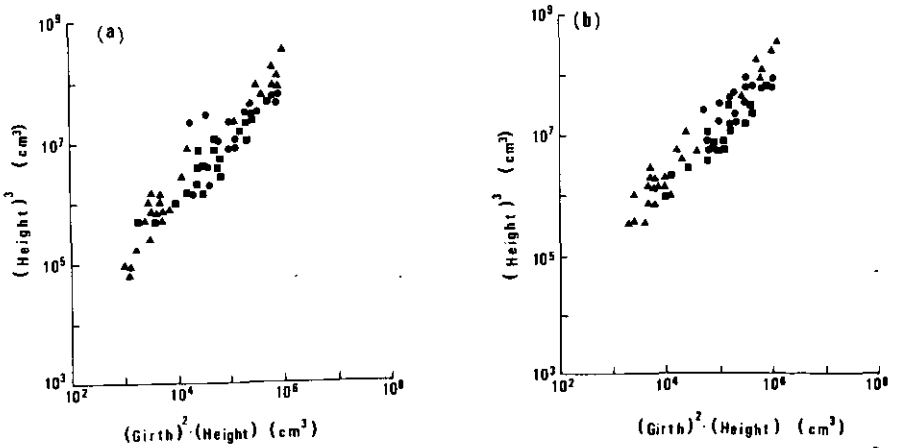

Fig. 4 Allometric relationships between (girth)^2(height) and (height)^3 of *Pinus thunbergii* grown at three sites: A (●), B (▲), and C (■)
Measurements were made on March 24, 1980 (a) and March 25, 1981 (b).

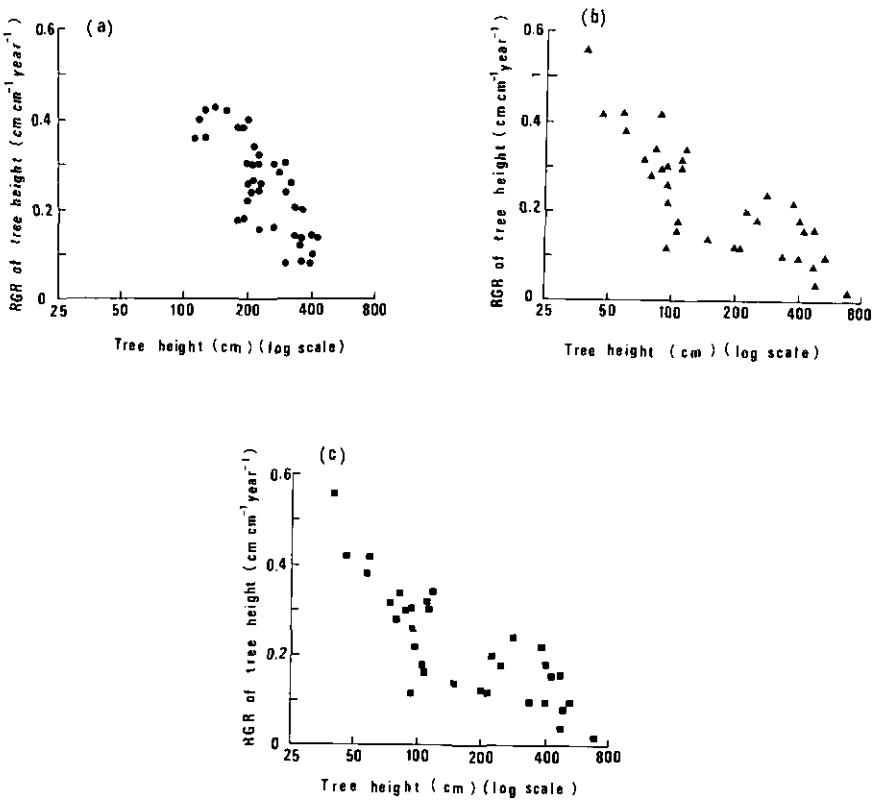

Fig. 5 Relationships between tree height (cm) and its RGR (cm/cm/year) of *Pinus thunbergii* grown in the industrial area in and around Kashima

(a), Site A; (b), Site B; (c), Site C.

$$Y = 0.772\,X + 0.651 \tag{19}$$

where X and Y are the normal logarithms of the tree height in 1980 and 1981, respectively. The maximal tree height is estimated as the X-coordinate of intersection between the regression line and the line Y = X. The value was 2.856 on a log-scale or $10^{2.856} = 718$ cm. When a linear relationship is obtained on a log-log plot as in Fig. 7, the growth can be described as the Gompertz equation (Kaufmann 1981 and see Methods). Since the Y-intersept where X = 0 in Fig. 7 was 0.651 in the common logarithms, it is transformed to the natural logarithms and putting the value to Eq (9);

$$1.50 = \ln[K[1 - \exp(-b)]] \tag{20}$$

Putting K = 718 cm into the above equation and solving for b:

$$b = 0.26. \tag{21}$$

Here let the tree height at t = 3 years be taken as 100 cm, Eq (7) becomes:

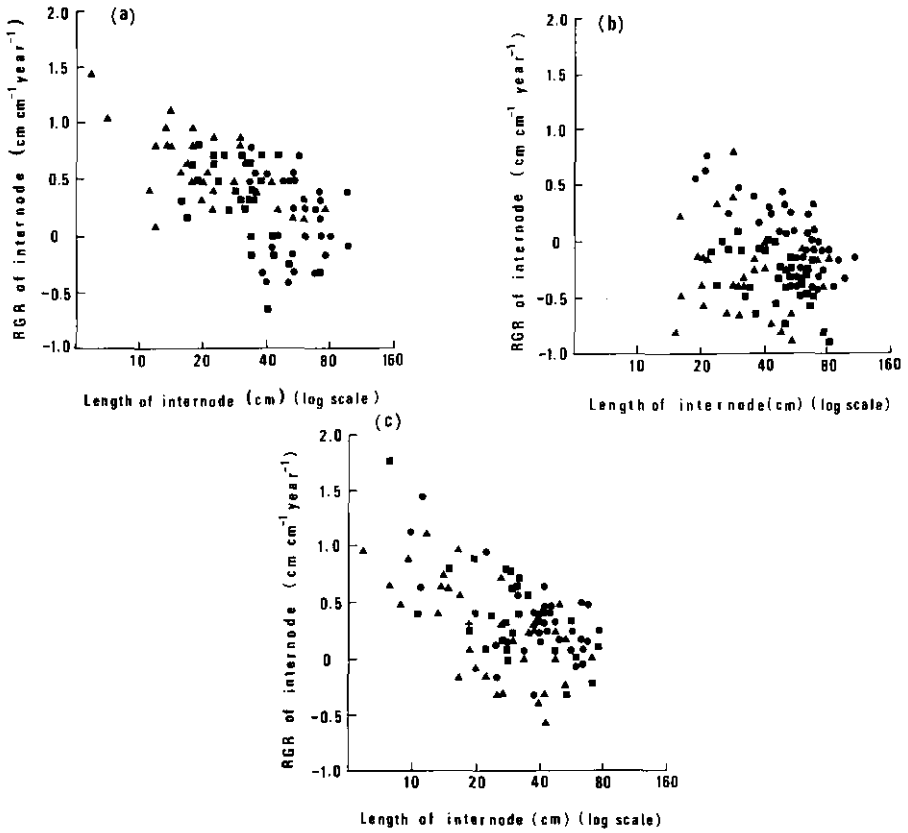

Fig. 6 Relationships between length of internodes (cm) and their RGRs of *Pinus thunbergii* grown in Si te A (●), Site B (▲), and Site C (■)
Years of development of internodes are 1980 (a), 1981 (b) and 1982 (c).

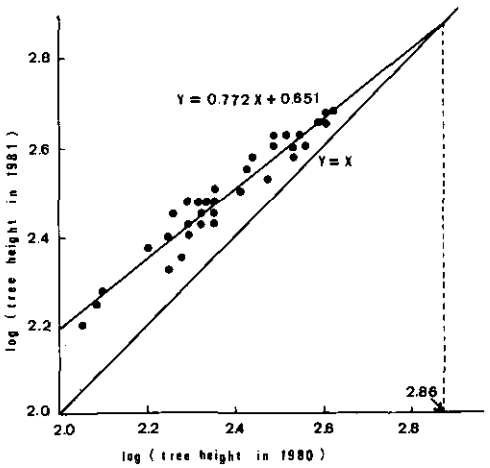

Fig. 7 Relationships between tree height (cm) of *Pinus thunbergii* in 1980 and 1981 in Site A
The X-coordinate of the intersection between the regression line and the line Y = X gives an estimated value of the maximum tree height.

$$\ln H_{t=3} = \ln K - a \exp(-3b) \quad (22)$$

Putting $H_{t=3}$, K = 718, b = 0.26 into the above equation and 'a' becomes 4.28. Thus the equations for the height growth at site A was obtained by putting the estimates of K, 'a' and 'b' to Eq (6):

$$H = 718 \exp -4.28^{\exp(-0.26t)} \quad (23)$$

Growth equations of tree height at the other two sites were estimated in the same manner:

$$H = 1005 \exp -3.46^{\exp(-0.13t)} \text{ for site B, and} \quad (24)$$

$$H = 580 \exp -3.22^{\exp(-0.20t)} \text{ for site C} \quad (25)$$

Fig. 8 shows the estimated growth curves for each site. At first the growth is faster at site A, but in advanced years the growth is relatively larger at site B.

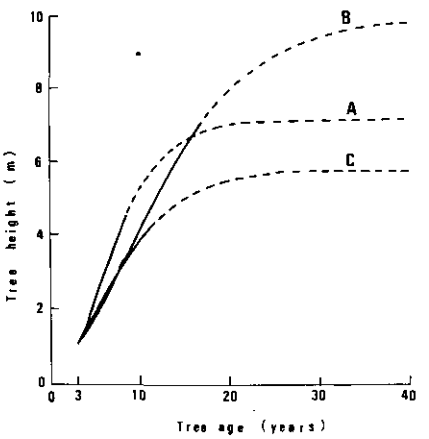

Fig. 8 Estimated growth curves fitted to the Gompertz equation for the tree height of *Pinus thunbergii* at three sites

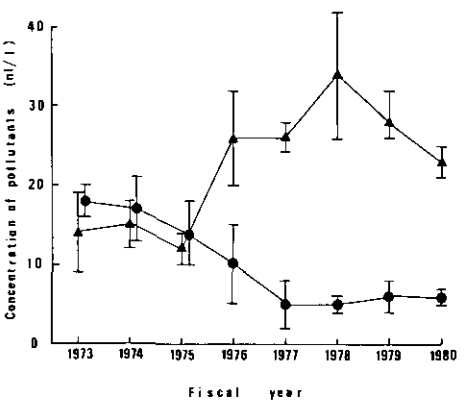

Fig. 9 Changes in annual mean concentrations of SO_2 (●) and NO_2 (▲) at five monitoring stations in the industrial area in Kashima. Vertical lines indicate 2 x standard deviation.

Discussion

Plant materials have been used for evaluating the atmospheric environments contaminated with phytotoxic air pollutants (Noble & Wright, 1958; Mandel *et al.*, 1973; LeBlanc & Rao, 1975; Manning & Feder, 1980; Matsushima, 1980; Steubing & Jager, 1982). However, direct comparisons of growth of woody plants at different sites are usually difficult, because the growth continues for many years. Furthermore, the growth is influenced by the plant size and also by the environmental conditions. In the present report, we tried to compare the patterns of height growth of pine trees, using a technique of curve-fitting to the plant growth. The obtained growth curve describes the average growth of individuals of different sizes under the same conditions. This averaging process by the curve-fitting is useful for comparing the same species growing at different sites.

A useful characteristic of the Gompertz equation is that the measure of the size does not change the type of curve to fit the data as long as the measures have an allometric relation to the original measure. Since the tree height could be expressed by the Gompertz curve, a measure of biomass [$(\text{girth})^2(\text{height})$ or $(\text{height})^3$] will also be fitted by the Gompertz curve with different parameters.

Care should be taken in estimating the initial size and the maximal size of plants by extraporating the fitted growth curve, because such an extraporation of data to either small or large values may predict erroneous values (Yamaguchi, 1975). Since we could obtain neither value, comparisons were made for the growth patterns during the periods when the collected data could be plotted. In Fig. 8, solid lines indicate those periods. During those periods growth rate of tree height at site A exceeded that at site B or C, while no significant differences were observed between sites B and C.

Yokobori (1975) presented an isopleth of air pollution in Kashima industrial area. Wind conditions exert a strong influence on the diffusion of air pollutants, because effects of air pollution on plants are greater on the leaward of a source of pollutants (Westman, 1974). Since prevailing wind direction in this region shows little changes from year to year, according to the isopleth of air pollution, degrees of air pollution would be in the order of site A > site B > site C. On the other hand, the growth curves of tree height showed that growth rate of *Pinus thunbergii* would be in the order of site A > site B ≒ site C for at least the first 10 years (Fig. 8). This result suggests that air pollution was not a primary factor which determined the height growth of *Pinus thunbergii*. Fig. 9 shows yearly fluctuations in average values of annual mean concentrations of NO_2 and SO_2 at the monitoring stations at Kashima-Cho and Kamisu-Cho from 1973 to 1980. The concentration of SO_2 shows a tendency to decline from 18 nl/l in 1973 to 5 nl/l in 1980, whereas the concentration of NO_2 showed a remarkable increase from 12 nl/l in 1975 to 33 nl/l in 1978 followed by a decrease to 22 nl/l in 1980. Thus average concentrations of air pollutants which would affect the growth of *Pinus thunbergii* seemed to have decreased in recent years. Yokobori and Ohta (1983) concluded that the air pollution affected the characteristics of tree rings of *Pinus densiflora* in 1975 and 1976, but thereafter the deteriorating effects have decreased to the non-detectable extent. Yokobori and Saruta (1974) suggested that *Pinus thunbergii* is more resistant to air pollutants than *Pinus densiflora.*

The estimated growth curves suggested that the growth during the period when the data were collected was larger at site A than at sites B or C. Differences in light and nutrient conditions among sites might contribute in part to these differences in growth rates among the three sites. Young trees at site A were under full sunlight conditions, whereas some of the young trees at site B were shaded by large ones. The soil nutrient conditions at site C might be poorer than those at site A, because the soils were composed of sands on coastal dunes, which

are known to be very infertile (Ito *et al.*, 1972).

Acknowledgments

We are very grateful to Dr. A. Furukawa of Terrestrial Ecology Section of NIES, for reviewing the early draft of this report. We wish to thank Drs. T. Yoneyama (National Institute of Agrobiological Resources), K. Okano, O. Ito, and Mr. H. Shimizu for assistance in the field survey, and Ms. H. Kitagawa for drawing figures.

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生長曲線の当てはめによる鹿島臨海工業地域における クロマツの樹高生長の解析

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茨城県鹿島臨海工業地域及びその周辺の大気汚染状況の異なる3地点に生育するクロマツについて1980年3月から1981年3月までの1年間の樹高生長を調査した。樹高と樹高の相対生長率の関係から各地点のクロマツの樹高生長をGompertz曲線に当てはめて推定した。得られた生長曲線は地点間で異なっていたが，大気汚染による樹高生長への影響は確認されず，樹高生長の差は主に土壌の栄養条件の違いと，光条件の違いによっていたと推察された。

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Res. Rep. Natl. Inst. Environ. Stud., Jpn., No. 65, 1984.

Effects of Air Pollutant Mixtures on Photosynthetic Electron Transport systems*

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After exposure of 0.5 ppm sulfur dioxide (SO_2), 4.0 ppm nitrogen dioxide (NO_2) and 0.1 ppm ozone (O_3) singly or in combination for 5–30 h to spinach plants, effects of the air pollutant mixtures on photosynthetic electron transport systems were analyzed about photosystem I and II by using chloroplasts isolated from the exposed spinach leaves.

1) On the exposure of the mixture of SO_2 and O_3, the inhibition of photosystem II reaction was not enhanced significantly than that with SO_2 alone. In the case of more than 30% inhibition, it was suggested that O_3 might protect photosystem II from SO_2. Photosystem I was not injured by the mixture.

2) On the exposure of the mixture of NO_2 and O_3, both reactions of photosystem I and II were inhibited significantly. Especially, the inhibition of photosystem II reaction was synergistic, whereas that with O_3 or NO_2 singly was not observed.

3) On the exposure of the mixture of SO_2 and NO_2, photosystem I reaction was enhanced at 10 h fumigation, but inhibited at 30 h fumigation. The inhibition of photosystem II reaction increased gradually with the time of fumigation and reached 50% after 30 h.

4) On the exposure of the mixture of SO_2, NO_2 and O_3, there was a tendency that the enhancement of photosystem I reaction, observed by fumigation with SO_2 and NO_2 for 10 h, was suppressed by O_3 participation. The inhibition pattern of photosystem II reaction almost resembled to that caused by the mixture of SO_2 and NO_2.

5) All of the mixtures containing NO_2 caused injury of photosystem I and II after 30 h fumigation, especially, the photosystem II reaction was inhibited severely.

From these results, possible mechanisms were discussed about the effects of the air pollutant mixtures on plants.

Key words: Air pollutant mixture, Electron transport, Chloroplasts, Photosystems

* A part of this study has been published in "Gaseous Air Pollutants and Plant Metabolism" edited by M. J. Koziol and F. R. Whatley, Oxford, 1984.

Recently, effects of an air pollutant on electron transport system have been studied (Shimazaki & Sugahara, 1979, 1980). The results demonstrated SO_2 inhibited photosystem II reaction specifically without inhibition of photosystem I reaction at the early stage of the fumigation. It may be possible to use the specific inhibition of SO_2 as an indicator in the analysis of the effects of pollutant mixture containing SO_2 on electron transport system.

In the meanwhile, there are only a few reports about the effects of air pollutant mixtures on physiological function in plants. Most of them are concerned with the visible foliar injury. The pollutant mixture showed either additive, synergestic or antagonistic effect of the single pollutant. The damage of plants depends on combination of the pollutants, concentration of each pollutant, exposure time, plant age and plant species. Further, the damage also depends on the threshold concentration of injury appearance by the single pollutant.

On these points of view, in the present study, the following concentrations of pollutants were selected: namely SO_2, 0.5 ppm; NO_2, 4.0 ppm; O_3, 0.1 ppm. The fumigation of plants with any one of the pollutants at these concentrations showed either no effect or a slight and gradual effect on electron transport with time. The results of fumigation by mixtures of these two or three pollutants are discussed.

Materials and Methods

Plant material

Spinach (*Spinacia oleracea* L. cv. New Asia) and lettuce (*Lactuca sativa* L. cv. Romaine) plants were grown in pots (115 mm diameter) containing vermiculite, peat moss, perlite and fine gravel (2:2:1:1, by volume) at 20°C d/15°C night temperatures with a relative humidity of 70% in a glasshouse under sunlight. As nutrients, 4 g Magamp K (NPK = 6:40:6, W. R. Grace Co., USA) and 8 g magnesia of lime were applied in dry form to each litre of soil mixture and 200 ml of a solution of 1 g l^{-1} Hyponex (NPK = 6.5:6:19) was supplied to each pot every five days thereafter. Plants were used for experimentation when four to six weeks old.

Fumigation conditions

Plants were fumigated with air pollutants in a growth cabinet (230 × 190 × 170 cm) at 20°C with a relative humidity of 75%; wind velocity in each cabinet was 0.22 m s^{-1}. Illumination was provided with heat-filtered stannous halide vapour lamps (Toshiba Yoko Lamp, 400W, Toshiba Co. Ltd, Tokyo, Japan) giving a light intensity of 25 000–35 000 lx at the leaf level. Plants were preconditioned for 2 h under illumination in the growth cabinet for clean air controls, after which half of the plants were transferred quickly into another growth cabinet receiving the appropriate concentration of pollutant gas or gas mixture. The lengths of the fumigation periods were varied and are given with the experimental results. Chloroplasts were isolated from plants from each growth cabinet as described below.

Preparation of chloroplasts

After pollutant fumigation, leaves were homogenized at 0°C in 0.05M Tricine-NaOH buffer (pH 7.5) containing 0.02M NaCl and 0.4M sucrose. After the homogenate had been filtered through four layers of gauze, the filtrate was centrifuged at 200 × g for 5 min and the chloroplasts were isolated from the supernatant by centrifugation at 1500 × g for 7 min.

Measurement of photosynthetic electron transport

The rates of dichloroindophenol (DCIP) and NADP photoreduction were determined

according to the method of Shimazaki and Sugaraha (1979). The reaction mixture for DCIP photoreduction contained in 4 ml final volume, 12.5 mM Tricine-NaOH buffer (pH 7.5), 100 mM sucrose, 5 mM NaCl, 50 μM DCIP and chloroplasts containing 20 μg chlorophyll. The reaction mixture (4 ml) for NADP reduction contained 12.5 mM Tricine-NaOH buffer (pH 7.5), 100 mM sucrose, 5 mM NaCl, 5 μM NADP, a saturating amount of spinach ferredoxin and chloroplasts containing 40 μg chlorophyll. The $DCIPH_2$–NADP system contained in addition 50 μM DCMU, 50 μM DCIP, 2.5 mM sodium ascorbate and 25 mM NH_4Cl (the last to act as an uncoupler).

The rate of O_2 exchange was determined with a Clark-type oxygen electrode according to the method of Shimazaki and Sugahara (1980).

Results

Effects of SO_2

Effects of SO_2 on the electron transport system have been reported elsewhere (Shimazaki & Sugahara, 1979, 1980).

The inhibitory action of SO_2 on the activities of photosystems I and II in chloroplasts are shown in Table 1. Electron flow from H_2O to DCIP was inhibited,

Table 1 Effect of SO_2 on electron transport activities[a]

Reaction measured	SO_2 fumigation (h)		
	0	2	4
	(μmol acceptor reduced mg^{-1} chlorophyll h^{-1})		
H_2O – NADP	170	107	66
$DCIPH_2$ – NADP (+ DCMU)	95	97	108
H_2O – DCIP	217	124	70

SO_2 fumigation was performed at 2.0 ppm; other conditions as described in the text.
[a] After Shimazaki and Sugahara 1979.

while that from reduced DCIP to NADP ($DCIPH_2$–NADP) was not affected when electron transport was uncoupled by 2 mM NH_4Cl. SO_2 inhibited the overall electron flow from H_2O to NADP to the same degree as the electron flow from H_2O to DCIP. From these results, we concluded that SO_2 inhibited the electron flow driven by photosystem II but not that by photosystem I.

Further analyses by using electron acceptors and inhibitors of electron transport or by fluorescence induction pattern of chloroplasts suggested that the site of SO_2 inhibition was the primary electron donor or reaction center itself in photosystem II (Shimazaki & Sugahara, 1980).

Experiments using low concentrations of SO_2 and/or NO_2 were conducted with perennial ryegrass (*Lolium perenne*) by Wellburn *et al.* (1981). Exposure to 0.25 ppm SO_2 for 11 days did not have any effect on either of the reactions of photosystem I and II. We found that in spinach exposed to 0.5 ppm SO_2 for 20–30 h photosystem II was inhibited but photosystem I

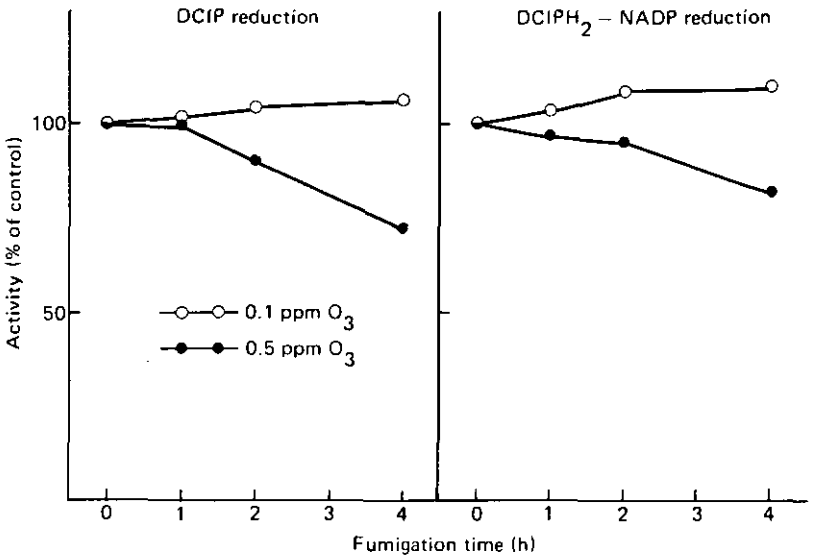

Fig. 1 Effects of O_3 on DCIP and $DCIPH_2$-NADP photoreduction. Experimental conditions are described in the text

was not (Fig. 2 and Fig. 4).

Effects of ozone

Fig. 1 shows the effect of O_3 on the photoreduction of DCIP by H_2O and of NADP by $DCIPH_2$; 0.1 ppm O_3 did not suppress the electron transport in either photosystem. On the other hand, 0.5 ppm O_3 inhibited the photoreactions in both photosystems after 4 h exposure. Unlike the effects of SO_2, O_3 did not preferentially inhibit photosystem II, but affected both photosystem reactions at the same time. Coulson and Heath (1974) also reported that O_3 bubbled into a suspension of isolated spinach chloroplasts inhibited electron transport in both photosystems. Murabayashi *et al.* (1981) and Suzuki, Murabayashi and Matsuno (1982) investigated the effect of O_3 on electron transport in spinach chloroplasts more closely. They performed experiments using both chloroplasts isolated from O_3-fumigated leaves and suspensions of normal isolated chloroplasts through which O_3 had been bubbled and found that electron transport in both photosystems was inhibited by O_3.

The maintenance of the normal permeability characteristics and integrity of the membranes of the chloroplast lamellae is necessary for the production of the proton gradient that is the driving force for ATP formation and O_3 may perhaps affect these.

Effects of nitrogen dioxide

In general, NO_2 fumigation does not affect plants severely, even at relatively high concentrations. As shown in Fig. 2 and Fig. 4, 4 ppm NO_2 caused little inhibition of electron transport in either photosystem I or II after 10 h fumigation, and only a slight inhibition was found after 20 h fumigation.

Wellburn *et al.* (1981) reported that long-term fumigation at a low concentration of NO_2 (0.25 ppm for 11 days) had no effect on electron transport in either photosystem, but did

enhance the production of ATP.

Effects of pollutant mixtures

In a series of experiments using pollutant mixtures, the following concentrations of pollutants were selected on the basis of the results obtained by exposure of plants to these pollutants singly: namely SO_2, 0.5 ppm; NO_2 4.0 ppm; O_3, 0.1 ppm. The fumigation of plants with any one of the pollutants at these concentrations showed either no effect or a slight and gradual effect on electron transport with time. The results of fumigation by mixtures at these concentrations are summarized in Fig. 2–7.

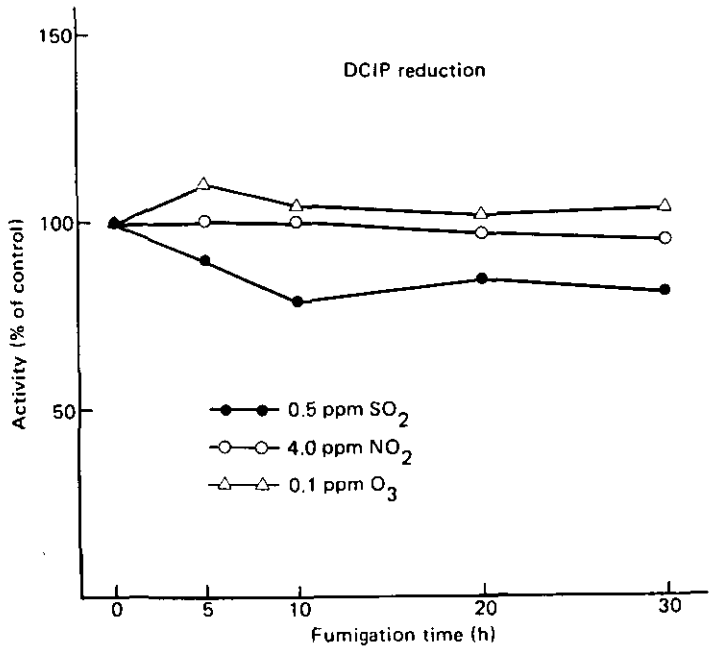

Fig. 2 Effects of exposure with SO_2, NO_2 or O_3 alone on DCIP photoreduction (PSII). Experimental conditions are described in the text

Sulphur dioxide and ozone

DCIP photoreduction was not inhibited by 0.1 ppm O_3 alone but was inhibited slightly by 0.5 ppm SO_2 alone after 10 h of fumigation (Fig. 2). The inhibition of photoreduction of DCIP by H_2O in response to SO_2 and O_3 given together was not significantly different from that observed with SO_2 alone (Fig. 3). Photoreduction of NADP by $DCIPH_2$ was not affected by 0.5 ppm SO_2 even after 30 h fumigation (Fig. 4). Fumigation with 0.1 ppm O_3 or a mixture of O_3 and SO_2 also had no effect on photosystem I (Fig. 5). Total photosystem activity (NADP photoreduction by H_2O) was enhanced by 0.1 ppm O_3 for the first 10 h of fumigation (Fig. 6). The gradual decrease in the rate of photoreduction of NADP by H_2O caused by exposure to 0.5 ppm SO_2 was not significantly increased by the additional presence of O_3 (Fig. 7).

Nitrogen dioxide and ozone

The photoreduction of DCIP was not inhibited by fumigation either with 4 ppm NO_2 or 0.1 ppm O_3 singly (Fig. 2), but in combination an inhibition was observed (Fig. 3). The activity

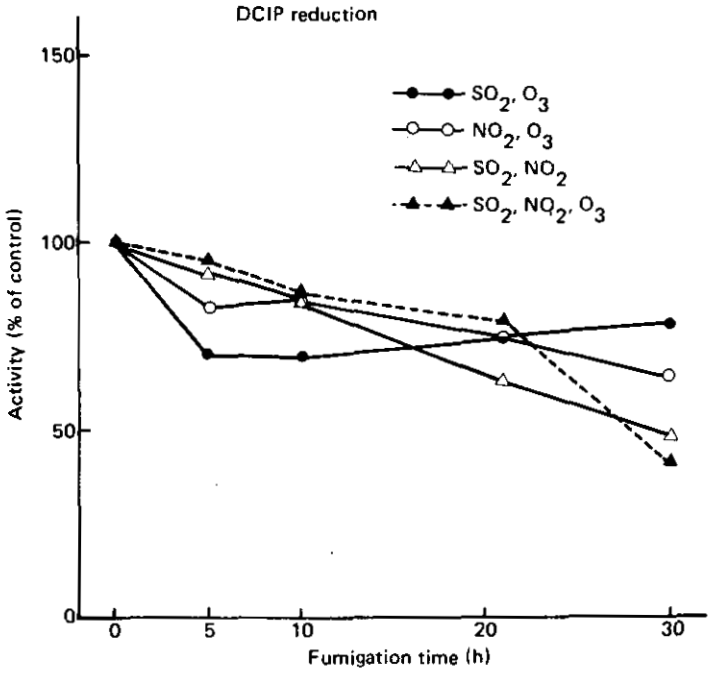

Fig. 3 Effects of exposure with SO_2, NO_2 and O_3 in combination on DCIP photoreduction (PSII)
Experimental conditions are described in the text

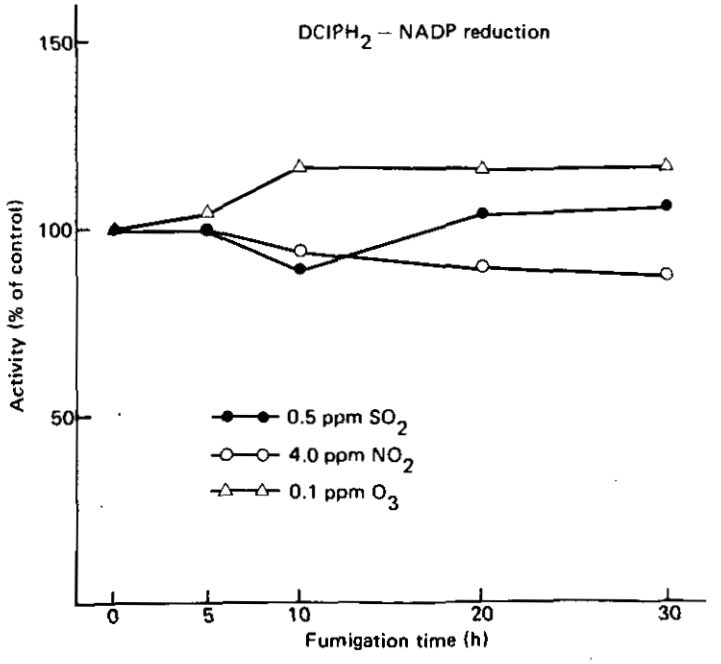

Fig. 4 Effects of exposure with SO_2, NO_2 or O_3 alone on $DCIPH_2$–NADP system (PSI)
NH_4Cl (25 mM) was added as an uncoupler; other conditions as described in the text

of photosystem I decreased only slightly after a 30 h exposure to 4 ppm NO_2 (Fig. 4). However, the inhibition of DCIP photoreduction by H_2O obtained by the mixture of NO_2 and O_3 was not reflected in any inhibition in photosystem I activity (Fig. 5). The total photosystem activity was inhibited gradually with the time in response to fumigation with 4 ppm NO_2; this inhibition was enhanced synergistically by the combination of NO_2 and O_3 (Fig. 6 and 7).

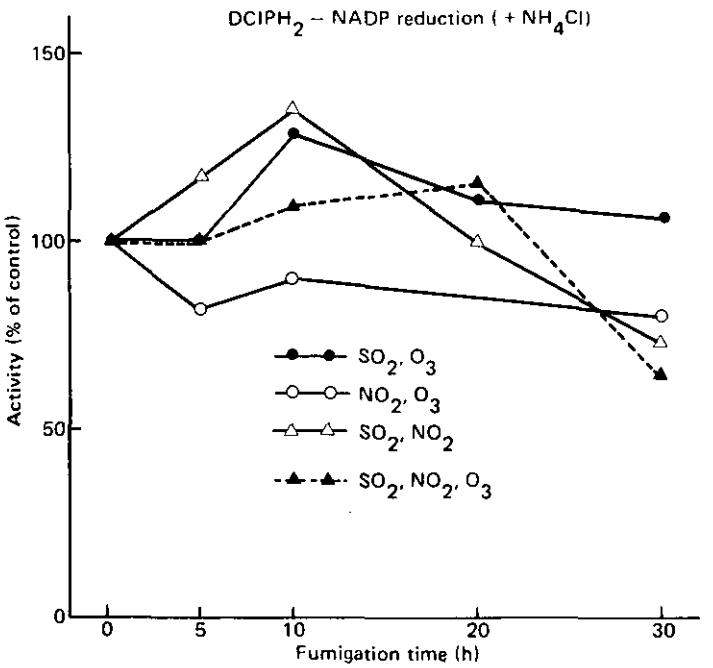

Fig. 5 Effects of exposure with SO_2, NO_2 and O_3 in combination on $DCIPH_2$-NADP system (PSI)
NH_4Cl (25 mM) was added as an uncoupler; other conditions are described in the text

Sulphur dioxide and nitrogen dioxide

In combination, 0.5 ppm SO_2 and 4 ppm NO_2 inhibited the photoreduction of DICP by H_2O after 20 h. On the other hand, this gas mixture increased the $DCIPH_2$–NADP photoreduction during the first 10 h of exposure, after which the photoreduction of $DCIPH_2$–NADP decreased with time to a level representing a significant inhibition after 30 h (Fig. 3 and 5). Exposure to the mixture of SO_2 and NO_2 also enhanced the total photosystem activity during the first 20 h of exposure although an inhibition appeared finally after 30 h fumigation (Fig. 7).

Sulphur dioxide, nitrogen dioxide and ozone

The inhibition by a mixture of SO_2, NO_2 and O_3 of photoreduction of DCIP by H_2O followed a similar pattern to the inhibition caused by a mixture of SO_2 and NO_2 (Fig. 3). Ozone did not enhance the inhibitions caused by SO_2 or NO_2, singly or in combination. However, in the photoreduction of NADP by $DCIPH_2$ it appears that the enhancement of the

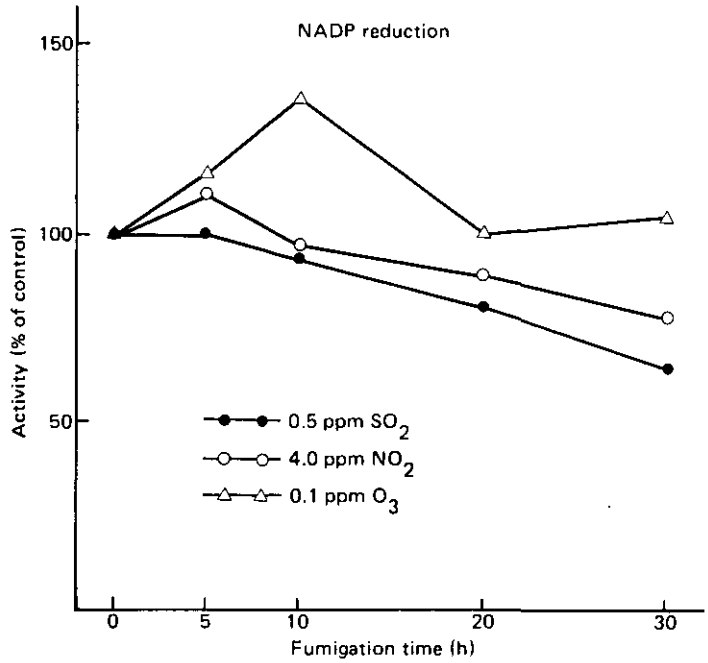

Fig. 6 Effects of exposure with SO_2, NO_2 or O_3 alone on NADP photoreduction (PSI+II)
Experimental conditions are described in the text

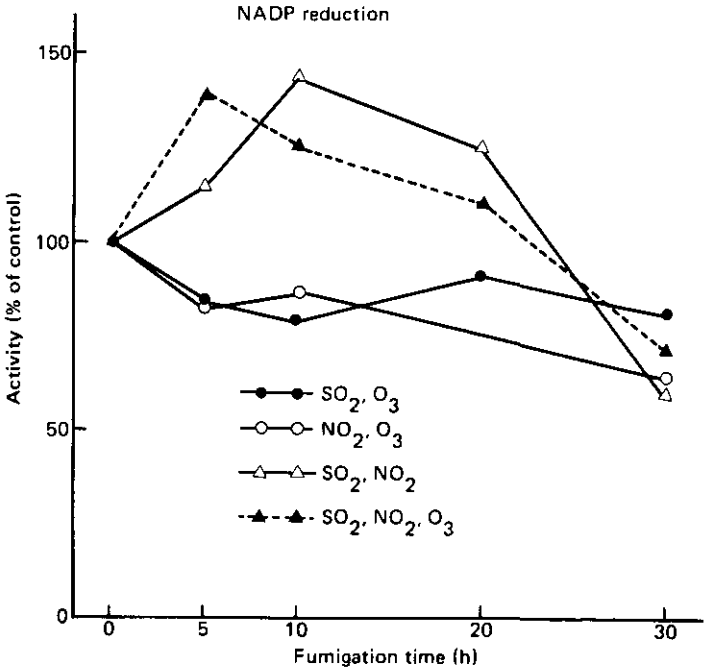

Fig. 7 Effects of exposure with SO_2, NO_2 and O_3 in combination of NADP photoreduction (PSI+II)
Experimental conditions are described in the text

activity observed by the fumigation with SO_2 and NO_2 in combination for 10 h was suppressed by O_3 treatment. When the overall photosystem was measured (NADP reduction by H_2O) the enhancement observed during 20 h fumigation with a mixture of SO_2 and NO_2 still remained.

Discussion

The inhibition of photosynthetic processes by air pollutants has been reported by many workers. Their results have shown that following exposure to low concentrations of these pollutants for long periods, no inhibition of photosynthetic electron transport occurred but there was a suppression of growth. This may indicate that membrane-associated light reactions were more resistant to the pollutants than dark reactions concerned with CO_2 fixation. Further, the inhibition of the light reaction was mostly irreversible and it took a long time to restore its activity. In the present study, we therefore used relatively high concentrations of air pollutants in order to get clear inhibitory effects on the activity of the light reaction.

SO_2 preferentially inhibited photosystem II. It was shown that the site of inhibition was at the primary electron donor site or at the reaction centre itself. However, O_3 and NO_2 inactivated electron transport in both photosystems I and II. This may suggest that inactivation of the reaction by O_3 or NO_2 was probably the result of denaturation or destruction of constituents contained in the membrane structure of both photosystems.

The effects of mixed pollutants on the light reactions were very complex. On fumigation with SO_2 and O_3 in combination, the inhibition of photosystem II, and of the total photosystem, did not increase beyond the level of 10 h inhibition even after 30 h fumigation. The result may indicate that from 10 to 30 h injury by SO_2 was prevented by the presence of O_3. A speculative mechanism of this 'protection' could be that SO_3^{2-} ion is accumulated in the cytoplasm at a relatively high concentration following SO_2 fumigation and that O_3 introduced into cytoplasm may react with SO_3^{2-} directly to produce the SO_4^{2-} ion, which is less toxic than SO_3^{2-}.

On exposure to a combination of O_3 and NO_2, both photosystem I and II reactions were inhibited significantly, although they were both slightly inhibited by 30 h exposure to NO_2 alone. It is possible that when O_3 is present with NO_2 the nitrite reductase system is inhibited and nitrite is accumulated to a toxic concentration. It is also possible that the combined effect of NO_2 and O_3 results in the formation of free radicals, which damage the chloroplast membrane.

On exposure to a mixture of SO_2 and NO_2, photosystem I reaction was enhanced at 10 h but had become inhibited at 30 h. The temporary enhancement was also observed when the total photosystem (PSI + PSII) was measured. Such an enhancement has never been observed in the effect of an air pollutant on photosynthetic processes in intact systems. The enhancement cannot be explained at present. We can only suggest that a new biochemical product was produced by the combined effect of sulphite and nitrite and believe that the transient phenomenon obtained by the fumigation with relatively high concentrations of SO_2 and NO_2 may occur even at low concentrations if the new product were accumulated in sufficient amount.

On exposure to a mixture of SO_2, NO_2 and O_3 an enhancement of photosystem I reaction was observed as well as a total photosystem reaction (PSI + PSII). The inhibition pattern of photosystem II reaction resembled that caused by the mixture of SO_2 and NO_2. No synergistic inhibition was observed.

All of the fumigations that included NO_2 caused injury in both photosystems I and II after

30 h. This implies that fumigation with 4 ppm NO_2 was more damaging to plants than exposure to the other pollutants in our experiments, and it is suggested that 4 ppm NO_2 exposure could have gone beyond the threshold of tolerance. If O_3 had been given at 0.2 ppm (double that in the present study) the effect of O_3 on photosynthetic reaction might have been more clearly observed.

The effects of the mixed pollutants on plant metabolism are very complex. It is necessary to perform the fumigation with several pollutants singly or in combination, to get more clear information on the mechanism of their effects.

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混合大気汚染物質の光合成電子伝達系に及ぼす影響

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大気汚染物質の二酸化硫黄（SO_2），0.5 ppm，二酸化窒素（NO_2），4.0 ppm 及びオゾン（O_3），0.1 ppm を，二種及び三種に混合して暴露したホウレンソウ葉から，葉緑体を単離して，光合成電子伝達反応への影響を光化学系Ⅰと系Ⅱの反応に分けて調べた。

1）SO_2+O_3 の複合影響では，SO_2 単一ガスで見られた系Ⅱの阻害が，O_3 が加わることによって大きな増加を示すことはなかった。むしろ，SO_2 による阻害が30％を越えるような30時間暴露の場合，阻害を弱めるような保護効果を示した。光化学系Ⅰの反応は，O_3 が存在しても全く阻害を受けなかった。

2）NO_2+O_3 の複合影響では，単一ガスでは全く阻害を与えない O_3 が，NO_2 と混在することにより明らかな活性阻害を引き起こした。この阻害は光化学系Ⅰ及びⅡの両方に及んでおり，光化学系のⅡの阻害は相乗的であった。

3）NO_2+SO_2 の複合影響では，光化学系のⅠの反応が暴露10時間前後に促進され，暴露が30時間を経ると阻害されることが示された。光化学系Ⅱの反応は暴露の時間の経過とともに阻害度を増し，30時間後には50％に達した。

4）$NO_2+SO_2+O_3$ の複合影響では，NO_2+SO_2 の影響として表れた暴露10時間前後の活性促進を，O_3 が抑制する傾向が見られた。光化学系Ⅱの反応の阻害は NO_2+SO_2 の場合に近似していた。暴露30時間について見ると，NO_2 の存在するすべての場合において光化学系Ⅰが阻害された。

以上の結果について，現在までに報告された知見による，これらの機作の解明の可能性を検討し，今後の問題点について考察した。

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Res. Rep. Natl. Inst. Environ. Stud., Jpn., No. 65, 1984.

Sulfite Inhibition of Photosystem II in Illuminated Spinach Leaves*

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PS II activity (dichlorophenolindophenol photoreduction) in chloroplasts isolated from sulfite-treated spinach leaves in light was inhibited but not in darkness. Sulfite treatment decreased the variable part of fluorescence induction and the fluorescence intensities of emissions at 685 and 694 nm at 77K, but it had no effect when sulfite was administered together with DCMU. These results indicate that sulfite inactivates the PS II reaction center when electron transport takes place.
Key words: Chloroplasts, Electron transport, PS II, Sulfite, Sulfur dioxide

Sulfur dioxide is a wide-spread air pollutant and has been known to affect photosynthesis (Hill & Thomas, 1933; Black, 1982). In a previous report, we showed that SO_2-fumigation inactivated PS II, but not PS I (Shimazaki & Sugahara, 1979). No inhibitory effect of SO_2 was found, however, when fumigation was done in the dark, probably because the entrance of SO_2 into leaf tissue was prevented by stomatal closure and/or by the requirement of light for this inhibition. We thus investigated the reasons for this phenomenon.

When SO_2 enters leaf tissue through the stomata it produces H^+, HSO_3^- and SO_3^{2-} on its dissolving in the water of the cells. We therefore assumed that sulfite serves as the toxicant in SO_2 phytotoxicity, and so administered sodium sulfite to spinach leaves by vacuum infiltration. The results of our experiments showed that sulfite inhibited the PS II reaction center only in light.

The spinach plants (*Spinacia oleracea* L. cv. New Asia) used were grown in a phytotron (Shimazaki & Sugahara, 1979). Sodium sulfite in 60 mM potassium buffer (pH 6) containing 2 mM EDTA was administered to spinach leaf disks (ϕ = 15 mm) under vacuum infiltration in the dark. Leaf disks maintained in fresh sulfite solution in Petri dishes (ϕ = 200 mm) were illuminated with heat-filtered white light from stannous halide vapor lamps (Toshiba Yoko lamps, 400 W) at 20°C. The light intensity was varied by covering the Petri dishes with lawn and was measured with a radiometer (Model LI-185, LI-COR Inc.). After illumination, chloroplasts were isolated quickly from the leaf disks and washed twice, after which they were

* This study had been published in Plant Cell Physiol., 25, 337-341 (1984) and was reproduced with permission of the publisher.
Abbreviations: DCIP, 2,6-dichlorophenolindophenol; DPC, diphenylcarbazide

suspended in isolation medium. The isolation medium consisted of 50 mM Tricine-NaOH (pH 7.5), 400 mM sucrose, 20 mM NaCl and 5 mM $MgCl_2$. Chl concentrations were determined from the absorption coefficients reproted by Mackinney (1941). DCIP photoreduction, O_2 uptake, and the transient change in Chl fluorescence were measured as described previously (Shimazaki & Sugahara, 1980). Low temperature (77K) emission spectra of Chl fluorescence in chloroplasts were obtained with a Hitachi MPF-4 fluorospectrophotometer equipped with a plastic cuvette in a Dewar flask containing liquid nitrogen.

Light was required for sulfite to inhibit photosynthetic electron transport (Fig. 1). When spinach leaves administered sodium sulfite were illuminated, photoreduction of DCIP in their chloroplasts was inhibited, but when the leaves were kept in darkness this activity was not affected. Apparently, light saturation took place at about 3,000 lx. Sulfite in a concentration range of 0.1 to 0.5 mM only slightly inhibited DCIP photoreduction on 1 h of illumination; but, when the concentration of sulfite exceeded 0.5 mM, there was strong inhibition (Fig. 2).

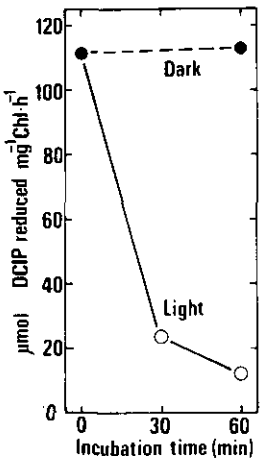

Fig. 1 Effect of sulfite treatment on DCIP photoreduction activity of spinach chloroplasts in light and darkness

Sodium sulfite was administered to spinach leaf disks at 2.5 mM then the disks were illuminated at 20,000 lx. DCIP photoreduction was assayed with chloroplasts isolated from these leaf disks. The reaction mixture (4 ml) contained 14 mM Tricine-NaOH (pH 7.5), 110 mM sucrose, 5.5 mM NaCl, 1.5 mM $MgCl_2$, 50 μM DCIP and chloroplasts (20 μg Chl).

Effects of sulfite-treatment on the activities of PS I and PS II are shown in Table 1A. Electron flow from water to DCIP was inhibited by sulfite-treatment, whereas that from reduced DCIP to methyl viologen was not. Inhibition of the whole-chain electron flow showed almost the same value as that of the electron flow driven by PS II. This means that sulfite inhibited the reaction driven by PS II, but not the reaction driven by PS I. The addition of diphenylcarbazide, an electron donor for PS II, restored some sulfite-inhibited DCIP photoreduction (Table 1B).

The effect of sulfite-treatment on fluorescence induction is shown in Fig. 3. Sulfite eliminated the variable part (I to P) of this induction, evidence that sulfite inhibited the photoreduction of Q, a primary electron acceptor of PS II, because it is accepted that a gradual increase in fluorescence yield (I to P) corresponds to the accumulation of reduced Q (Duysens & Sweers, 1963). On the addition of DCMU to control chloroplasts, the fluorescence intensity

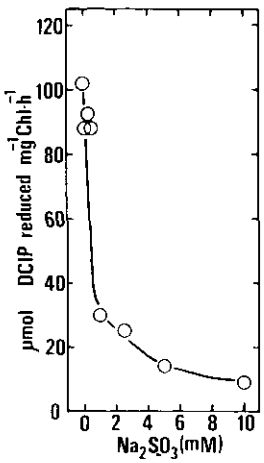

Fig. 2 Effect of sulfite concentration on DCIP photoreduction activity of spinach chloroplasts

Sodium sulfite was administered at the indicated concentrations to leaf disks, then the disks were illuminated at 23,000 lx for 1 h. Spinach DCIP photoreduction was measured with chloroplasts isolated from these leaf disks. The reaction mixture was the same as that in Fig. 1.

Table 1 Effects of sodium sulfite-treatment on electron transport activities in spinach chloroplasts

Reaction measured		Na_2SO_3 concentration (mM)		
		0	1	2.5
		μmol acceptor reduced mg^{-1} Chl·h^{-1}		
A)	$H_2O \rightarrow$ DCIP	127	23	10
	$DCIPH_2 \rightarrow$ MV (+DCMU)	116	115	115
	$H_2O \rightarrow$ MV	255	58	40
B)	$H_2O \rightarrow$ DCIP	130	7	5
	$H_2O \rightarrow$ DCIP (+DPC)	135	18	17

Sodium sulfite was administered to spinach leaf disks at the concentrations indicated. The disks then were illuminated for 1 h at a light intensity of 20,000 lx. Electron transport activities were measured in chloroplasts isolated from the leaf disks. A) The reaction mixture (4 ml) for the determination of DCIP photoreduction contained 14 mM Tricine-NaOH (pH 7.5), 110 mM sucrose, 17 mM NaCl, 1.3 mM $MgCl_2$, 50 μM DCIP and chloroplasts (20 μg Chl). The rate of O_2 exchange was determined with a Rank oxygen electrode (Rank Bros., Bottisham, England). The basal reaction mixture (4 ml) contained 15 mM Tricine-NaOH (pH 7.5), 133 mM sucrose, 17 mM NaCl, 1 mM NaN_3, 0.1 mM methyl viologen and chloroplasts (40 μg Chl). To measure the PS I-driven O_2 uptake, we added 50 μM DCIP, 0.5 mM sodium ascorbate and 10 μM DCMU to the basal reaction mixture. B) Experimental conditions were the same as shown in (A). Where indicated, 0.25 mM DPC was added to the basal reaction mixture.

rose rapidly, but the yield did not increase. The addition of DCMU to sulfite-treated chloroplasts, however, restored the fluorescence yield although the restored yield was much lower than that of the controls. On the addition of sodium dithionite to chloroplasts with DCMU, the fluorescence yield increased in both samples (Fig. 3), but the yield was much higher for the controls than for sulfite-treated chloroplasts. In contrast, when sulfite was administered to spinach leaf disks together with DCMU, sulfite had no effect on fluorescence induction (Fig. 4). This suggests that sulfite inactivates the reaction center of PS II only when electron transport takes place.

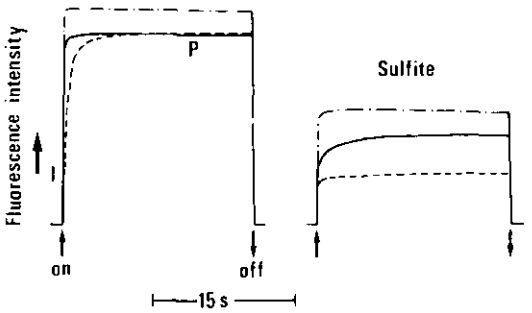

Fig. 3 Effect of sulfite treatment on the time course of the fluorescence transient of spinach chloroplasts

Sodium sulfite at 1 mM was administered to spinach leaf disks. The disks then were illuminated for 1 h at 25,000 lx, and their chloroplasts isolated. The fluorescence transient was recorded (Technicorder F type 3052, Yokogawa), No addition (– – –); 10 μM DCMU (———); and 10 μM DCMU and a few grains of sodium dithionite (—•—•). The actinic blue-light was 7,000 erg $cm^{-2} \cdot s^{-1}$. The reaction mixture (4 ml) contained 50 mM Tricine-NaOH (pH 7.5), 400 mM sucrose, 20 mM NaCl, 5 mM $MgCl_2$ and chloroplasts (10 μg Chl). Chloroplasts were kept in the dark for 5 min before illumination.

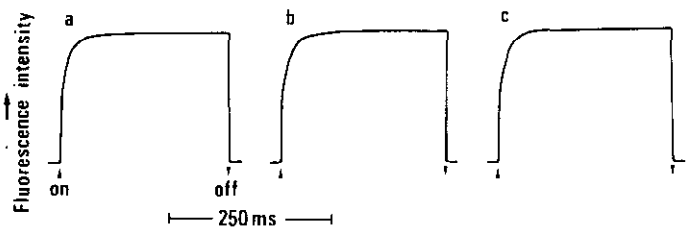

Fig. 4 Effect of sulfite treatment on the time course of the fluorescence transient of spinach chloroplasts in the presence of DCMU

DCMU at 0.5 mM was administered to spinach leaf disks together with sodium sulfite at 0 (a), 1 (b) and 2.5 mM (c). The treated disks were illuminated at 25,000 lx for 1 h, after which chloroplasts were isolated. The fluorescence transient was measured with a digital transient recorder (Model TM 1410, Kawasaki Electronica) in the presence of 10 μM DCMU. The reaction mixture was the same as in Fig. 3, but contained DCMU. The actinic blue-light intensity was 15,000 erg $cm^{-2} \cdot s^{-1}$.

Clear evidence of injury to PS II by sulfite was obtained from the low-temperature (77K) fluorescence emission spectra of the spinach chloroplasts (Fig. 5). Spectra of the control chloroplasts had maxima at 685, 694 and 734 nm. Sulfite-treatment depressed both the emission bands at 685 and 694 nm derived from PS II (Murata, 1968), to similar extent when spectra were adjusted in terms of the fluorescence at 735 nm.

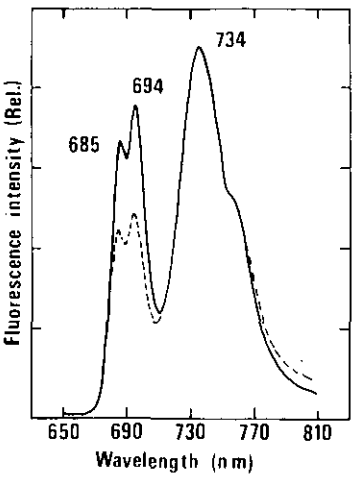

Fig. 5 Effect of sulfite treatment on the low temperature (77K) fluorescence emission spectra of spinach chloroplasts

Sodium sulfite at 1 mM was administered to spinach leaf disks which then were illuminated at 25,000 lx for 1 h. Fluorescence emission spectra of sulfite-treated (– – –) and non-treated (———) spinach chloroplasts at liquid nitrogen temperature (77K) were measured in chloroplasts isolated from the leaf disks. The reaction mixture (1.5 ml) contained 60% glycerol, 1 mM Tricine-NaOH (pH 7.5), 0.2 mM NaCl, 8 mM sucrose, 0.1 mM $MgCl_2$ and chloroplasts (4 μg Chl). The shoulders found at approximately 750 nm in the emission spectra were instrumental artifacts.

Next, we examined the effect of sulfite on electron transport in chloroplasts isolated from spinach leaves. Prior illumination (24,000 lx) of chloroplasts in the presence of 5 mM sodium sulfite for 10 min at pH 6 inhibited DCIP photoreduction by 10 to 30% but no effect was observed in darkness. In the absence of sulfite, however, a 10-min illumination caused photoinhibition of about 25%. There have been contradictory observations (Asada *et al.*, 1965; Silvius *et al.*, 1975) on the effect of sulfite on photosynthetic electron transport. Asada *et al.* (1965) indicated that the addition of sulfite to chloroplasts had no inhibitory effect on electron flow. The difference in results is due to the light-dependence of inhibition demonstrated in our study reported here. In the study of Asada *et al.* (1965), chloroplasts with sulfite were illuminated only during measurements. Light-dependent inhibition of sulfite also is one reason why SO_2 damage to plants is severe in the daytime (Wislicenus, 1914).

Why is light required for sulfite inhibition of PS II? There are two possible explanations of our results. The sulfite inhibition requires (a) electron transport which leads to the production of toxic substances such as active species of oxygen (O_2^-, H_2O_2, $^1O_2$) (Asada & Kiso, 1973; Shimazaki *et al.*, 1980; Tanaka & Sugahara, 1980; Tanaka *et al.*, 1982), or (b) a conformational change in PS II is induced by electron transport, and this may expose protein moieties to sulfite.

Sulfite is known to modify protein molecules by sulfitolysis (Baily & Cole, 1959). If (a) is the case, O_2 should participate in the inhibition; but, the anaerobic condition maintained during the illumination did not arrest the effect of sulfite. If (b) is the case, sulfite should bind to thylakoid membranes when spinach leaf disks are illuminated. We found much more binding of ^{35}S to the thylakoid membranes in light than in darkness when ^{35}S-sulfite was administered to our spinach leaf disks. The binding accelerated by light was suppressed strongly by DCMU. Possibly, sulfite exerts its inhibitory effect by combining proteins in the vicinity of PS II, but we could not identify whether the bound ^{35}S was a sulfite or some other metabolite. Our next step is to determine the quantitative relationship between ^{35}S incorporation into the thylakoid membranes and the degree of PS II inhibition.

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光照射ホウレンソウ葉における亜硫酸の光化学系Ⅱ阻害

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ホウレンソウ葉に亜硫酸を加え，光照射を行うと葉緑体の光化学Ⅱが阻害された。しかし，亜硫酸処理葉を暗中に置くとこの阻害は認められなかった。亜硫酸と同時に電子伝達の阻害剤DCMUを加えておくと，光照射を行っても光化学系Ⅱは阻害されなかった。

葉緑体クロロフィル蛍光の低温（77K）スペクトル及び電子供与体の添加効果から，亜硫酸の阻害部位は光化学系Ⅱの反応中心であることが示された。

以上の結果は，電子伝達系の作動時に亜硫酸による光化学系Ⅱ反応中心の阻害が起こることを示している。

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Res. Rep. Natl. Inst. Environ. Stud., Jpn., No. 65, 1984.

Reversible Inhibition of the Photosynthetic Water-splitting Enzyme System by SO_2 Fumigation Assayed by Chlorophyll Fluorescence and EPR Signal *in vivo**

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The effect of SO_2 fumigation (2 ppm, v/v) on photosynthesis in spinach leaves *in vivo* was investigated by measuring Chl *a* fluorescence (OIDP transient) and the electron paramagnetic resonance (EPR) signal I. SO_2 fumigation raised the I level to yield the ID dip and suppressed the DP transient before any visible damage occurred in the leaf. In SO_2-fumigated leaves, the time course of EPR signal I indicates that reduction of P700 by white light illumination was inhibited but dark reduction of P700 was not significantly affected. Photosynthetic O_2 evolution was also inhibited by SO_2 fumigation. All of these effects were reversible after removal of SO_2. The variable part of the fluorescence in the presence of DCMU was only slightly affected and decreased as the fumigation time increased. We concluded that SO_2 fumigation reversibly inhibits the photosynthetic water-splitting enzyme system and it injures the reaction center of PS II *in vivo* when the fumigation time is prolonged.

We discussed the role of possible toxicants derived from SO_2 within the leaf on the basis of the SO_2 action on Chl *a* fluorescence.

Key words: Chl fluorescence, EPR signal, Oxygen evolution, P700, PS II, Sulfur dioxide

Sulfur dioxide is a widespread air pollutant which damages plants (Barett & Benedict, 1970; Bell & Mudd, 1976; Hällgren, 1978; Wellburn, 1982), mainly by suppressing photosynthesis (Sij & Swanson, 1974; Furukawa *et al.*, 1979; Shimazaki & Sugahara, 1979; Tanaka *et al.*, 1982b). SO_2 may inactivate photosynthetic electron transport or Calvin cycle enzymes or cause stomatal closure (Kondo & Sugahara, 1978). Shimazaki and Sugahara (1980) showed that SO_2 fumigation injures the reaction center of PS II in spinach leaves. Tanaka *et al.* (1982b) demonstrated that SH-enzymes in the Calvin cycle, NADP-glyceraldehyde-3-phosphate dehydrogenase, ribulose-5-phosphate kinase and fructose-1, 6-bisphosphatase are inactivated

* This study had been published in Plant Cell Physiol., 25, 795-803 (1984) and was reproduced with permission of the publisher.

Abbreviation: EPR, electron paramagnetic resonance

rapidly by SO_2 fumigation of spinach leaves. These workers studied chloroplasts and enzymes isolated from fumigated leaves. However, an apparent inhibition may have occurred in these studies during sample preparation because of the chloroplasts or enzymes coming into contact with secondary toxicants formed in the cytoplasm by SO_2 fumigation (Heath, 1980). Another possibility which must be considered is that inhibitions that had taken place *in vivo* were removed during the preparation. Thus, the effect of SO_2 on photosynthesis needed to be studied *in vivo* without disrupting the plant tissue. Suitable indicators are Chl *a* fluorescence induction (Kautsky & Appel, 1960; Papageorgiou, 1975; Hällgren, 1978) and the kinetics of EPR signal I (Andreeva, 1982), which are altered by changes in the photosynthetic apparatus.

Our present study showed that short-term SO_2 fumigation reversibly inhibits the water-splitting enzyme system according to analysis of Chl *a* fluorescence induction and EPR signal I kinetics *in vivo*. A reversible inhibition of CO_2-dependent photosynthetic O_2 evolution in spinach leaves was also found together with evidence that prolonged SO_2 fumigation damages PS II reaction centers *in vivo*, thus confirming our previous conclusion based on experiments with chloroplasts isolated from fumigated leaves (Shimazaki & Sugahara, 1980). The preliminary results of these experiments have been presented elsewhere (Shimazaki *et al.*, 1979).

Materials and Methods

Plant materials

Spinach (*Spinacia oleracea* L. cv. New Asia) plants were cultivated in a greenhouse under sunlight as described previously (Shimazaki & Sugahara, 1979). The artificial soil was composed of vermiculite, peat moss, perlite and fine gravel (2:2:2:1, v/v). Nutrients containing 4 g/liter Magamp K and 8 g/liter magnesia lime were applied initially and 1 g/liter Hyponex was supplied every 5 days. Plants used were 4–6 weeks old.

SO_2 fumigation

Spinach plants were fumigated with 2.0 ppm (v/v) SO_2 in a growth cabinet (230 × 190 × 170 cm) with a relative humidity of 70% at 20°C under artificial light. Plants were preconditioned for 1 to 2 h in light and then transferred quickly to the growth cabinet in which 2 ppm of SO_2 had been prepared for fumigation. Illumination was provided from stannous halide vapor lamps (Toshiba Yoko Lamps, 400W). The light intensity was 2.0×10^5 erg·cm^{-2}·s^{-1} at the leaf level.

Chlorophyll a fluorescence

To measure Chl *a* fluorescence induction, a leaf strip (0.8 × 2.5 cm) without the main veins was excised quickly from a fumigated plant and sandwiched between a pair of plastic plates which was placed diagonally in a four-sided transparent cell (1 × 1 × 4 cm). To prevent desiccation of the leaf strip, water was supplied at the bottom of the cuvette. The leaf was kept in darkness for the indicated periods of time and then a definite area of leaf was illuminated with a beam of light at an angle of 45° to the leaf surface. The fluorescence emitted from the leaf at right angle to the actinic light was detected by a photomultiplier (Hitachi R-375) through a red cut-off filter (Corning 2030, >650 nm) and an interference filter (683 nm, half band width 10 nm). Signals from the photomultiplier were traced on a strip chart recorder (Yokogawa Technicorder F 3052) or on a digital transient recorder (Model TM-1410 Kawasaki

Electronica). The actinic light was obtained from a 100W halogen lamp operated on a d. c. stabilizer. The beam passed through two glass filters (Corning 9782). Light intensity was measured with a radiometer (Model LI-185, LI-COR Inc.). All measurements were carried out at 22°–24°C.

EPR measurement

EPR measurements were made using a JES-FE-3X spectrometer at room temperature. A leaf strip (0.25 x 1.5 cm) of spinach was placed in a quartz cuvette after fumigation. The $P700^+$ EPR signal (a *g* value of 2.00 and peak to peak width of 7.9 to 8.4 G) was induced by far-red light through a red cut-off filter (Hoya R-72, >700 nm) combined with two heat-absorbing filters (Hoya HA 30). The intensities of far-red and white light were 7.5×10^5 and 1.0×10^6 $erg \cdot cm^{-2} \cdot s^{-1}$, *respectively. The light source* was a 1 kW Xenon lamp (Ushio Electric).

O_2 exchange

The rate of O_2 exchange in spinach leaves was determined with a Clark-type electrode in aqueous media as described previously (Shimazaki & Sugahara, 1979). After SO_2 fumigation, respiratory O_2 uptake was measured in darkness, then photosynthetic O_2 evolution was determined in white light. The light intensity was 2.2×10^5 $erg \cdot cm^{-2} \cdot s^{-1}$.

Results

Visible damages

During the first hour after SO_2 fumigation, no visible damage to the spinach leaves was observed. Later a few leaves showed water-soaked visible damages. For the measurements of Chl fluorescence, EPR signals and O_2 evolution, we used leaves which exhibited no visible damage.

Effect of SO_2 fumigation on Chl a fluorescence induction

When a dark-adapted leaf is illuminated, fluorescence intensity rises rapidly from the initial level (O) to an intermediary level (I), then shows a slow decline (D) and again rises to a peak (P) (Papageorgiou, 1975). These transient changes (OIDP transient) in fluorescence are closely correlated with the partial reactions of photosynthesis. In unfumigated spinach leaves, fluorescence intensity showed a typical OIDP transient (Fig. 1a). SO_2 fumigation raised the I level and obscured the O level probably due to elevation of the O level (Fig. 1b,c). Since the fluorescence yield during the early induction period is regulated by the redox state of Q, the primary electron acceptor of PS II (Duysens & Sweers, 1963), and since the increase in the yield corresponds to the accumulation of reduced Q, the initial rapid rise to a high I level in SO_2-fumigated leaves indicates that portions of Q and PQ were present in their reduced states in darkness. This implies that SO_2 fumigation might cause plant tissue anaerobiosis (see Discussion).

In SO_2-fumigated leaves, the fluorescence yield showed a rapid quenching from the high I level with a clear ID dip (Fig. 1b,c). The ID decline became larger with the time of SO_2 fumigation. Since the ID decline corresponds to the oxidation of Q and PQ by PS I (Munday & Govindjee, 1969; Satoh & Katoh, 1981), a quick ID decline indicates that oxidation of these electron carriers progressed rapidly in SO_2-fumigated leaves. The result suggests that SO_2 fumigation did not inhibit the electron flow between PS II and PS I. In unfumigated plants, no prominent ID dip was observed (Fig. 1a). This may indicate that Q and PQ were mostly in their oxidized states in unfumigated dark-adapted plants (Rühle & Wild, 1979).

SO_2 fumigation suppressed the DP rise (Fig. 1b,c) and completely eliminated it with 1 h fumigation (Fig. 1c). Since the DP rise corresponds to the photoreduction of Q by the PS II reaction linked to the water-splitting enzyme system (Munday & Govindjee, 1969), the result indicates inactivation of the water-splitting enzyme system and/or of the PS II reaction center. To locate the inactivation site more precisely, we isolated PS II from PS I by administering DCMU to the fumigated leaves by vacuum infiltration. In the presence of DCMU, the variable part of the fluorescence diminished slightly in 1-h fumigated leaves (Fig. 2A), in which the DP rise did not occur in the absence of DCMU (Fig. 1c). This result suggests that short-term SO_2 fumigation did not significantly affect the PS II reaction center. From these results, we conclude that short-term SO_2 fumigation inactivates the water-splitting enzyme system. However, as the fumigation time increases, the variable part of the fluorescence decreases in the presence of DCMU, indicating that the reaction center of PS II becomes damaged (Fig. 2A).

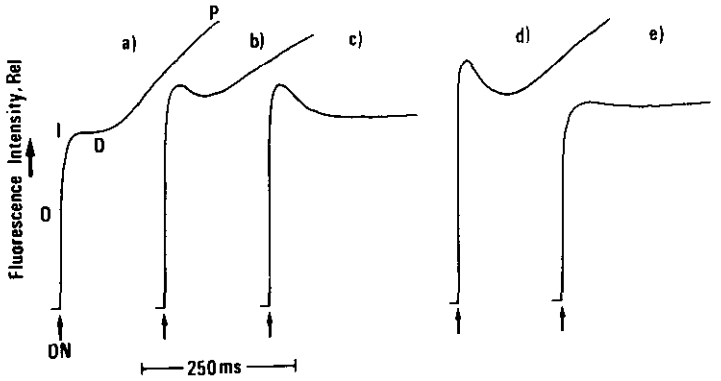

Fig. 1 Effects of SO_2 fumigation on Chl *a* fluorescence induction in spinach leaves

SO_2 of 2 ppm was fumigated. a,d, Unfumigated leaf. b, Leaf fumigated for 30 min. c,e, Leaf fumigated for 1 h. a,b,c, Leaf kept in carkness for 15 min before fluorescence measurement. d,e, Fluorescence measured 10 s after preillumination for 1 s. The intensity of the actinic and preillumination beam was 6.5×10^4 erg·cm^{-2}·s^{-1}. Fluorescence transients; O, initial fluorescence level; I, an intermediary level; D, a dip; P, peak.

Further support for the inactivation of the water-splitting enzyme system was also obtained. Fig. 1d shows the fluorescence transient in an unfumigated leaf which had been illuminated for 1 s, followed by dark incubation for 10 s prior to measurement. An elevated I level and an ID dip were found (Fig. 1d). This suggests that the preillumination time of 1 s reduces Q and PQ partially through PS II linked to the water-splitting enzyme system. The 1-s preillumination of fumigated leaves, however, induced neither a high I level nor an ID dip (Fig. 1e). The I level was much lower in fumigated leaves (Fig. 1e) than in unfumigated leaves (Fig. 1d) after the preillumination. These results suggest that the photoreduction rate of Q and PQ by PS II is lower in fumigated than in unfumigated leaves. In addition, we found a prominent DP rise in unfumigated leaves after preillumination (Fig. 1d) but not in fumigated leaves (Fig. 1e). All these results strongly suggest that SO_2 fumigation inactivates the water-splitting enzyme system *in vivo*.

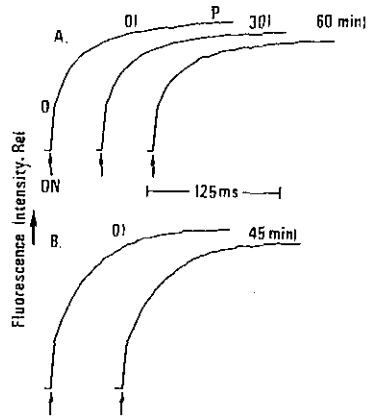

Fig. 2 Effects of SO_2 fumigation on Chl *a* fluorescence induction of spinach leaves in the presence of DCMU

Fumigation with SO_2 was conducted at 2 ppm for the periods indicated at the top of the figure. DCMU, 0.5 mM, was administered to the spinach leaf by vacuum infiltration. The leaf was kept in darkness for 10 min before the measurement. A: Immediately after SO_2 fumigation, DCMU was administered to the leaf, then fluorescence was measured. B: After SO_2 fumigation, spinach leaves were kept in darkness for 20 h at 20°C in SO_2-free air, then fluorescence was measured in the presence of DCMU. Fluorescence was traced on a digital transient recorder (Model TM 1410 Kawasaki Electronica). Actinic light intensity was 10,000 erg·cm^{-2}·s^{-1}.

Recovery of fluorescence induction after SO_2 fumigation

Fluorescence induction affected by SO_2 fumigation recovered when the fumigated leaf was kept in darkness in SO_2-free air. The time course of the recovery was expressed in terms of the increase in the ratio of the DP magnitude to the D level in fluorescence yield (Fig. 3). The magnitude of the DP transient was decreased to one-fifth of the control value by SO_2 fumigation for 45 min. No recovery was observed for 1 h after SO_2 removal, instead, there was a slight drop in the ratio (Fig. 3). Then recovery started and progressed to reach approximately 70% of the control level (Fig. 3). The result indicates that inactivation of the water-splitting enzyme system by SO_2 fumigation is largely reversible. A similar incomplete recovery of fluorescence induction was observed when the fumigated leaf was kept in light in SO_2-free air (not shown); light apparently had little effect on the recovery process.

Following recovery of fluorescence induction (20 h after the SO_2 fumigation), the variable fluorescence in the presence of DCMU was diminished slightly (Fig. 2B), indicating irreversible damage to the PS II reaction center. This damage may be reflected in a suppression of the DP rise (Fig. 3).

Effect of SO_2 fumigation on light-induced redox changes of P700

In plant leaves, P700, the primary electron donor of PS I, is normally in the reduced form because of its high redox potential (Kok, 1961) and is oxidized by far-red light which activates PS I predominantly and $P700^+$, the oxidized form of P700, exhibits an EPR signal (signal I) (Ke *et al.*, 1974). Thus, the oxidation-reduction kinetics of P700 *in vivo* can be determined by monitoring the EPR signal I as shown by Andreeva (1982). Fig. 4 shows the time courses of light-induced changes of the EPR signal I. The increase and decrease of the signal correspond to the oxidation and reduction of P700, respectively. In unfumigated plants, P700 was oxidized

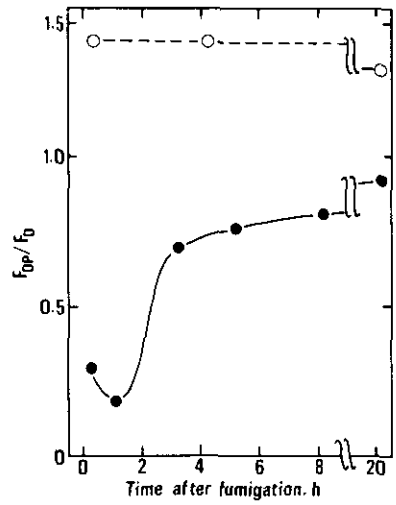

Fig. 3 Recovery of Chl *a* fluorescence induction of SO_2-fumigated spinach leaves

SO_2 of 2 ppm was fumigated for 45 min. ○, Unfumigated leaf. ●, Fumigated leaf. F_D and F_{DP} indicate the fluorescence intensities of the D level and the chage in fluorescence intensity from D to P, respectively. Recovery of the fluorescence induction was determined by incubating the fumigated leaf strips in darkness for the indicated periods. In each measurement, the leaf was preilluminated for 1 min, and 15 min after the dark incubation, fluorescence was recorded on a strip chart recorder (Model 3052 Yokogawa Technicorder F). Actinic light intensity was 10,000 erg·cm^{-2}·s^{-1}.

by far-red light and reduced gradually in darkness (Fig. 4a). On second excitation by far-red light, P700 was reoxidized, then reduced rapidly upon replacement of the far-red light with white light which activates both photosystems, although a small portion of P700 remained in the oxidized state (Fig. 4a). Neither the rate of dark reduction nor photo-oxidation was affected significantly by SO_2 fumigation for 45 min (Fig. 4b). However, the fumigation slowed the reduction of P700 by white light (Fig. 4b). These results are consistent with the fluorescence data which indicated inhibition of the water-splitting. When a dark-adapted unfumigated leaf was excited with white light only, a small transient due to photo-oxidation of P700 appeared (Fig. 4c) indicating that $P700^+$ was rapidly re-reduced by electron transport through PS II. In SO_2-fumigated leaves, a large transient $P700^+$ signal was elicited by white light (Fig. 3d), suggesting that electron flow through PS II was impaired.

As reported above, the effect of SO_2 fumigation on fluorescence induction was largely reversible (Fig. 3). We found that inhibition of P700 reduction by white light in SO_2-fumigated leaf was largely reversed when the leaf was kept in the dark in SO_2-free air (data not shown). Thus, the results of EPR experiments agree with those of fluorescence experiments and strengthen our conclusion that SO_2 fumigation reversibly inactivates the water-splitting enzyme system.

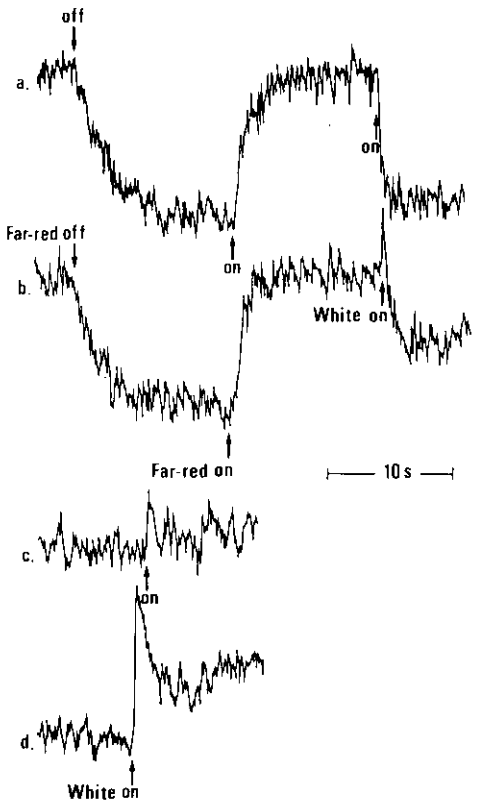

Fig. 4 Effects of SO_2 fumigation on the time courses of light-induced changes in the EPR signal I

After SO_2 fumigation at 2 ppm for 45 min, the leaf strip (0.25 × 1.5 cm) was excised quickly and the light-induced change of the EPR signal was measured. The EPR signal was photoinduced by far-red light (>700 nm), which was obtained by passing white light through a red cut-off filter (Hoya R-72). White light was provided by rapid removal of the red cut-off filter (Hoya R-72) or by using a shutter. a,c, Unfumigated leaf. b,d, Fumigated leaf. In a,b, the leaf was first illuminated by far-red light and kept in darkness (first downward arrows), and then re-illuminated with the same light (second upward arrows), followed by white light illumination (third upward arrows). c,d, Leaf strip excited by white light. Instrumental conditions: microwave power 10 mW, modulation amplitude 8G, and time constant 0.03 s. The magnetic field was fixed during measurement of the time course of EPR signal. Intensities of the far-red and white light were 7.5×10^5 and 1.0×10^6 erg·cm^{-2}·s^{-1}, respectively. The light source was a 1 kW Ushio Xenon lamp.

Effect of SO_2 fumigation on photosynthetic O_2 evolution

Fig. 5 shows the effect of SO_2 fumigation on O_2 uptake by respiration and on O_2 evolution by photosynthesis in a spinach leaf. The evolution was decreased to 40% of the control level by 0.5-h fumigation and to 15% by 1-h fumigation. O_2 uptake was only slightly affected (Fig. 5). The inhibition of O_2 evolution was in large part reversible; the O_2 evolution rate, which had been suppressed to 12 μmol O_2 mg^{-1} Chl h^{-1} by fumigation increased gradually and reached 31 μmol O_2 mg^{-1} Chl h^{-1} (70% of control value) at 3 h after the removal of SO_2.

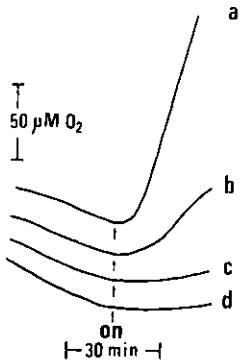

Fig. 5 Effects of SO_2 fumigation on the rate of O_2 exchange in spinach leaves

SO_2 of 2 ppm was fumigated. a, Unfumigated leaf. b, Leaf fumigated for 0.5 h. c, Fumigated for 1 h. d, Fumigated for 2 h. After SO_2 fumigation, O_2 uptake and evolution were measured immediately using fumigated leaves (1.2 x 5 cm) at 20°C in aqueous media. Rates of respiratory O_2 uptake and photosynthetic O_2 evolution in unfumigated plant were 8 and 69 μmol O_2 mg^{-1} Chl·h^{-1}, respectively. The reaction mixture (25 ml) contained 38 mM potassium phosphate buffer (pH 7.0) and 25 mM $NaHCO_3$. Photosynthesis was started by illumination with white light (2.2 x 10^5 erg·cm^{-2}·s^{-1}).

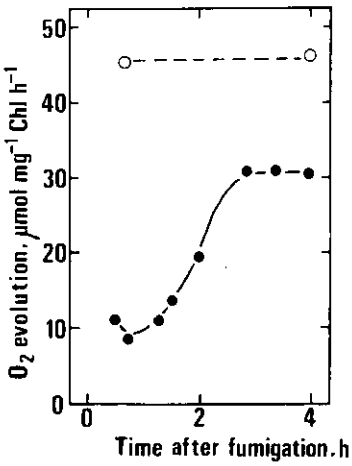

Fig. 6 Recovery of SO_2-inhibited O_2 evolution in spinach leaves after fumigation

SO_2 of 2 ppm was fumigated for 1 h. Photosynthetic O_2 evolution, measured continuously for 4 h during the illumination, remained constant in the unfumigated plant but increased gradually in the fumigated plant and could be determined from the slope of the O_2 evolution curve at the indicated times. Other experimental conditions were the same as in Fig. 5.

Discussion

The effects of SO_2 fumigation on photosynthesis were investigated using Chl *a* fluorescence *in vivo* as a parameter. Short-term (30–60 min) SO_2 fumigation not only inhibited the water-splitting enzyme system but also caused reduction of Q and PQ. These effects were reversible since dark incubation of the fumigated leaves in SO_2-free air restored the fluorescence induction. However, the recovery rate was slow, suggesting that toxicants formed in SO_2-fumigated leaves diminished only slowly. In a previous study (Shimazaki & Sugahara, 1979), we found no inhibition of the electron flow when cholorplasts were isolated from spinach leaves after short-term (30–60 min) fumigation. Toxicants causing the inhibition might have been removed during preparation of the chloroplasts from the leaves.

When the fumigation time was prolonged, damage to the reaction center of PS II became prominent. Our earlier work (Shimazaki & Sugahara, 1979; 1980) showed that chloroplasts isolated from long-fumigated plants had imparied PS II centers. It has been suggested that chloroplasts may be damaged during isolation by secondary toxic substance formed in the cytoplasm during fumigation (Heath, 1980). However, the present results offer evidence that SO_2 fumigation itself injures the reaction center of PS II *in vivo*, thus supporting our previous conclusion.

There is some discrepancy between the results of our present and previous studies. Chl *a* fluorescence from leaves exhibited an inhibition of PS II reaction centers even after short-term (30–60 min) SO_2 fumigation although the inhibition was slight (Fig. 2a). However, in this fumigation period we detected no inhibition when chloroplasts were isolated from the fumigated leaves. We detected PS II inhibition in chloroplasts only when the fumigation time exceeded 1 h (Shimazaki & Sugahara, 1979). This time difference may be due to differences in chloroplast populations measured, as Chl *a* fluorescence is mainly derived from chloroplasts located near the leaf surface whereas isolated chloroplasts were obtained uniformly from the entire chloroplast population. Thus the delayed inhibition in the isolated chloroplasts may have been an expression of a time delay before PS II centers of a sizable portion of the chloroplast population became affected.

Chl *a* fluorescence induction yielded information on the redox-state of the primary electron acceptor of PS II but little on that of the reaction center of PS I. This was part of the reason that we investigated the effect of SO_2 on the oxidation-reduction kinetics of P700 by measuring the light-induced EPR signal I *in vivo*. The redox behavior of P700 was consistent with the reversible inactivation of the water-splitting enzyme system being the major effect of SO_2. There was no indication that the PS I reaction center or the dark electron flow from PS II to PS I was inhibited by SO_2 fumigation.

SO_2 fumigation inhibited CO_2-dependent photosynthetic O_2 evolution in spinach leaves (Fig. 5). The inhibition may be ascribed to the inactivation of the water-splitting enzyme system. If this is the case, the O_2 evolution activity should be restored after SO_2 fumigation as observed with Chl *a* fluorescence. The activity recovered gradually with a time course similar to that of the recovery of fluorescence induction (Fig. 3 and 6). Moreover, the recovery of O_2 evolution was partial (Fig. 6) as with fluorescence induction (Fig. 3). Most of the irreversible inhibition of O_2 evolution is probably due to the damage to PS II reaction centers.

SO_2 fumigation reversibly inhibits photosynthetic CO_2-fixation activity (Sij & Swanson, 1974; Furukawa *et al.*, 1979; Tanaka *et al.*, 1982b). In a recent study, Tanaka *et al.* (1982b) demonstrated that the inhibition of photosynthetic CO_2 fixation during SO_2 fumigation is caused by reversible inactivation of SH-enzymes in the Calvin cycle, especially at the beginning of fumigation. This raises the possibility that the depression of O_2 evolution shown in the

present study is due to inactivation of the Calvin cycle enzymes.

Although the toxicants exerting the adverse effect on the water-splitting enzyme system, were not identified, sulfide may be the main inhibitor. SO_2 entering the leaf tissue through the stomata would yield H^+, HSO_3^- and SO_3^{2-} ions upon dissolving in water. In light, O_2^- formed on the reducing side of PS I initiates an aerobic chain oxidation of sulfite to yield a large number of active oxygen species such as O_2^-, $^1O_2$, H_2O_2 and OH· (Asaka & Kiso, 1973; Shimazaki *et al.*, 1980; Tanaka *et al.*, 1980, 1982a). Part of the sulfite is photoreduced to H_2S (Cormis, 1968; Silvius *et al.*, 1979). Among these toxicants, sulfide (S^{2-}) is relatively stable in leaves and its effect on Chl fluorescence is similar to that of SO_2 fumigation (Oren *et al.*, 1979). It inhibits the water-splitting enzyme system without affecting the PS II reaction center and raises the I level to produce the ID dip due to decreased oxygen tension caused by chemical oxidation of sulfide (Oren *et al.*, 1979). Low levels of H_2S (30–300 ppb) in the air inhibit plant growth (De Kok *et al.*, 1982) and a positive correlation has also been observed between the capability of H_2S emission (removal of H_2S from the leaf tissue) into the atmosphere and SO_2 resistance in cucumber (Sekiya *et al.*, 1982).

In the present investigation, we examined the effect of short-term fumigation with SO_2 at concentrations higher than ambient. Using Chl *a* fluorescence technique, reversible and irreversible inhibitions of electron transport associated with PS II were demonstrated. These results are consistent with reversible and irreversible inhibitory effects on photosynthesis of fumigation with low levels of SO_2 (Saxe, 1983), suggesting that the results are relevant to the phytotoxicity of SO_2 pollution.

Acknowledgment

We wish to thank Mr. M. Kunugi, Chemistry and Physics Division of our institute for preparing the EPR spectrometer. We also thank the members of the Engineering Division of our institute for their adept cultivation of plants and maintenance of the growth cabinet for fumigation.

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SO_2 暴露による光合成水分解系の可逆的阻害のクロロフィルけい光及び電子スピン共鳴法による *in vivo* 測定

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SO_2 暴露の光合成に及ぼす影響をクロロフィルけい光及び電子スピン共鳴法により *in vivo* で調べ，以下のことが明らかになった。

1）短時間の SO_2（2ppm）暴露により光合成水分解系が阻害され，暴露時間が長くなると光化学系Ⅱ反応中心も阻害された。

2）短時間暴露を行ったホウレンソウ葉を明又は暗条件におくと阻害された水分解系はゆっくりと回復した。

以上の結果は SO_2 暴露により，光合成水分解が可逆的に阻害され，また暴露時間が長くなると光化学系Ⅱ反応中心が損傷を受けることを示している。

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Res. Rep. Natl. Inst. Environ. Stud., Jpn., No. 65, 1984.

Accumulation of Hydrogen Peroxide in Chloroplasts of SO_2-fumigated Spinach Leaves*

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Illuminated chloroplasts isolated from SO_2-fumigated spinach leaves accumulated more H_2O_2 than those from non-fumigated ones. This H_2O_2 formation was dependent on light and was inhibited by DCMU. It also was depressed by cytochrome *c* and superoxide dismutase (EC 1.15.1.1). The addition of sulfite to ruptured chloroplasts isolated from non-fumigated leaves caused an H_2O_2 accumulation that accompanied O_2 uptake. Spinach leaves lost their catalase (EC 1.11.1.6), ascorbate peroxidase and glutathione reductase (EC 1.6.4.2) activities at the beginning of SO_2 fumigation, when H_2O_2 was accumulated. These results suggest that the accumulation of H_2O_2 in SO_2-fumigated spinach leaves is caused by the increase in O_2^- production, the precursor for H_2O_2, with a sulfite-mediated chain reaction at the reducing site of photosystem I, and by inactivation of the H_2O_2 scavenging system.

Key words: Ascorbate peroxidase, Catalase, Glutathione reductase, Hydrogen peroxide, Sulfur dioxide, Spinach.

When the fumigation of higher plants with SO_2 is begun, one of the most remarkable phytotoxicities at the physiological level is the depression of photosynthesis, which always precedes visible injury. Most studies of the depression of light-dependent CO_2 uptake with SO_2 have done with an infrared gas analyzer (Furukawa *et al.* 1979); there have been only a few physiological studies on the inhibitory mechanism of CO_2 fixation with SO_2. Ziegler (1972) reported that ribulose-1,5-bisphosphate carboxylase was inhibited by sulfite ion competitively with $NaHCO_3$. But, there is no reliable evidence that this inhibition occurs in SO_2-fumigated leaves. Shimazaki and Sugahara (1979, 1980) demonstrated that photosystem II in chloroplasts isolated from SO_2-fumigated spinach leaves was inhibited, and that this damage occurred just before or when visible injury appeared.

SO_2 toxicity may be grouped into the direct and indirect effects of sulfite on plants. In isolated chloroplasts, sulfite itself almost perfectly inhibits CO_2-fixation at 1 to 10 mM (Ziegler, 1972; Silvius *et al.*, 1975). But, it is doubtful whether sulfite accumulates at such high

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concentrations in chloroplasts because sulfite is photooxidized rapidly to a less toxic sulfate (Asada & Kiso, 1973; Miller & Xerikos, 1979). Tanaka and Sugahara (1980), Shimazaki *et al.* (1980), and Peiser and Yang (1979) have proposed that the active species of oxygen produced in chloroplasts of SO_2-fumigated leaves are reponsible for SO_2 toxicity. The superoxide radical causes plant damage at 0.01 to 0.1 μM (Asada *et al.*, 1977), and hydrogen peroxide strongly inhibits CO_2-fixation in intact chloroplasts (80% inhibition at 10 μM) (Kaiser, 1976). The present paper describes hydrogen peroxide accumulation in chloroplasts from SO_2-fumigated spinach leaves and gives a preliminary report on the possible participation of H_2O_2 in the inhibition of photosynthesis at the initial stage of SO_2 fumigation.

Materials and Methods

Plant materials and SO_2 fumigation

Spinach plants (*Spinacia oleracea* L. cv. New Asia) were grown in a phytotron and fumigated with 2 ppm SO_2 in a growth cabinet, as described previously (Tanaka & Sugahara, 1980).

Preparation of chloroplasts

After SO_2 fumigation, the spinach leaves (50 to 70 g) were homogenized in a National blendor (MX-50s) with 130 ml of grinding medium (pH 6.5), consisting of 50 mM MES-NaOH, 0.4 M sorbitol, 2 mM EDTA, 5 mM $MgCl_2$ and 5 mM NaCl at three 2-sec bursts at top speed. The homogenate was immediately filtered through four layers of cotton cloth after which the filtrate was centrifuged at 750 × *g* for 30 s. The pellet was twice washed with 100 ml of 50 mM HEPES-NaOH (pH 7.6), 0.4 M sorbitol, 2 mM EDTA, 5 mM $MgCl_2$ and 5 mM NaCl by centrifugation at 750 × *g* for 40 s. Under these conditions, 50 to 80% intact chloroplasts were obtained from the control leaves, but the intactness of chloroplasts from SO_2-fumigated leaves was very low. The plastid pellet was resuspended in 2 ml of the washing medium.

Chlorophyll was estimated by the method of Arnon (1949). Protein was determined by the method of Lowry *et al.* (1951). The percentage of intact chloroplasts with envelopes was estimated by the method of Heber and Santarius (1970).

Measurement of H_2O_2 and O_2 in chloroplasts

The standard reaction mixture for measurements of H_2O_2 formation and O_2 uptake contained (final volume 1 ml) 50 mM HEPES-NaOH (pH 7.6), 0.4 M sorbitol, 10 mM $NaHCO_3$ and chlorplasts equivalent to 80 to 120 μg chlorophyll. O_2 uptake was measured at 25°C under saturated while light (−1,000 $W \cdot m^{-2}$) in a Hansatech oxygen electrode vessel and portions of the reaction mixture were sampled with a microsyringe to determine the H_2O_2 contents periodically. H_2O_2 was measured according to the method of Asada *et al.* (1974) using a reaction mixture which contained (total volume 2 ml) 100 mM HEPES-NaOH (pH 7.6), 5 mM homovanillic acid. 50 nM peroxidase and chloroplasts. The fluorescence intensity at 425 nm was determined with a Hitachi MPF-4 spectrofluorophotometer when the sample was excited at 315 nm.

Measurement of H_2O_2, enzymes, ascorbate, GSH and GSSG in leaves

Ten to twenty leaf discs (1.5 cm in diameter) were excised within 3 min at the times indicated during SO_2 fumigation. Ten leaf discs contained approximately 0.73 mg chlorophyll

and 5.9 mg protein. These discs were homogenized immediately with a Polytron (Kinematica PT 10/35) in 5 ml of various extracting solutions that suited each purpose. The extracting solutions were 0.1 M Tris-HCl (pH 7.8) for enzyme activities; 5% metaphosphoric acid for ascorbate; 80% ethanol containing 5 mM sodium phosphate buffer (pH 7.8) and 0.25 mM EDTA for GSH and GSSG; 80% ethanol containing 5 mM sodium phosphate buffer (pH 7.5), 0.25 mM EDTA and 2 mM *N*-ethylmaleimide for GSSG; and 0.1 M phosphate buffer (pH 6.0) containing 5 mM homovanillic acid and 0.8 μM peroxidase in the presence or absence of 1,000 unit catalase for H_2O_2. In all cases the homogenates were centrifuged at 18,000 × *g* for 30 min after which the clear supernatants were used in the determination for each substance. H_2O_2 was measured fluorometrically as described above. Enzyme activities in crude extracts were measured after dialyzing the extracts against 20 volumes of 10 mM Tris-HCl (pH 7.8) with four changes overnight; ascorbate peroxidase and dehydroascorbate reductase activities were measured without dialysis.

Superoxide dismutase and guaiacol peroxidase (EC 1.11.1.7) were assayed as described previously (Tanaka & Sugahara, 1980). Catalase was assayed at 25°C by the oxygen evolution from H_2O_2 with a reaction mixture (1 ml) that contained 50 mM Tris-HCl (pH 7.8), 5 mM H_2O_2 and crude enzyme. Glutathione reductase was assayed by monitoring the oxidation of NADPH with GSSG (Foster & Hess, 1980). Ascorbate peroxidase and dehydroascorbate reductase (EC 1.8.5.1) were assayed according to Nakano and Asada (1981) with a slight modification. Ascorbate peroxidase was assayed at 25°C with a reaction mixture (1 ml) that contained 50 mM MES-NaOH (pH 6.3), 1 mM ascorbate, 0.2 mM H_2O_2 and enzyme. Dehydroascorbate reductase was assayed at 25°C with a reaction mixture (1 ml) that contained 50 mM MES-NaOH (pH 6.3), 2 mM dehydroascorbate, 5 mM GSH and enzyme.

In the assays of ascorbate peroxidase and dehydroascorbate reductase, the decrease and increase in absorbance at 290 nm due to ascorbate with a reference wavelength at 310 nm (an absorbance coefficient of 2.8 $mM^{-1}cm^{-1}$) were measured with a Hitachi 557 dual-wavelength spectrophotometer. The determinations of GSH and GSSG were performed according to Tietze (1969). Ascorbate was determined at 25°C by measuring the ascorbate oxidase-dependent O_2 uptake with a Hansatech oxygen electrode. The reaction mixture (1 ml) contained 100 mM MES-NaOH (pH 6.3), 20 units of ascorbate oxidase and the extracts adjusted to pH 6.3 with 1 M Tris-base. The reaction was started by adding ascorbate oxidase.

Chemicals

Horseradish peroxidase (type II' RZ: 1.4), horse heart cytochrome *c* (type III), bovine erythrocyte superoxide dismutase and homovanillic acid were obtained from Sigma. Milk xanthine oxidase and cucurbita species ascorbate oxidase were products of Boehringer. Beef liver catalase from P-L Biochemical Inc. was purified further on a column of Sepharose 6B to remove the contaminating superoxide dismutase. Dehydroascorbate was purchased from Pfaltz and Bauer Inc.

The superoxide dismutase preparation of Sigma was purified further on a column (1.5 × 15 cm) of hydroxyapatite gel equilibrated with 10 mM phosphate buffer (pH 7.8). The superoxide dismutase (30 mg in 3 ml of 10 mM phosphate buffer) was charged onto the gel and the column washed with 100 ml of 10 mM phosphate buffer (pH 7.8). The enzyme was eluted with 100 ml of a linear 10 to 100 mM phosphate buffer (pH 7.8). The most active fraction was showed to be homogeneous by polyacrylamide gel disc electrophoresis at pH 8.3 (Davis 1964). The amount of superoxide dismutase was determined from the absorbance at 258 nm (Asada *et al.*, 1973).

Results

Illuminated chloroplasts isolated from SO_2-fumigated spinach leaves accumulated H_2O_2 at a higher rate than those from non-fumigated ones. The rate of H_2O_2 accumulation increased during SO_2 fumigation and reached a maximum after 2 hr-fumigation (Fig. 1). Assuming that the average concentration of chlorophyll in intact chloroplasts is 25 mM (Nobel 1973) and that all the H_2O_2 stays in the chloroplasts, the rate of H_2O_2 accumulation in chloroplasts from 2-h-fumigated leaves was 11.3 μmol H_2O_2 formed mg^{-1} chlorophyll h^{-1} and corresponds to 260 mM H_2O_2 for 1 h. Therefore, only 0.14 s of illumination is enough to accumulate 10 μM H_2O_2, which leads to 50% inhibition of photosynthesis (Kaiser, 1976).

The accumulation of H_2O_2 was accompanied by an increased O_2 uptake (Fi g. 1) and was completely inhibited by 10 μM DCMU (data not shown). These results are evidence that H_2O_2 is produced by the photoreduction of O_2 in chloroplasts. Since Asada and Kiso (1973) demonstrated that chloroplasts illuminated in the presence of sulfite ion produced much O_2^- at the reducing site of photosystem I, it is perfectly conceivable that this increased production of O_2^- caused the accumulation of H_2O_2. When broken chloroplasts prepared from non-fumigated leaves were illuminated in the presence of sulfite ion, an H_2O_2 accumulation accompanied by O_2 uptake was found (Fig. 2). Because H_2O_2 reacts with sulfite ion, the rate of H_2O_2 accumulation became saturated at a lower concentration of sulfite than did that of O_2 uptake.

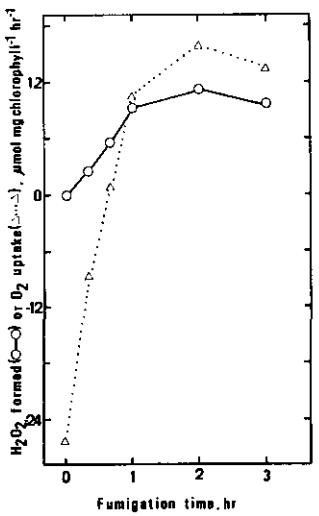

Fig. 1 H_2O_2 formation and O_2 uptake in chloroplasts isolated from spinach leaves fumigated with 2.0 ppm SO_2 for the periods indicated

After illumination for 10 min, a sample was transferred to a cuvette for the assay of H_2O_2.

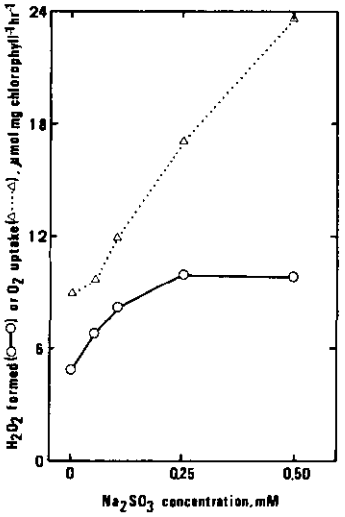

Fig. 2 Effects of Na_2SO_3 on H_2O_2 formation and O_2 uptake in ruptured chloroplasts prepared from nonfumigated leaves

Chloroplasts isolated from nonfumigated leaves were ruptured in an oxygen electrode vessel in 10 volumes of 50 mM HEPES-NaOH (pH 7.8) containing 2 mM EDTA, 5 mM NaCl and 5 mM $MgCl_2$. Other conditions were the same as described in Fig. 1, except that the Na_2SO_3 concentration was varied as indicated. The photoreactions of H_2O_2 formation and O_2 uptake were followed for 5 min.

The accumulation of H_2O_2 in chloroplasts from SO_2-fumigated leaves was depressed by superoxide dismutase and cytochrome *c* (Fig. 3). Fig. 3b and c show the dependence of the depression of H_2O_2 formation on the superoxide dismutase and cytochrome *c* concentrations. The inhibition of H_2O_2 formation with cytochrome *c* is explained by the reduction of cytochrome *c* by O_2^-, a precursor of H_2O_2. Superoxide dismutase may depress H_2O_2 formation by removing the O_2^- that initiates the sulfite-mediated chain reaction.

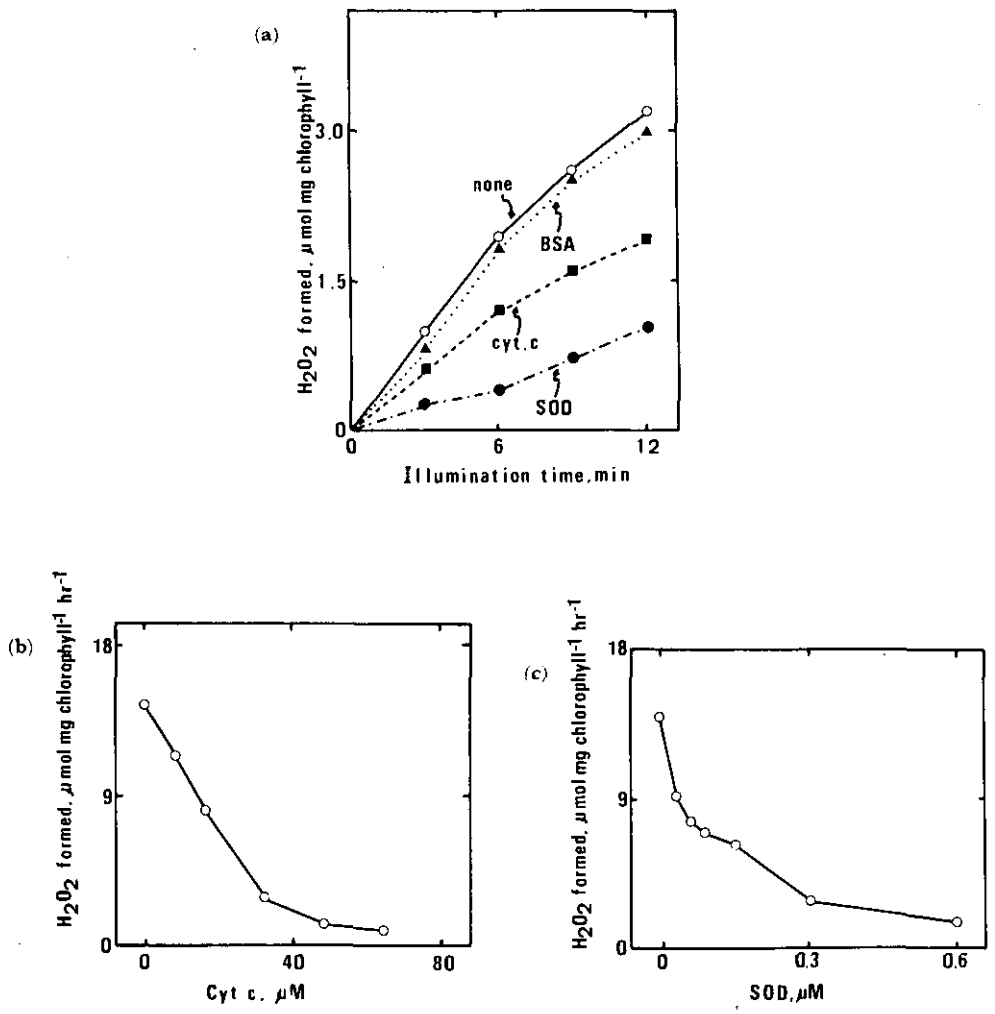

Fig. 3 Effects of cytochrome *c* and superoxide dismutase on H_2O_2 formation in illuminated chloroplasts isolated from SO_2-fumigated leaves

Chloroplasts were prepared from leaves fumigated for 1 h. The photoreaction was the same as described in Fig. 1, except for the presence of 16 μM cytochrome *c*, 0.15 μM superoxide dismutase and 7.5 μM bovine serum albumin (a). In b and c, the superoxide dismutase and the cytochrome *c* concentrations were varied as indicated.

To examine whether H_2O_2 also accumulates in spinach leaves during SO_2 fumigation, we homogenized fumigated leaves just after sampling in phosphate buffer containing homovanillic acid and peroxidase in the presence or absence of catalse. Samples prepared from SO_2-fumigated leaves had higher fluorescence intensity due to H_2O_2 than non-fumigated ones did (Fig. 4). In contrast the fluorescence intensity decreased when catalase was added during the extraction. Thus, H_2O_2 did accumulate in leaf cells during SO_2 fumigation.

The relationships for chloroplast integrity of SO_2-fumigated spinach leaves, H_2O_2 formation and O_2 uptake are shown in Fig. 5. The destruction of chloroplast integrity in spinach leaves during SO_2 fumigation also may be related to H_2O_2 formation. This agrees with observations that H_2O_2 accumulates in broken chloroplasts more actively than in intact ones (Allen, 1978a, b; Robinson *et al.*, 1980; Nakano & Asada, 1980). But, the accumulation of H_2O_2 in broken chloroplasts prepared from SO_2-fumigated leaves also was greater than in chloroplasts from non-fumigated ones. These results mean that the increased accumulation of H_2O_2 may be caused not only by decreased chloroplast integrity, but by other conditions such as the accumulation of sulfite and inactivation of enzymes that scavenge H_2O_2 as described below.

Activities of enzymes that scavenge active species of oxygen were followed during SO_2 fumigation (Fig. 6). Catalase, ascorbate peroxidase and glutathione reductase were inactivated, but superoxide dismutase, guaiacol peroxidase and dehydroascorbate reductase were scarcely affected. Nakano and Asada (1980, 1981) recently demonstrated that intact spinach chloroplasts can scavenge H_2O_2 at a high rate under light. Because the scavenging rate of H_2O_2 is higher than the formation rate of H_2O_2 in chloroplasts, the H_2O_2 produced in the chloroplasts would be scavenged not by the catalase in the peroxisome, but by the chloroplasts themselves.

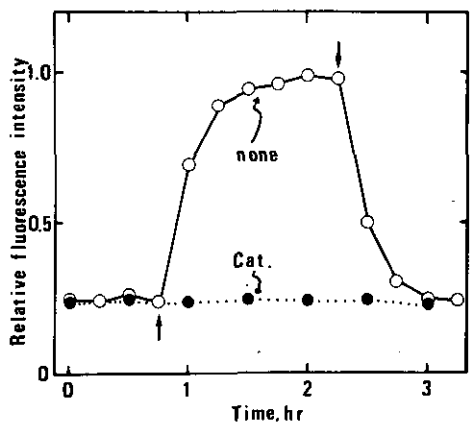

Fig. 4 Formation of H_2O_2 in SO_2-fumigated spinach leaves .

Ten leaf discs were cut from leaves fumigated for the periods indicated. The fluorescence of the clear extract was measured 3 h after homogenization. At the indicated tumes, SO_2 was passed in (↓) or out (↑).

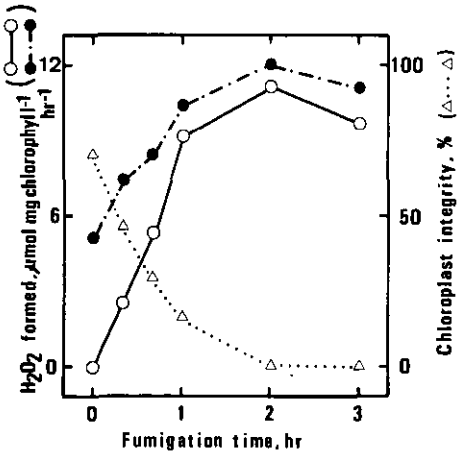

Fig. 5 Relationship between H_2O_2 formation and chloroplast integrity in chloroplasts prepared from SO_2-fumigated spinach leaves

H_2O_2 formation in chloroplasts (○) isolated from leaves fumigated for the periods indicated and in ruptured chloroplasts (△), as described in Fig. 2, was measured. The photoreaction of H_2O_2 formation took place for 10 min.

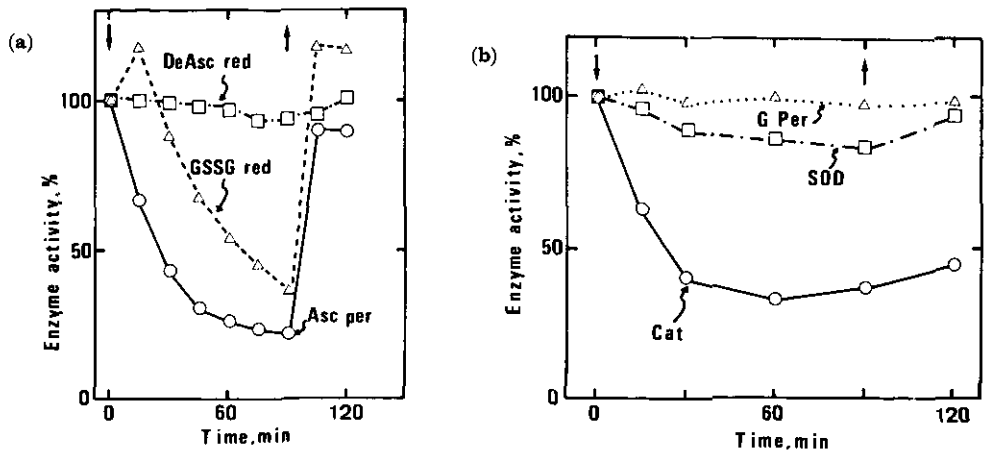

Fig. 6 Effects of SO_2 fumigation on several enzymes that scavenge active species of oxygen

Spinach plants in pots were fumigated with 2.0 ppm SO_2 for the periods indicated, then 15 leaf discs were excised. The enzyme activity 100 corresponds to 0.546 μmol of ascorbate oxidized cm^{-2} leaf area min^{-1} for ascorbate peroxidase (Asc per); 0.025 μmol dehydroascorbate reduced cm^{-2} leaf area min^{-1} for dehydroascorbate reductase (Deasc red); 9.6 μmol O_2 evolved cm^{-2} leaf area min^{-1} for catalase (Cat); 2.4 unit cm^{-2} leaf area for superoxide dismutase (SOD) and 0.44 unit cm^{-2} leaf area for guaiacol peroxidase (G per). Where indicated, SO_2 was passed in (↓) or out (↑). The definition of an enzyme unit has been described previously (Tanaka & Sugahara 1980).

The presence of ascorbate (Gerhardt, 1964) and glutathione (Foyer & Halliwell, 1976) at high concentrations in chloroplasts, and the localization of ascorbate peroxidase, dehydroascorbate reductase (Nakano & Asada, 1981), and glutathione reductase (Foyer & Halliwell, 1976) in the chloroplast stroma support the NADP → glutathione → ascorbate → H_2O_2 system for the scavenging of H_2O_2 in chloroplasts. Conceivably, the inactivation of ascorbate peroxidase and glutathione reductase may be more relevant to the accumulation of H_2O_2 than that of catalase.

H_2O_2 disappeared immediately after the removal of SO_2 (Fig. 4). The rapid recovery of ascorbate peroxidase and glutathione reductase and the almost nonrecovery of catalase after the removal of SO_2 support the position that the accumulation of H_2O_2 was brought about by inactivation of the chloroplast H_2O_2-decomposing system in addition to stimulation of H_2O_2 production. The contents of ascorbate and glutathione, which participate as substrates in the chloroplast-H_2O_2-decomposing system, and which also react with the superoxide radical (Asada *et al.*, 1977), were not affected by SO_2-fumigation (Fig. 7).

Discussion

It is widely accepted that gaseous SO_2 turns to SO_3^{2-} and HSO_3^- within leaf tissues (Hällgren, 1978; Thompson, 1967). Only a few percent of these ion species are incorporated into the sulfur metabolites in plants (Hällgren, 1978). Most of the SO_3^{2-} and HSO_3^- is

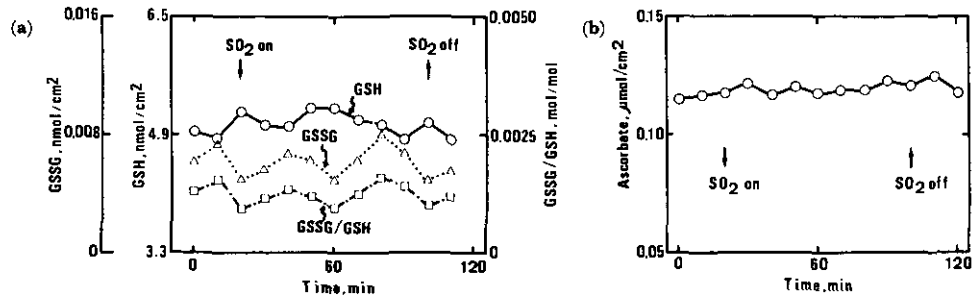

Fig. 7 Effects of SO_2 fumigation on the contents of glutathione and ascorbate in spinach leaves

Fifteen leaf discs were used for the determinations of glutathione (a) and ascorbate (b). Where indicated, 2.0 ppm SO_2 was passed in (↓) and out (↑).

photooxidized to the less toxic SO_4^{2-} in chloroplasts; this photooxidation is accompanied by propagation of the superoxide radical (O_2^-) (Asada & Kiso, 1973). The superoxide radical is dismutated to H_2O_2 and O_2 either spontaneously or by chloroplast superoxide dismutase. Interactions among the H_2O_2, O_2^- and chloroplast components produces the hydroxyl radical (OH·) and singlet oxygen ($^1O_2$) (Asada *et al.*, 1977). These active species of oxygen are highly deleterious to cell components. Therefore, a major reason for the SO_2 toxicities may be the production of active species of oxygen in higher plants.

Among the active species of oxygen produced on the thylakoids, $^1O_2$ and OH· would not diffuse to the stroma because of their short lives and high reactivity with such thylakoid membrane components as tocopherol, carotenoids and lipids (Asada *et al.*, 1977; Foote, 1976; Fridovich, 1978, Takahama & Nishimura, 1975, 1976). Similarly, O_2^- could hardly encounter stromal components due to its scavening by superoxide dismutase and other membrane bound electron transfer components such as cytochrome *f* (Tanaka *et al.*, 1978), plastocyanin (Takahashi *et al.*, 1980) and ferredoxin (Allen, 1975). But, H_2O_2 having a weaker reactivity and a longer life than the other active oxygen can react with stromal components. Although it is conceivable that inhibition of photosynthesis on SO_2 fumigation is caused by H_2O_2, there has been no report of how much H_2O_2 is accumulated in chloroplasts during SO_2 fumigation.

Chloroplasts isolated from 2 hr-SO_2-fumigated leaves would produce 260mM H_2O_2 in 1 hr of illumination, assuming that no H_2O_2 escapes from the chloroplasts (Fig. 1). Increased H_2O_2 accumulation could be detected after only 20 min of fumigation. The rates of H_2O_2 accumulation are high enough to depress photosynthesis. SO_2 fumigation for more than 2 hr decreased the rate of H_2O_2 formation. This decrease may be caused by inhibition of photosystem II by SO_2 (Shimazaki & Sugahara, 1979, 1980). H_2O_2 formation was accompanied by an increase in the light-dependent O_2 uptake (Fig. 1 and 2). The increases in the H_2O_2 formation and O_2 uptake were suppressed by DCMU (data not shown). The fact that both superoxide dismutase and cytochrome *c* depressed H_2O_2 formation (Fig. 3) is evidence that H_2O_2 was produced via O_2^-, and that an increase in the O_2^- concentration caused by the sulfite-mediated chain reaction might take place in chloroplasts from SO_2-fumigated leaves.

The SO_2 fumigation to spinach leaves gave chloroplasts which produced conditions for the

increase of O_2^- concentration other than by sulfite-mediated chain reaction. Chloroplast integrity was impaired by SO_2 fumigation (Fig. 5). Destruction of the chloroplast envelope stimulates the formation of O_2^- by accelerating the interaction of ferredoxin and/or P-430 with O_2 (Asada & Nakano, 1978; Lien & San Pietro, 1979; Robinson *et al.*, 1980; Allen, 1978a b) which results in the accumulation of H_2O_2. Of the enzymes that scavenge active oxygen, ascorbate peroxidase, glutathione reductase and catalase were more sensitive to SO_2 fumigation (Fig. 6). This suggests that the accumulation of H_2O_2 may originate not only from acceleration of its formation, but by a decrease in its decomposition as well.

A transient increase in glutathione reductase activity after a 15-min-fumigation with 2 ppm SO_2 also was observed in cotton leaves exposed to 75% O_2 (Foster & Hess, 1980). The effect of low concentrations of SO_2 on the enzyme is now under investigation. The relationship between the inhibition of photosynthesis and the inactivation of chloroplast SH enzymes with the H_2O_2 produced during SO_2 fumigation is the topic of another paper (Tanaka *et al.*, 1982).

Acknowledgment

We thank the members of the Engineering Division of this Institute for their operation of the growth cabinet and for the cultivation and supply of plant materials. We also are indebted to Drs. K. Asada and M. Takahashi of the Research Institute for Food Science, Kyoto University for their valuable discussions of the work. This study was supported by a grant from the Environmental Agency of Japan.

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二酸化イオウ暴露ホウレンソウ葉緑体における過酸化水素の蓄積

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二酸化イオウ暴露したホウレンソウから単離した葉緑体は光照射すると260mM h^{-1} という高速度で過酸化水素を蓄積した。一方，非暴露葉緑体の過酸化水素生成は全く起こらなかった。暴露葉緑体の過酸化水素生成はDCMU，チトクロム c，スーパーオキシド　ジスムターゼで抑制された。植物体における過酸化水素分解に関与する酵素系であるカタラーゼ，アスコルビン酸パーオキシダーゼ，グルチオン還元酵素活性は二酸化イオウ暴露初期に減少した。非暴露葉緑体に亜硫酸イオンを加えて光照射したときにも酸素吸収を伴う過酸化水素生成が検出された。以上の結果から二酸化硫黄暴露時の過酸化水素の蓄積は葉緑体光化学系Ⅰにおける亜硫酸イオンによるスーパーオキシド・ラジカル（過酸化水素の前駆体）の連鎖反応的生成と過酸化水素分解系の阻害の両方が原因で起こると推察した。

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Res. Rep. Natl. Inst. Environ. Stud., Jpn., No. 65, 1984.

Participation of Hydrogen Peroxide in the Inactivation of Calvin-Cycle SH Enzymes in SO_2-Fumigated Spinach Leaves*

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In SO_2-fumigated spinach leaves under light, chloroplast SH enzymes, glyceraldehyde-3-phosphate dehydrogenase (NADP-GAPD) (EC 1.2.1.13), ribulose-5-phosphate kinase (Ru5PK) (EC 2.7.1.19) and fructose-1,6-bisphosphatase (FBPase) (EC 3.1.3.11) were more remarkably inactivated than other chloroplast enzymes. Their activities recovered after removal of SO_2. The inactivation paralleled light-dependent CO_2-fixation in spinach leaves. In illuminated chloroplasts isolated from SO_2-fumigated spinach leaves, NADP-GAPD and Ru5PK were more specifically inactivated than other chloroplast enzymes. These two enzymes could be protected from the inactivation by adding catalase. The NADP-GAPD inactivation was suppressed by DCMU, cytochrome *c* or anaerobic conditions. By adding thiol compounds, the NADP-GAPD inactivation was discharged and the activity increased. In chloroplasts or crude extracts from non-fumigated spinach leaves, NADP-GAPD and Ru5PK were more strongly inhibited by externally added H_2O_2 than other chloroplast enzymes. All results supported the idea that the suppression of photosynthesis at the beginning of SO_2 fumigation was caused by the reversible inhibition of chloroplast SH enzyme with H_2O_2.

Key words: CO_2-fixation (spinach), Fructose-1,6-bisphosphatase, Hydrogen peroxide, NADP-glyceraldehyde-3-phosphate dehydrogenase, Ribulose-5-phosphate kinase, Sulfur dioxide.

Although higher plants are known to lose the faculty for CO_2 fixation at the beginning of SO_2 fumigation, the mechanism has remained unclear. Several workers proposed an important role of active oxygen in SO_2 toxicity (Tanaka & Sugahara, 1980; Shimazaki *et al.*, 1980; Peiser & Yang, 1979). In a previous paper, we showed that H_2O_2 accumulated in illuminated chloroplasts of SO_2-fumigated leaves (Tanaka *et al.*, 1982). H_2O_2 at low concentration (10 to 100 μM) is inhibitory to chloroplast SH enzymes (Kaiser, 1979; Robinson *et al.*, 1980; Forti &

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Abbreviations: FBPase, fructose-1,6-bisphosphatase; G6PD, glucose-6-phosphate dehydrogenase; NADP-GAPD, NADP-glyceraldehyde-3-phosphate dehydrogenase; PGAK, 3-phosphoglycerate kinase; RuBPC, ribulose-1,5-bisphosphate carboxylase; Trans A, Transaldolase; Trans K, Transketolase.

Gerola, 1977; Heldt *et al.*, 1978). Therefore, it is conceivable that the inhibition of photosynthesis in SO_2-fumigated higher plants is caused by H_2O_2.

This paper reports findings that chloroplast SH enzymes, NADP-GAPD, Ru5PK and FBPase, in SO_2-fumigated leaves are more strongly inactivated than other enzymes, and that the inactivation may be caused by the oxidation of sulfhydryl groups of these enzymes and the suppression of their light activation process with H_2O_2.

Materials and Methods

Preparation of spinach chloroplasts

Spinach (*Spinacia oleracea* L. cv. New Asia) plants were grown in a phytotron and fumigated with 2.0 ppm SO_2 under light ($-1{,}000 W \cdot m^{-2}$) in a growth cabinet as previously described (Tanaka & Sugahara, 1980). Chloroplasts were isolated in an isotonic sorbitol medium without ascorbate from the SO_2-fumigated leaves (Tanaka *et al.*, 1982). Chloroplasts were stored in the dark for 2 hr before experiments. Part of the stocked chloroplasts (100 to 200 μg chlorophyll) was transferred to a test tube containing 0.4 M sorbitol, 50 mM HEPES-NaOH (pH 7.6) and 10 mM $NaHCO_3$, in a total volume of 1 ml, and incubated under light ($-1{,}000 W \cdot m^{-2}$) at 25°C. Chloroplasts in the test tube were periodically transferred to the reaction cuvette for enzyme assay.

The CO_2 uptake of spinach plants in a pot was measured in a plexiglass assimilation chamber (50 x 50 x 95 cm) at 20°C, 75% relative humidity and a flow rate of 10 liter air (340 ppm CO_2) min^{-1} under light ($-1{,}000 W \cdot m^{-2}$). Prior to SO_2 fumigation, the plants were preilluminated for more than 2 hr. The rate of CO_2 uptake was determined by measuring the difference between the inlet and outlet of the assimilation chamber using an infrared CO_2 analyzer (Shimadzu URA2S).

Preparation of crude enzymes

Ten leaf discs (1.5 cm in diameter) were excised as quickly as possible (within 2 min) at indicated times during 2.0 ppm SO_2 fumigation. Within 3 min after the excision, the leaf discs were homogenized with 5 ml of 0.1 M Tris-HCl (pH 7.8) using a Polytron (Kinematica PT 10/35). The homogenate was centrifuged at 15,000 x *g* for 30 min and the supernatant was dialyzed four times against 20 volumes of 10 mM Tris-HCl (pH 7.8) overnight. After centrifugation of the dialyzed solution at 15,000 x *g* for 30 min, the clear supernatant was used to determine enzyme activities and protein. Protein and chlorophyll were determined according to Lowry *et al.* (1951) and Arnon (1949), respectively. Ten leaf discs contained approximately 0.73 mg chlorophyll and 5.9 mg protein.

Enzyme assays

Spectrophotometric assays were carried out at 25°C. Most enzymes were assayed by continuous recording with a Hitachi 557 dual-wavelength spectrophotometer. Thiol compound was omitted from the assay mixture unless otherwise stated. Enzyme assays were conducted in a total volume of 1 ml and started by adding enzymes, unless otherwise stated.

RuBPC (EC 4.1.1.39) was assayed according to Lorimer *et al.* (1976). The preincubation mixture contained 100 mM Tris-HCl (pH 7.8), 20 mM $MgCl_2$, 5 mM $NaH^{14}CO_3$ (2.8×10^5 cpm μmol^{-1}) and the enzyme. After preincubation of 15 min at 25°C, the reaction was initiated by adding 1 mM RuBP and run for 10 min at 25°C. It was stopped by adding HCl in a final

concentration of 1 N and the incorporation of ^{14}C into acid-stable products was measured with a Packard 3255 liquid scintillation counter. SBPase (EC 3.1.37) was assayed according to Racker and Schroeder (1958). The reaction mixture contained 100 mM HEPES-NaOH (pH 7.2) and 2.1 mM SBP. The reaction was done for 20 min at 35°C and stopped by the addition of 0.1 ml of 10% trichloroacetic acid. Phosphate released from SBP was determined according to Chen *et al.* (1958). PGAK (EC 2.7.2.3) was assayed by coupling with NAD-GAPD (Fuller *et al.* 1961). The reaction mixture contained 100 mM Tris-HCl (pH 7.8), 5 mM ATP, 5 mM PGA, 10 mM $MgCl_2$, 0.2 mM NADH and 1 unit of NAD-GAPD. FBPase was measured according to Kelly *et al.* (1976). The reaction mixture contained 100 mM Tris-HCl (pH 7.8), 1 mM FBP, 1 mM EDTA, 0.4 mM $NADP^+$, 10 mM $MgCl_2$, 4 units of glucosephosphate isomerase and 2 units of G6PD. G6PD (EC 1.1.1.49) was assayed according to Muto and Uritani (1970). The reaction mixture contained 10 mM Tris-HCl (pH 7.8), 1.7 mM G6P, 1.7 mM 6-phosphogluconate and 1 mM $NADP^+$. The activity of 6-phosphogluconate dehydrogenase was measured by omitting G6P from the above reaction mixture and subtracted from the activity obtained in the above reaction. Ru5PK was measured according to the modified method of Wara-Aswapati *et al.* (1980). The reaction mixture contained 100 mM Tris-HCl (pH 7.8), 0.5 mM Ru5P, 5 mM phosphoenolpyruvate, 0.2 mM NADH, 1 mM ATP, 50 mM KCl, 10 mM $MgCl_2$, 10 units of pyruvate kinase and 10 units of lactate dehydrogenase. Trans A (EC 2.2.1.2) was measured by coupling with α-glycerophosphate dehydrogenase (Latzko & Gibbs, 1969). The reaction mixture contained 100 mM Tris-HCl (pH 7.8), 0.2 mM NADH, 2 mM FBP and 0.2 unit of α-glycerophosphate dehydrogenase. Trans K (EC 2.2.1.1.) was measured according to Cooper *et al.* (1958). The reaction mixture contained 10 mM Tris-HCl (pH 7.8), 0.2 mM NADH, 0.12 μM thiamine pyrophosphate, 2.4 units of triosephosphate isomerase 0.5 mM ribose5P, 0.5 mM xylulose5P and 0.2 unit of α-glycerophosphate dehydrogenase. NADP-malic dehydrogenase (EC 1.1.1.37) was assayed according to the modified method of Hatch and Slack (1969). The reaction mixture contained 100 mM Tris-HCl (pH 7.2), 5 mM oxalacetate, 3 mM $MgCl_2$ and 0.1 mM $NADP^+$. NADP-GAPD was assayed according to Wolosiuk and Buchanan (1976). The reaction mixture contained 10 mM Tris-HCl (pH 7.8), 5 mM PGA, 1 mM ATP, 10 mM $MgCl_2$, 0.2 mM NADPH and 1 unit of PGAK.

Chemicals

Yeast NAD-GAPD, PGAK (type X), glucosephosphate isomerase (type III), bakers' yeast G6PD (type XV), rabbit muscle pyruvate kinase (type III), lactate dehydroganase (type II), α-glycerophosphate dehydrogenase (type III), and triosephosphate isomerase (type X) were obtained from Sigma. Beef liver catalase from P-L Biochemical Inc. was further purified with a column of Sepharose 6B in order to remove the contaminating superoxide dismutase. All other reagents were of the highest quality commercially available.

Results

Spinach plants were fumigated with 2 ppm SO_2 under light and the activities of ten chloroplast enzymes were followed (Fig. 1). Among them, FBPase, Ru5PK and NADP-GAPD were more remarkably inactivated than the others. These three SO_2-sensitive enzymes have sulfhydryl groups necessary for their activities (Wolosiuk & Buchanan, 1976; Buchanan *et al.* 1967, 1971; Latzko *et al.*, 1970). During SO_2 fumigation, their inactivation proceeded in parallel with the decrease of photosynthetic CO_2-fixation (Fig. 2). After removal of SO_2 their activities recovered almost completely within 10 min, while photosynthetic CO_2-fixation only

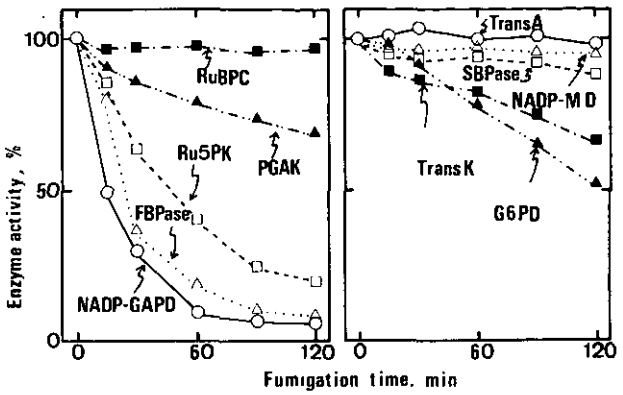

Fig. 1 Changes in activities of chloroplast enzymes in spinach leaves with SO_2 fumigation

The enzyme activities before SO_2 fumigation (100%) were 82.3 for NADP-GAPD, 70.3 for FBPase, 280.4 for Trans K, 823 for PGAK, 402 for Trans A, 132 for Ru5PK, 23.4 for NADP-malic dehydrogenase (NADP-MD), and 24.3 for G6PD in nmol NADPH, $NADP^+$ or NAD^+ formed mg $protein^{-1}$ min^{-1}, 53 nmol phosphate released mg $protein^{-1}$ min^{-1} for SBPase and 242 nmol $^{14}CO_2$ fixed mg $protein^{-1}$ min^{-1} for RuBPC.

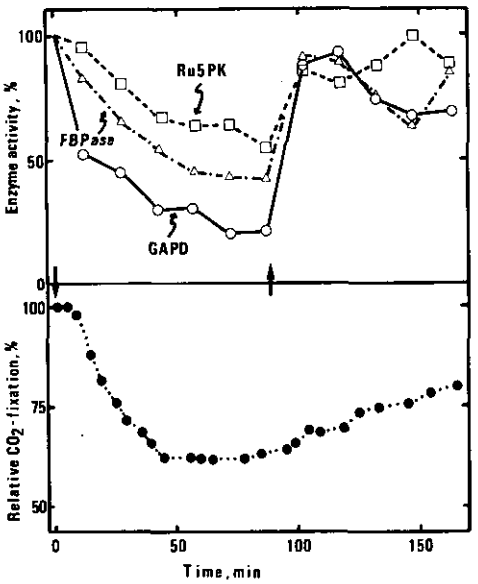

Fig. 2 Time couse of photosynthesis and activities of chloroplast SH enzymes during 2.0 ppm SO_2 fumigation

The SH enzyme activities in SO_2-fumigated leaves were followed. The light-dependent CO_2 uptake of spinach plants in a pot was measured as described in Materials and Methods. The total chlorophyll content in spinach leaves was 33.1 mg. The rate of light-dependent CO_2 uptake just before SO_2 fumigation was 168 μmol CO_2 mg $chlorophyll^{-1}$ hr^{-1}. The CO_2-evolution of soil itself in a pot (636 μmol hr^{-1}) was not changed by SO_2-fumigation and this CO_2-evolution was corrected when net photosynthesis of spinach leaves was calculated. At the indicated times, SO_2 fumigation was started (↓) or stopped (↑). Enzyme units (defined in Fig. 1) before SO_2 fumigation (100%) were 88.4 for NADP-GAPD, 72.3 for EBPase and 143 for Ru5PK.

partially recovered. Therefore, the decrease in photosynthetic CO_2-fixation at an early stage of SO_2 fumigation may be caused by the reversible inhibition of SH enzymes. Shimazaki and Sugahara (1979, 1980) reported irreversible impairment of photosystem II in spinach leaves fumigated with 2 ppm SO_2 just before or when visible damage appeared (about 1 to 2 hr after 2 ppm SO_2 fumigation). The incomplete recovery of photosynthetic CO_2-fixation after removal of SO_2 might be due to this inhibition.

Previously, we reported that chloroplasts isolated from SO_2-fumigated spinach leaves produced much more H_2O_2 than control ones (Tanaka *et al.*, 1982). Since H_2O_2 has a high reactivity with the SH enzyme, these enzymes may be inactivated by H_2O_2 produced during SO_2 fumigation. To check this, we followed the activities of seven enzymes in chloroplasts isolated from SO_2-fumigated spinach leaves under illumination (Fig. 3). NADP-GAPD and Ru5PK activities were depressed remarkably during illumination, but not as much if catalase was present. When H_2O_2 was added in the dark to chloroplasts isolated from non-fumigated spinach leaves, NADP-GAPD and Ru5PK activities also decreased more remarkably than those of the other enzymes (Fig. 4). Also, the addition of sulfite (1, 5 and 10 mM pH 5.0, 6.0 and 7.6) to chloroplasts or crude extracts from non-fumigated spinach plants had no effect on the activities of Ru5PK and NADP-GAPD. Therefore, the inactivation of the SH enzymes may have been caused not by sulfite ion but H_2O_2.

Further evidence for the participation of H_2O_2 in the inactivation of NADP-GAPD in SO_2-fumigated leaves is found in the results in Fig. 5 to 8. NADP-GAPD inactivation in illuminated chloroplasts isolated from SO_2-fumigated leaves was most marked around pH 6.3 (Fig. 5). This pH profile agreed well with that of H_2O_2 accumulation. NADP-GAPD

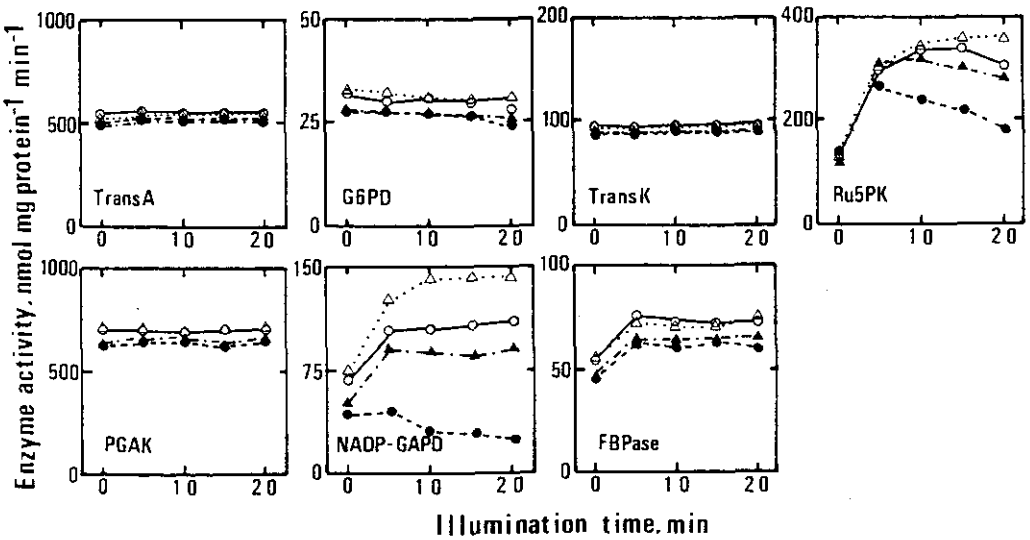

Fig. 3 Effects of catalase on enzyme activities in illuminated chloroplasts isolated from SO_2-fumigated and nonfumigated spinach leaves

Chloroplasts were isolated from spinach leaves fumigated with 2.0 ppm SO_2 for 1 hr and were illuminated. A part of the illuminated chloroplasts was periodically transferred to the cuvette for enzyme assay. For details, see **Meterials and Methods**. Enzyme units are defined in Fig. 1. (○, △), enzyme activity in chloroplasts from non-fumigated leaves; (●, ▲), enzyme activity in chloroplasts from SO_2-fumigated leaves; (△, ▲), in the presence of 200 units of catalase; (○, ●), no addition.

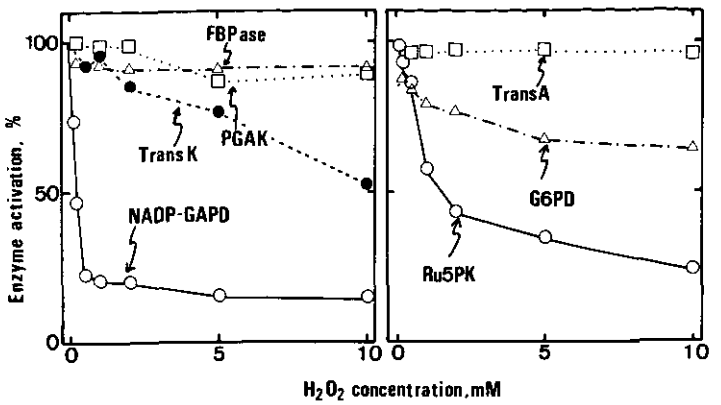

Fig. 4 Effects of H_2O_2 on activities of enzymes in chloroplasts isolated from non-fumigated spinach leaves

Chloroplasts (equivalent to 140 μg) were incubated for 10 min at 0°C with 50 mM HEPES-NaOH (pH 7.6), 0.4 M sorbitol, 10 mM $NaHCO_3$, 0.4 mM NaN_3 and the indicated concentrations of H_2O_2 in a total volume of 1 ml in the dark. Enzyme units before adding H_2O_2 (100%) were 91.0 for NADP-GAPD, 77.4 for FBPase, 1242 for PGAK, 123 for Ru5PK, 112 for Trans K, 212 for Trans A and 40.8 for G6PD, respectively. Enzyme units are defined in Fig. 1.

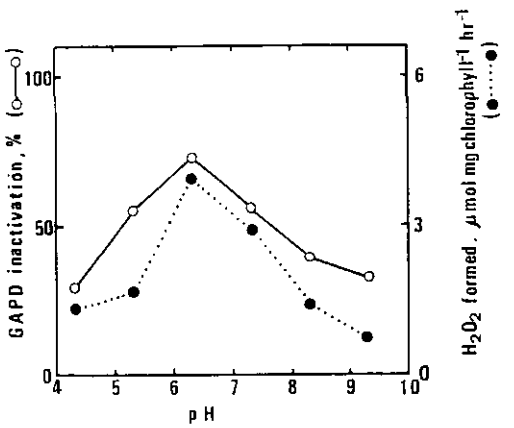

Fig. 5 Relationship between the inactivation of NADP-GAPD and the formation of H_2O_2 at various pH values in illuminated chloroplasts from SO_2-fumigated leaves

Plants were fumigated for an hour. Parts of chloroplasts illuminated for 10 min were transferred to the cuvettes for enzyme or H_2O_2 assay. The pH values were varied with 0.1 M Tris-malate. The NADP-GAPD activity before light illumination was regarded as 100%. The H_2O_2 formation was measured as described previously (Tanaka *et al.*, 1982).

inactivation was suppressed by cytochrome *c* and DCMU (Fig. 6), which have been shown to inhibit H_2O_2 formation (Tanaka *et al.*, 1982). No inactivation of NADP-GAPD activity under anaerobic conditions also supports the inactivation of this enzyme with H_2O_2 (Fig. 7). The NADP-GAPD photoinactivation wa protected by catalase. The photoinactivated NADP-GAPD was reactivated by dithiothreitol (Fig. 8). The NADP-GAPD activity was also recovered with the addition of 2 mM cysteine, 2 mM 2-mercaptoethanol of 2 mM GSH. These results show that

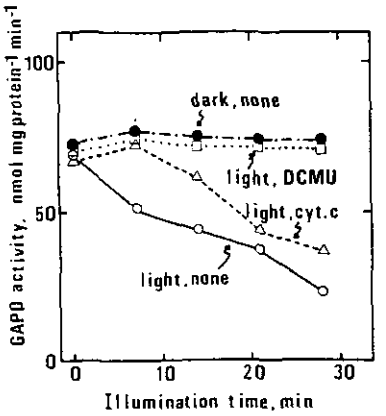

Fig. 6 Effects of cytochrome *c* and DCMU on the inactivation of NADP-GAPD in chloroplasts isolated from 2.0 ppm SO_2-fumigated spinach leaves

Plants were fumigated for an hour. The reaction mixture contained 16 μM cytochrome *c* or 10 μM DCMU.

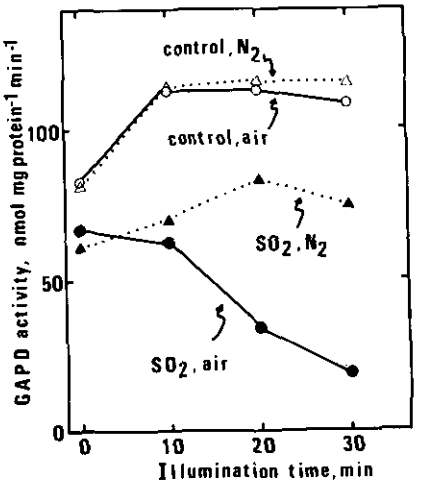

Fig. 7 Oxygen requirement for the inactivation of NADP-GAPD in illuminated chloroplasts isolated from 2.0 ppm SO_2-sumigated spinach leaves

Spinach plants were fumigated for an hour. Part of the illuminated chloroplasts was periodically transferred to the cuvette for enzyme assay. The reaction mixture was bubbled with N_2 or air for 10 min before and during photoreaction.

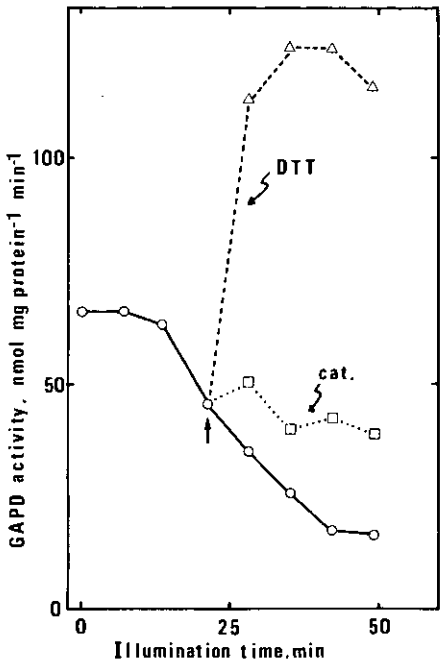

Fig. 8 Effect of dithiothreitol on the inactivation of NADP-GAPD in illuminated chloroplasts isolated from 2.0 ppm SO_2-fumigated spinach leaves

Plants were fumigated for an hour. At the indicated times, 2 mM dithiothreitol or 200 units of catalase was added.

the photoinactivation of NADP-GAPD in chloroplasts is caused by the oxidation of SH-groups necessary for the enzyme activity. Because dithiothreitol raised NADP-GAPD activity, it seemed to interact directly with SH groups of the enzyme and/or a dithiol compound (Anderson & Avron, 1976) in the process of photoactivation of the enzyme.

Both NADP-GAPD and Ru5PK are activated by light in chloroplasts (Anderson & Avron, 1976). This activation has been attributed to a dithiol compound produced in the reducing site of photosystem I. We tested whether H_2O_2 attacks these SH enzymes themselves or the process of photoactivation. As shown in Fig. 9, the photoactivation of NADP-GAPD and Ru5PK was also inhibited by H_2O_2. Therefore, H_2O_2 affected not only these SH enzymes themselves but their photoactivation also. It should be noted that the sensitivity of photoactivation of FBPase to H_2O_2 is very low. This result may support the proposal of Wolosiuk *et al.* (1979, 1980) that the mechanism of photoactivation of FBPase differs from that of NADP-GAPD and Ru5PK.

Discussion

We previously reported that spinach plants accumulated much H_2O_2 during SO_2 fumigation, and suggested that H_2O_2 thus produced in chloroplasts might participate in the suppression of photosynthesis during SO_2 fumigation (Tanaka *et al.*, 1982). As summarized in Fig. 1, chloroplast SH enzymes, NADP-GAPD, Ru5PK and FBPase, were strongly inactivated in

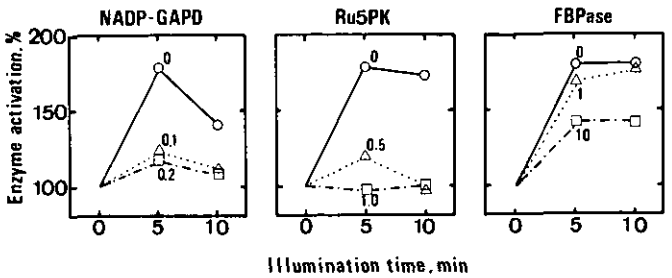

Fig. 9 Effects of H_2O_2 on the process of light activation of chloroplast SH enzymes

Chloroplasts isolated from non-fumigated spinach leaves were incubated with the indicated concentrations of H_2O_2 as described in Fig. 4 and illuminated for the indicated times. Enzyme units before illumination (100%) were 78.3 (no addition), 64.2 (0.1 mM H_2O_2) and 42.1 (0.2 mM H_2O_2) for NADP-GAPD, 145.2 (no addition), 129.4 (0.5 mM H_2O_2) and 112.2 (1.0 mM H_2O_2) for Ru5PK, and 86.4 (no addition), 82.1 (1.0 mM H_2O_2) and 84.9 (10.0 mM H_2O_2) for FBPase, respectively. Enzyme units and defined in Fig. 1. The numbers in the figures represent H_2O_2 concentrations (mM).

SO_2-fumigated spinach leaves. It has been proposed from the change in the substrate level of the photosynthetic carbon pentose phosphate cycle that these enzymes might be specifically sensitive to H_2O_2 (Kaiser, 1979; Heldt *et al.*, 1978). In addition to NADP-GAPD, Ru5PK and FBPase, Trans K, PGAK and G6PD activities in SO_2-fumigated leaves were also depressed slightly (Fig. 1). These enzymes were more or less sensitive to externally added H_2O_2 (Fig. 4). Therefore, the inactivation of these three enzymes during SO_2 fumigation may be also caused by H_2O_2. The question remains of which enzyme inactivation is actually responsible for the decrease in CO_2-fixation rate. Thus, we are studying the change in the substrate level of the reductive pentose phosphate cycle in leaves during SO_2 fumigation.

Unexpectedly, FBPase activity was not affected while NADP-GAPD and Ru5PK were inactivated in illuminated chloroplasts isolated from SO_2-fumigated leaves, which produced H_2O_2 at a high rate (Fig. 3). We also confirmed that NADP-GAPD and Ru5PK in chloroplasts were inhibited by externally added H_2O_2 but FBPase was insensitive to H_2O_2 (Fig. 4). Charles and Halliwell (1980) reported that thiol-treated FBPase having a low K_m for FBP was severely inhibited by H_2O_2 but the nontreated enzyme having a high K_m for it was insensitive to H_2O_2. We also observed that the increased activity of FBPase with dithiothreitol decreased to the original activity by adding H_2O_2 (data not shown). Therefore, the question of why FBPase in SO_2-fumigated leaves was inactivated (Fig. 1 and 2) but the enzyme in chloroplasts isolated from SO_2-fumigated leaves was not (Fig. 3 and 4), may be answered by the different sensitivities of the two forms of FBPase to H_2O_2.

Why higher plant photosynthetic CO_2-fixation is depressed at the first stage of SO_2 fumigation has not been known. Some workers supposed that it is caused by the competitive SO_3^{2-} inhibition of RuBPC with respect to HCO_3^-. Ziegler (1972) reported that RuBPC in crude spinach extract was competitively inhibited by sulfite ion with respect to HCO_3^-. Others have observed that the inhibition of CO_2-fixation with SO_2 fumigation can be relieved by raising the CO_2 concentration (Furukawa *et al.*, 1979; Majernik & Mansfield, 1972). This inhibition by SO_2 has been attributed to the competitive inhibition of RuBPC by SO_3^{2-} with respect to HCO_3^-. However, no evidence has been presented that this inhibition occurs in SO_2-fumigated leaves. The photooxidation of sulfite to sulfate at an extremely high rate in

cloroplasts (Asada & Kiso, 1973) might oppose the above view.

We propose here another possibility for the reversal of the inhibition of photosynthesis with SO_2 by the increase in CO_2 concentration. It is widely known that the photosynthesis is inhibited by the increase of O_2 concentration (Warburg, 1920; Ellyard & Gibbs, 1969) and several lines of evidence have been presented showing that this phenomenon is caused by the competition between CO_2 and O_2 for electrons from water (Asada & Nakano, 1978; Nakano & Asada, 1980; Radmer & Ollinger, 1980). Previously, we reported that the light-dependent O_2 uptake in chloroplasts isolated from SO_2-fumigated spinach leaves was greater than control ones (Tanaka *et al.*, 1982). Therefore, SO_2 fumigation of higher plants may stimulate flows of electrons to O_2, followed by the formation of a reduced species of oxygen. Under such conditions, supply of more CO_2 decreases the production of active oxygen. Therefore, we inferred that CO_2 lessens the inhibition of photosynthesis in SO_2-fumigated leaves by decreasing the production of active species of oxygen due to increased flow of electrons to CO_2.

When spinach plants in pots were fumigated with 0.5 ppm O_3 under the same condition as SO_2 fumigation, the activities of the thiol enzymes (NADP-GAPD, Ru5PK and FBPase) in leaves did not change at all at least for 3 h. This result indicates that O_3 fumigation may cause no accumulation of H_2O_2 in leaves. Further, since it is widely known that O_3 is a strong oxidizer for thiol groups, it can be thought that O_3 does not react directly with stromal components. Sakaki and Kondo showed that lipids composing chloroplast thylakoids were affected by O_3 fumigation (unpublished data). Therefore, the target for O_3 attack in chloroplasts may be thylakoids and O_3 may be thoroughly consumed there.

Acknowledgment

We wish to thank the members of the Engineering Division of this Institute for their operation of the growth cabinet and the cultivation and supply of the plant materials. We are also indebted to Drs. K. Asada and M. Takahashi of the Research Institute for Food Science, Kyoto University for valuable discussion during this work. This work was supported by a grant from the Environmental Agency.

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二酸化イオウ暴露ホウレンソウ葉における炭酸固定系チオール酵素の失活への過酸化水素の関与

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二酸化イオウ暴露したホウレンソウ葉の炭酸固定系酵素活性を測定すると，グリセルアルデヒドリン酸脱水素酵素，リブロースリン酸キナーゼ，フルクトースニリン酸フォスファターゼが顕著に失活していた。これら3酵素の失活は同時に測定した光合成の低下と並行して起こった。二酸化イオウ暴露したホウレンソウから単離した葉緑体を光照射したときの炭酸固定系酵素活性を追跡したときにもチオール酵素の特異的な失活が見られた。この葉緑体におけるチオール酵素の失活は過酸化水素生成と並行して起こり，DCMU，チトクロム c，嫌気条件下で抑制された。非暴露葉緑体の抽出液に過酸化水素を添加したときも上記チオール酵素が特異的に失活した。一方，同じ条件で亜硫酸イオンを加えてもチオール酵素活性は変化しなかった。以上の結果から，二酸化イオウ暴露初期の光合成阻害の原因は暴露時，副次的に生成した過酸化水素が炭酸固定系チオール酵素を失活させることであると考えた。

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Res. Rep. Natl. Inst. Environ. Stud., Jpn., No. 65, 1984.

Further Evidence for Inactivation of Fructose-1,6-bisphosphatase at the Beginning of SO_2 Fumigation

Increase in Fructose-1, 6-bisphosphate and Decrease in Fructose-6-phosphate in SO_2 - Fumigated Spinach Leaves*

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The substrate level of the photosynthetic reductive pentose phosphate cycle in spinach leaves during SO_2 fumigation was surveyed. At the beginning of SO_2 fumigation, fructose-1,6-bisphosphate increased and fructose-6-phosphate decreased, while ribulose-1,5-bisphosphate remained unchanged and 3-phosphoglyceric acid rapidly decreased. These results suggested that the inhibition of photosynthesis in spinach leaves with SO_2 might be due to inactivation of fructose-1,6-bisphosphatase.

Key words: CO_2 fixation, Fructose-1,6-bisphosphatase, NADPH/$NADP^+$ ratio, Ribulose-1,5-bisphosphate carboxylase, Sulfur dioxide.

Although the depression of photosynthesis is one of the most remarkable phytotoxic phenomena on the physiological level at the beginning of SO_2 fumigation, the mechanism is not clearly understood. RuBPC in isolated chloroplasts has been reported to be inhibited by sulfite competitively with $NaHCO_3$ (Ziegler, 1972). However, there has been no definite evidence to show that this inhibition occurs in SO_2-fumigated higher plants. Shimazaki and Sugahara (1979, 1980) demonstrated that photosystem II in SO_2-fumigated spinach leaves was impaired, but this damage occurred after the photosynthetic CO_2 uptake had been depressed.

Recently, we suggested that hydrogen peroxide accumulated in spinach leaves and their

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Abbreviations: FBPase, fructose-1, 6-bisphosphatase; F6P, fructose-6-phosphate; MTT, 3-(4, 5-dimethyl thiazolyl-2)-2, 5-diphenyltetrazolium bromide; PES, 5-ethyl phenazium ethyl sulfate; PGA, 3-phosphoglycerate; RuBPC, ribulose-1, 5-bisphosphate carboxylase.

chloroplasts immediately after SO_2 fumigation and inactivated thiol enzymes of the reductive pentose phosphate cycle such as NADP-glyceraldehyde-3-phosphate dehydrogenase, ribulose-5-phosphate kinase and FBPase, which resulted in depression of photosynthetic CO_2 uptake (Tanaka *et al.*, 1982a, b). To clarify further the inhibitory site in the photosynthetic reductive pentose phosphate cycle with SO_2 fumigation, we determined the amounts of several photosynthetic intermediates in SO_2-fumigated spinach leaves.

Yeast 5-phosphogluconate dehydrogenase (type V), baker's yeast glucose-6-phosphate dehydrogenase (type XV), rabbit muscle phosphoglucomutase, yeast glucose-6-phosphate isomerase (type III), *Tolura* yeast fructose-1,6-bisphosphatase, rabbit muscle triosephosphate isomerase (type X), rabbit muscle α-glycerol-3-phosphate dehydrogenase (type III), yeast glyceraldehyde-3-phosphate dehydrogenase, yeast phosphoglycerate kinase (type X) and MTT were obtained from Sigma.

Spinach (*Spinacia oleracea* L. cv. New Asia) plants were grown in a phytotron and fumigated with 2.0 ppm SO_2 under light (~1,000 W/m^2) in a growth cabinet as previously described (Tanaka *et al.*, 1982a). In the assay of photosynthetic intermediates, spinach leaf segments (1.5 g) were excised as quickly as possible and immersed in liquid N_2 under illumination. These were homogenized in 5 ml of 5% $HClO_4$ with a Polytron (Kinematica PT 10/35) in the presence of a little liquid N_2. The homogenates, with the pH adjusted to 7.8 with 1 M Tricine/5 M KOH, were centrifuged at 18,000 × *g* for 30 min and the clear supernatants were used for the enzymic determination. RuBP and PGA were measured enzymatically from the oxidation of NADH according to the method of Latzko and Gibbs (1972). Tobacco (*Nicotiana tabacum* L. cv. xanthii) RuBPC was twice crystallized according to the method of Kung *et al.* (1980) and this enzyme sample was free of transaldolase and transketolase which interfere with the measurement of RuBP and PGA. FBP and F6P were determined from the reduction of $NADP^+$ enzymatically according to the method of Latzko and Gibbs (1972).

In the assay of NADPH and $NADP^+$, ten spinach leaf discs (1.5 cm in diameter) were excised and immersed in liquid N_2 as quickly as possible. These were homogenized in either 5 ml of 0.1 M NaOH (to destroy the oxidized pyridine nucleotides and retain the reduced ones) or 0.1% $HClO_4$ (to destory the reduced pyridine nucleotides and retain the oxidized ones). After centrifugation, the clear supernatants were used for the determination of NADPH and $NADP^+$ according to the method of Matsumura and Miyachi (1980). The assay cuvette for NADPH ($NADP^+$) contained, in a total volume of 1 ml, 100 mM Tricine-NaOH (pH 8.0), 5 mM glucose-6-phosphate, 0.42 mM MTT, 0.17 mM PES, 20 mM Tricine (20 mM Tris), 0.2 ml of the clear supernatants and 2 units of glucose-6-phosphate dehydrogenase. The increase of absorbance at 570 nm was measured at 22°C with a Hitachi 557 dual wavelength spectrophotometer.

We previously demonstrated that chloroplast SH enzymes such as NADP-glyceraldehyde-3-phosphate dehydrogenase (NADP-GAPD), FBPase and ribulose-5-phosphate kinase (Ru5PK) were inactivated by H_2O_2 accumulated in chloroplasts at the beginning of SO_2 fumigation to spinach plants (Tanaka *et al.*, 1982a, b). Since FBPase has the lowest specific activity in chloroplasts among these SH enzymes and Heldt *et al.* (1978) found changes in the substrate level of the photosynthetic carbon cycle which showed that the inhibition of CO_2 fixation caused by adding H_2O_2 to intact chloroplasts might be due to the decrease in FBPase activity rather than in NADP-GAPD and Ru5PK activities, we expected the inhibition of CO_2 fixation by SO_2 fumigation to be accompanied by an increase in the stromal levels of FBP and a decrease of those of F6P. As shown in Fig. 1, there was a large increase in FBP concomitant with a decrease in F6P after SO_2 fumigation.

Although addition of sulfite to isolated chloroplasts has been reported to cause inhibition

of RuBPC (Ziegler, 1972), we have found no loss of RuBPC activity in spinach leaves fumigated with SO_2 (Tanaka *et al.*, 1982b). In order to clarify further the insensitivity of RuBPC to SO_2 fumigation, we determined the levels of RuBP and PGA in SO_2-fumigated leaves. As shown in Fig. 2, the RuBP level did not change and PGA rapidly decreased during SO_2 fumigation. Portis *et al.* (1979) reported that the inhibition of CO_2 fixation caused by lowering the stromal Mg^{2+} was due to a decrease in FBPase activity. They observed increases in FBP, ATP and NADPH, no change in RuBP, and decreases in F6P, PGA, ADP and $NADP^+$. As PGA and triosephosphate are nearly in equilibrium with NADPH and ATP, they speculated that the rapid decrease in PGA might be a consequence of the changes in the nucleotide levels. As shown in Fig. 3, the NADPH/$NADP^+$ ratio in SO_2-fumigated leaves increased. Similarly, Shimazaki *et al.* (unpublished data) have observed that the ATP level increases by 20 to 30% at 45 min after 2 ppm SO_2 fumigation of spinach leaves. These changes in the nucleotide levels in SO_2-fumigated leaves might cause a decrease in PGA. A decrease in the total content of NADPH plus $NADP^+$ observed after 15 min of SO_2 fumigation might be due to destruction of the structure of these compounds by certain reactive species produced in SO_2-fumigated leaves. RuBPC in leaves may be insensitive to SO_2-fumigation because of rapid photooxidative extinction of sulfite ion by photosystem I (Asada & Kiso, 1973). The increases in NADPH/$NADP^+$ ratio and ATP level also suggest that the inhibition of photosynthetic CO_2 fixation at the beginning of SO_2 fumigation is caused by inactivation of CO_2 fixation rather than the photosystem.

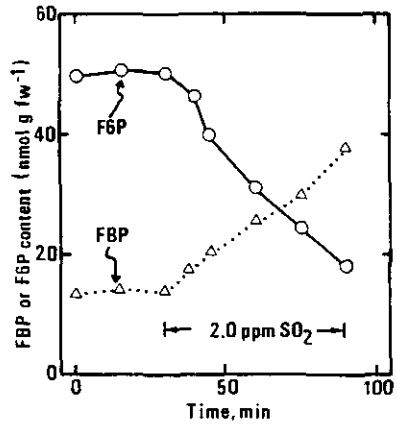

Fig. 1 Effects of SO_2 on FBP and F6P levels in spinach leaves
Where indicated, SO_2 was applied.

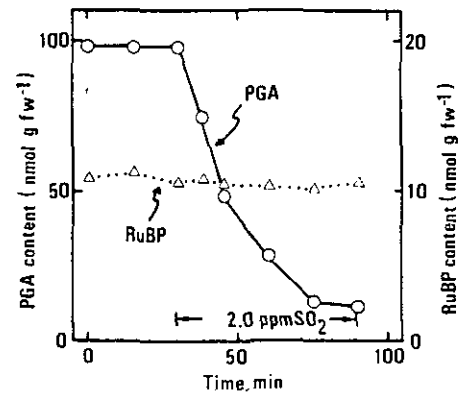

Fig. 2 Effects of SO_2 on RuBP and PGA levels in spinach leaves
Where indicated, SO_2 was applied.

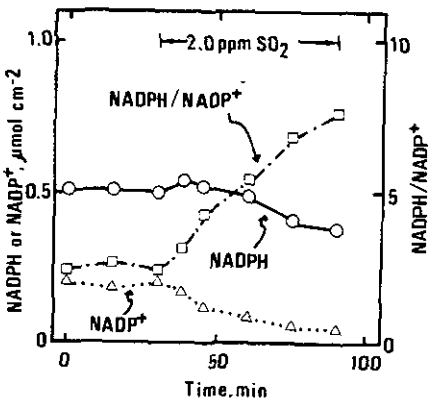

Fig. 3 Effects on NADPH and $NADP^+$ levels in spinach leaves
Where indicated, SO_2 was applied.

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二酸化イオウ暴露初期のフルクトースニリン酸フオスフアターゼ失活の証明，暴露葉中のフルクトースニリン酸の増加とフルクトースニリン酸の減少

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二酸化イオウ暴露初期におけるホウレンソウ葉中の光合成基質レベルの変動を調べた。フルクトースニリン酸の増加及びフルクトースニリン酸の減少が認められた。一方，リブロースニリン酸は変化せず，フオスフオグリセリン酸は急速に減少した。またNADPHとNADP$^+$の比を調べたところ，増大が認められた。以上の結果から二酸化イオウ暴露初期の光合成阻害は光化学系よりも炭酸固定系が律速になり，この阻害はフルクトースニリン酸フオスファターゼの失活により起こると推察した。

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Res. Rep. Natl. Inst. Environ. Stud., Jpn., No. 65, 1984.

Breakdown of photosynthetic pigments and lipids in spinach leaves with ozone fumigation: Role of active oxygens*

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In spinach (*Spinacia oleracea* L. cv. New Asia) plants fumigated with ozone in light, destruction of chlorophylls and carotenoids and formation of malondialdehyde (MDA), an indicator of lipid peroxidation, were observed. Chlorophyll *a* and carotenoids in leaves started to be broken down 6–8 h after the commencement of 0.5 ppm ozone fumigation, whereas MDA formation in leaves increased linearly for the initial 8 h of fumigation followed by a more rapid increase. In leaf discs excised from 6-h fumigated plants, destruction of chlorophyll *a* and carotenoids and MDA formation proceeded in the light but were almost completely suppressed under an anaerobic condition. Effect of exogenously applied scavengers of active oxygen species suggest that active oxygens, especially superoxide radical (O_2^-), participated in both the destruction of chlorophyll *a* and carotenoids and the formation of MDA. Ozone fumigation reduced the levels of endogenous scavengers of O_2^-, superoxide dismutase (SOD) and L-ascorbate, in leaves to one-half the initial levels each by 3.5 and 8 h fumigation, respectively. The results indicate that the photosynthetic pigments and lipids were broken down by active oxygens accumulated in leaves as a result of the ozone-induced destruction of physiological defense against oxygen toxicity.

Activity of polyphenol oxidase in chloroplast membranes of 4-h fumigated leaves increased to 240% of the initial level, suggesting that the thylakoid membranes had been affected severely before the pigment destruction. The relations between the pigment destruction and the disintegration of thylakoids were discussed.

Key words: Active oxygen, Chlorophyll destruction, Lipid destruction, Malondialdehyde, Ozone, *Spinacia oleracea*

Ozone is a major atmospheric pollutant, the level of which has often been reported to reach 0.1–0.5 ppm in the urban areas in Japan (Akimoto, 1972). Exposure to ozone causes visible foliar injuries, e.g., chlorosis and necrosis, in many species of plants. Numerous studies

* This study was published in Physiol. Plant. 59, 28-34 (1983).

Abbreviations: DABCO, 1,4-diazabicyclo-[2,2,2]-octane; DOPA, DL-dihydroxyphenylalanine; EDU, N-[2-(2-oxo-1-imidazolidinyl)ethyl]-N'-phenylurea; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; MDA, malondialdehyde; $^1O_2$, singlet molecular oxygen; O_2^-, superoxide radical, •OH, hydroxyl radical; SOD, superoxide dismutase; tiron, 1,2-dihydroxybenzene-3,5-disulfonate.

have documented the decrease in chlorophyll content in ozone-exposed plants (Todd & Arnold 1961; Knudson *et al.*, 1977). Ozone is a reactive oxidizing agent and destroys various cellular components. Chlorophyll in organic solvents was destroyed by ozone bubbling (Nobel, 1974). In the case of bean leaves fumigated with ozone, maximum chlorophyll reduction occurred 4 days after the end of ozone fumigation (Knudson *et al.*, 1977). This suggests that some biochemical reaction(s) induced by ozone fumigation may also be responsible for the chlorophyll destruction in plants.

Ozone has been shown to cause metabolic alterations by affecting various enzyme activities and metabolite levels. Enzymes associated with metabolic oxidation process such as peroxidase (Tingey *et al.*, 1975; Curtis *et al.*, 1976) and polyphenol oxidase (Tingey *et al.*, 1975) were activated by fumigation with ozone. It has been shown that various reagents including reducing substances and anti-oxidants can protect plants against ozone injuries (Rich, 1964). These results suggest that some oxidative reactions would participate, at least in part, in the appearance of visible foliar injuries caused by ozone.

Ozone damages cellular membrane systems as a result of lipid destruction. Exposure to ozone caused the formation of MDA, an indicator of lipid peroxidation, in plant cells (Tomlinson & Rich, 1970; Frederick & Heath, 1975). Recently, the vesiculation and swelling of chloroplast thylakoids in leaves were observed in electron micrographs taken after short exposure to ozone (Athanassious, 1980; Miyake *et al.*, 1981). Thus disintegration of chloroplast membranes could occur upon fumigation with ozone, and this may have deleterious effect on the associated pigments.

In the present work, we studied the relationship between the loss of photosynthetic pigments and the destruction of lipids in ozone-fumigaged spinach leaves. We found that both of these phenomena proceeded in the light and in the presence of oxygen even if ozone fumigation was stopped, and that active oxygens, especially O_2^-, took part in these destruction processes. In addition, it was suggested that the disintegration of thylakoid membranes induced by ozone preceded the massive breakdown of the pigments.

Materials and Methods

Plant materials

Spinach plants (*Spinacia oleracea* L. cv. New Asia) were grown from seeds in pots which were placed in an environment-controlled glass house maintained at 20 ± 0.5°C in the daytime and 15 ± 0.5°C at night with a relative humidity of 70 ± 5% under natural light. A composition of soil in pots, nutrients, and irrigation to plants were the same as previously reported (Kondo & Sugahara, 1978). The 5–7 week old plants were used through the experiments.

Ozone fumigation

Plants were fumigated with ozone in a growth cabinet (230 × 190 × 170 cm) controlled at 20 ± 0.5°C with a relative humidity of 75 ± 3%. The light was provided from 24 metal halide lamps (Yoko Lamp. 400 W; Toshiba) with an intensity of 430 – 580 μmol $m^{-2}s^{-1}$ PAR at leaf level, and measured with a quantameter (Model LI-185; Lambda). Mean wind velocity in the cabinet was 0.22 m s^{-1}. Pots of plants were transferred from a glass house to the cabinet in the morning and preconditioned for about 2 h; then ozone was flushed into the cabinet. The concentration of ozone in the cabinet rose within 5 min to the desired level. The ozone was generated with a UV lamp from dry oxygen and was diluted by mixing with the filtered fresh air.

The concentration of ozone in the cabinet was maintained at 0.5 ± 0.02 ppm (v/v) in most of the experiments and at 0.1 ± 0.01, 0.2 ± 0.01, and 0.3 ± 0.02 ppm in the experiment of Fig. 4 according to the continuous monitoring with a chemiluminescent ozone analyzer (Model 806; Kimoto).

Treatment of leaf discs

Leaf discs (15 mm in diameter), cut from the interveinal areas of leaves fumigated with 0.5 ppm ozone for 6 h, were floated on 40 mM potassium phosphate buffer (pH 6.0) in the growth cabinet at 20°C and 470–510 μmol m^{-2} s^{-1} PAR. Reagents dissolved in 40 mM phosphate buffer (pH 6.0) were supplied to leaf discs by vacuum infiltration. Leaf discs were incubated in the same medium. In an O_2-free experiment, leaf discs were floated on the buffer in 100-ml Erlenmeyer flasks which had previously been flushed with N_2, and further subjected to continuous N_2 flush throughout the experiment.

Measurement of photosynthetic pigments

Leaf discs were excised from control plants and from plants subjected to ozone fumigation and were homogenized in 80% acetone with a glass homogenizer. After filtration, chlorophyll *a* and *b* were determined from the absorption at 663 and 645 nm according to the methods of Mackinney (1941). Total carotenoids were estimated in the same 80% acetone extract from the absorption at 480 nm after correction for chlorophyll interference (Kirk & Allen, 1965). When photosynthetic pigments were chromatographically separated, pigments in 80% acetone extract were transferred to diethyl ether, spotted on a microcrystalline cellulose plate (Avicel SF), developed with *n*-hexane:acetone (9:1, v/v) in the ascending manner.

Measurement of lipid peroxidation

MDA content in leaves was assayed according to Heath & Packer (1968) to determine the amount of lipid peroxidized. The discs excised from leaves were homogenized in distilled water. The homogenates were mixed with thiobarbituric acid and trichloroacetic acid at the final concentrations of 0.3 and 12.5% (w/v), respectively, and then incubated in a boiling water for 30 min. After centrifugation, MDA contents in the supernatants were determined from the difference between the absorbances at 532 and 600 nm.

Measurement of L-ascorbate and dehydro-L-ascorbate

Contents of L-ascorbate and dehydro-L-ascorbate in leaves were determined according to the method of Shigeoka *et al.* (1979). Leaf discs were homogenized in 5% (w/v) metaphosphoric acid with a Polytron (PT 10/35; Kinematica) and centrifuged at 15,000 g for 20 min. To convert L-ascorbate to dehydro-L-ascorbate, 1 mM 2,6-dichlorophenolindophenol (DCIP) was added to the supernatants. Thereafter, the supernatants were mixed with 2, 4-dinitrophenyhydrazine, thiourea, and sulfuric acid at the final concentrations of 2.9 mM, 0.3% (w/v), and 0.66 M, respectively, and then incubated at 50°C for 60 min. After the reaction was terminated by cooling in an ice bath, same volume of 15.4 M sulfuric acid was added. Dehydro-L-ascorbate content in the samples was determined from the absorption at 520 nm. The content of L-ascorbate was obtained from the difference between the contents of dehydro-L-ascorbate in the sample with and without the addition of DCIP.

Enzyme assays

For the determination of SOD activity, leaf discs were homogenized with a Polytron in 0.1 M potassium phosphate buffer (pH 7.8) and centrifuged at 10,000 g for 30 min. The

supernatant obtained was dialyzed against 10 mM phosphate buffer (pH 7.8) and used in the enzyme assays. The estimation of SOD activity was based on the inhibition of cytochrome *c* reduction caused by O_2^- as previously described (Tanaka & Sugahara, 1980). The reaction mixture contained 50 mM phosphate buffer (pH 7.8), 0.1 mM EDTA, 0.1 mM cytochrome *c*, 0.1 mM xanthine and 10 μg xanthine oxidase in a total volume of 1.0 ml. After the addition of xanthine oxidase the increase in absorbance at 550 nm was followed at 25°C.

Polyphenol oxidase activity was estimated from DOPA-dependent O_2 uptake of osmotically shocked chloroplasts according to Golbeck and Cammarata (1981) with a slight modification. Leaves were blended with a homogenizer (Universal Homogenizer, HB; Nihon Seiki) in a cold medium containing 50 mM Tricine-NaOH buffer (pH 7.5), 20 mM NaCl, and 400 mM sucrose. The homogenate was filtered through 4 layers of gauze and the filtrate was centrifuged at 150 g for 2 min and the pellet discarded. The supernatant obtained was recentrifuged at 3,000 g for 5 min. The resulting pellet was osmotically shocked in 25 mM Tricine-NaOH buffer (pH 7.2) for 1 h at 4°C and this suspension was used in the assays. Polyphenol oxidase activity was determined polarographically with Rank Brothers O_2 electrode at 25°C in the dark. The air-saturated reaction mixture contained 50 mM HEPES-NaOH buffer (pH 7.5) and 13.0 mM DOPA in a final volume of 1.0 ml. After the O_2 electrode had been equilibrated the reaction was started by addition of 0.1 ml of chloroplast suspension containing 0.8–1.0 mg chlorophyll.

Protein was determined according to Lowry *et al.* (1951).

Chemicals

D_2O (99.9%) was obtained from Merck. Cytochrome *c* from horse heart (Type III) was purchased from Sigma, and xanthine oxidase from milk was obtained from Boehringer.

Results

In spinach plants fumigated with 0.5 ppm ozone in the light, water-soaked spots appeared on both adaxial and abaxial surfaces of the leaves 1–2 h after the beginning of the fumigation and then spread over the interveinal areas. After 7–9 h the affected leaves had wilted and thereafter changed from green to white or light brown.

Fig. 1 shows the changes of pigment contents in leaves during the fumigation with ozone. The content of chlorophyll *a* did not change during about 8 h of fumigation but subsequently decreased rapidly (see also Fig. 3). After 14.5 h, chlorophyll *a* content was 76% of the initial level. Chlorophyll *b* content was unchanged for about 12 h and thereafter gradually decreased. The content of carotenoids started to decrease after about 6 h of fumigation and reached 59% of the original value 14.5 h after the initiation of the fumigation. The pigments extracted from 14.5-h fumigated leaves were chromatographically separated and their Rf-values were compared with those of non-fumigated leaves. No pigment different from those in non-fumigated leaves was detected in the ozone-fumigated leaves (data not shown).

Fig. 1 also shows the accumulation of MDA in leaves. MDA content slowly increased for the initial 8.5 h of fumigation, followed by a subsequent drastic rise. MDA contents after 8.5 and 14.5 h of ozone fumigation were 155 and 490% of the initial level, respectively.

In the next experiment, the plants were exposed to ozone in the light for various periods and then kept in light or darkness for 24 h without ozone. The longer the leaves were fumigated, the less chlorophyll *a* was retained in the light (Table 1). In the plants prefumigated for 6–8 h, the amount of chlorophyll *a* was reduced by 24 h illumination to 36–39% of that of

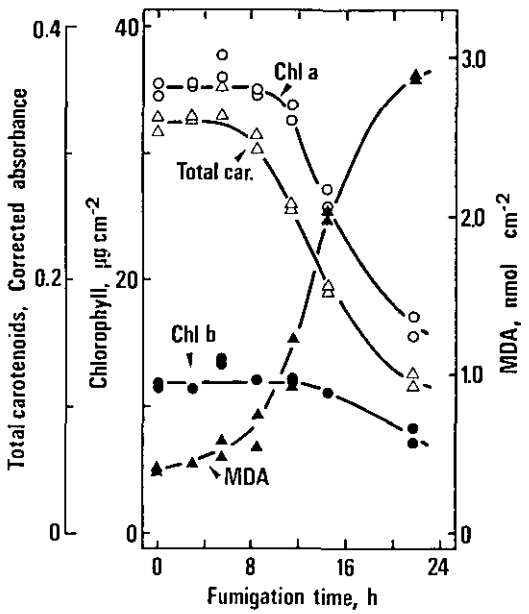

Fig. 1 Changes in contents of photosynthetic pigments and MDA with fumigation of ozone

Exposure of spinach plants to 0.5 ppm ozone was started at 0 time after about 2 h of preconditioning in the growth cabinet. At the indicated times, leaf discs were cut from plants and immediately homogenized to determine the pigments and MDA. Each point of the pigments and MDA was obtained from 10 and 5 leaf discs, respectively.

non-fumigated plants. However, the pigment was not destroyed in the dark, even after 8 h fumigation. Illumination increased the MDA content in the fumigated leaves (Table 1) whereas little increase in MDA content was observed during dark incubation. In control plants MDA content was higher in the light than in darkness. Thus illumination was a prerequisite for both chlorophyll *a* destruction and MDA formation in ozone-treated plants.

The characteristics of the destruction of pigments and lipids were further investigated using the discs excised from leaves subjected to 6 h fumigation with 0.5 ppm ozone. The contents of chlorophyll *a* and carotenoids in the leaf discs were decreased drastically by illumination (Table 2), which agreed with the observation on whole plants (Talbe 1). Chlorophyll *a* and carotenoids were retained almost completely under nitrogen (Table 2). The breakdown of pigments and lipids in the leaf discs was modified by additon of various reagents to incubation medium (Table 2). Tiron and L-ascorbate, which are the scavengers of O_2^- (Greenstock & Miller, 1975; Nishikimi, 1975), effectively protected pigments from the destruction. DABCO, a scavenger of $^1O_2$ (Ouannes & Wilson, 1968), had essentially no effect on the destruction of pigments, and D_2O, which lengthens the lifetime of $^1O_2$ (Merkel *et al.*, 1972), was also without effect. Benzoate and formate, which are ·OH scavengers (Neta & Dorfman 1968; Harbour & Bolton, 1978), had no effect on the destruction of chlorophyll *a* and carotenoids. These results suggest that O_2^- plays an important role in the destruction of these pigments.

The contents of MDA increased to 236% of the initial level when leaf discs cut from ozone-treated plants were incubated in the light for 5.5 h, whereas it decreased to 66% in the

Table 1 Effects of fumigation time and post-illumination on the chlorophyll *a* and MDA contents of spinach leaves

Duration of ozone fumigation (h)	Chlorophyll *a* ($\mu g\ cm^{-2}$)		MDA ($nmol\ cm^{-2}$)	
	light	dark	light	dark
0	26.6	23.2	0.59	0.26
1	18.9	–	0.99	–
3	14.1	–	1.87	–
6	9.5	–	1.92	–
8	10.5	24.0	1.96	0.72

Spinach plants fumigated with 0.5 ppm ozone in the light for the indicated periods of time, were kept for 24 h in two growth cabinets with or without lighting. Chlorophyll *a* and MDA contents were determined from the average of two values obtained each from 10 discs. Maximum differences of the two values in chlorophyll *a* and MDA contents were 11 and 10% of the presented average values, respectively. The contents of chlorophyll *a* and MDA in leaves immediately after fumigation for 8 h were 25.5 $\mu g\ cm^{-2}$ leaf area, and 0.95 $nmol\ cm^{-2}$ leaf area, respectively.

Table 2 Effect of illumination, nitrogen stream, and some reagents on the contents of pigments and MDA in leaf discs cut from leaves fumigated with ozone for 6 h

Treatment	Chlorophyll *a*		Total carotenoids		MDA	
	$\mu g\ cm^{-2}$	(%)	Corrected $A_{480\ nm}$	(%)	$nmol\ cm^{-2}$	(%)
Initial (Ozone, 6 h)	31.7 ± 1.2	(100)	0.384 ± 0.010	(100)	0.92 ± 0.02	(100)
Dark (5.5 h)	32.7 ± 2.4	(103)	0.361 ± 0.009	(94)	0.61 ± 0.15	(66)
Light (5.5 h)	16.3 ± 1.9	(51)	0.155 ± 0.017	(40)	2.17 ± 0.12	(236)
Light (5.5 h), N_2	33.5 ± 0.8	(106)	0.371 ± 0.025	(97)	0.60 ± 0.04	(65)
Light (5.5 h), tiron, 5 mM	24.6 ± 1.7	(78)	0.212 ± 0.014	(55)	1.13 ± 0.21	(123)
Light (5.5 h), tiron, 50 mM	28.7 ± 1.2	(91)	0.271 ± 0.008	(71)	0.79 ± 0.09	(86)
Light (5.5 h), L-ascorbate, 1 mM	21.3 ± 1.6	(67)	0.185 ± 0.011	(48)	1.73 ± 0.24	(188)
Light (5.5 h), L-ascorbate, 10 mM	31.1 ± 0.5	(98)	0.331 ± 0.003	(86)	0.50 ± 0.03	(54)
Light (5.5 h), DABCO, 10 mM	19.2 ± 1.4	(61)	0.164 ± 0.021	(43)	1.83 ± 0.26	(199)
Light (5.5 h), DABCO, 100 mM	16.5 ± 1.8	(52)	0.151 ± 0.012	(39)	1.65 ± 0.13	(179)
Light (5.5 h), D_2O	18.1 ± 0.3	(57)	0.129 ± 0.004	(34)	2.38 ± 0.12	(259)
Light (5.5 h), Benzoate, 10 mM	17.8 ± 1.8	(56)	0.150 ± 0.028	(39)	2.06 ± 0.22	(224)
Light (5.5 h), Formate, 10 mM	15.9 ± 2.6	(50)	0.135 ± 0.019	(35)	2.21 ± 0.11	(240)

The discs were floated on a 40 mM phosphate buffer (pH 6.0) and incubated for 5.5 h as described in Materials and Methods. The value is the mean ± SD of 3 experiments with 7 discs each.

dark (Table 2). Incubation under nitrogen, and addition of tiron and L-ascorbate retarded the light-dependent MDA accumulation. Moreover, DABCO treatment was partially effective in reducing the MDA formation, and D_2O slightly stimulated it ($P<0.1$). Benzoate and formate had no effect on the MDA accumulation. Thus both O_2^- and $^1O_2$ seem to participate in the light-dependent lipid peroxidation in ozone-treated leaves.

The effect of ozone fumigation on endogenous scavengers of O_2^- is shown in Figs 2, 3, and 4. Fig. 2 presents the effect of ozone of the activity of SOD, which dismutates O_2^- to H_2O_2 and O_2, in leaves. SOD activity was reduced to 47% of the original level after 3.5 h of fumigation. During this time the protein content did not change (Fig. 2). The content of L-ascorbate decreased to 49% of the initial level in 8 h of fumigation with a corresponding increase in dehydro-L-ascorbate, an oxidized product of L-ascorbate (Fig. 3). Only after 8 h, chlorophyll *a* started to be destroyed (Fig. 3), as shown previously in Fig. 1.

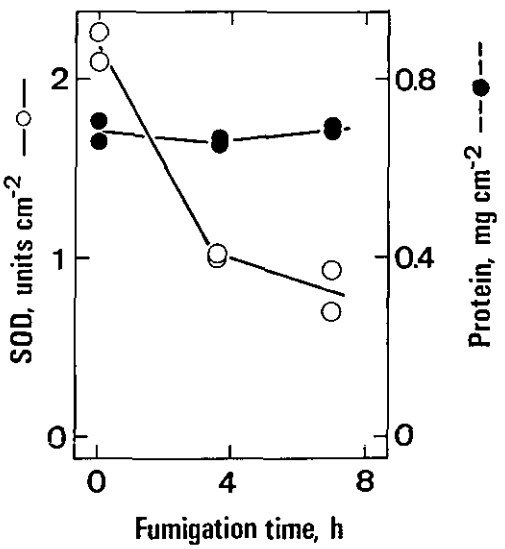

Fig. 2 Effect of ozone fumigation on the endogenous SOD activity of spinach leaves

Twenty leaf discs excised from ozone-treated plants were used to obtain one point. One unit activity of SOD was defined as described previously (Tanaka & Sugahara, 1980).

Table 3 shows the effect of ozone on polyphenol oxidase activity. This enzyme is known to be bound to the thylakoid membranes in the latent state (Golbeck & Cammarata, 1981). The activity increased to about 240% of the initial level during 4 h fumigation with ozone.

Effects of various concentrations of ozone on the contents of chlorophyll, MDA, L-ascorbate and dehydro-L-ascorbate and SOD activity in leaves were examined (Fig. 4). Visible damage was observed in 5–10% of the total leaf area after 24 h of ozone fumigation at 0.3 ppm, but not observed in the plants fumigated with 0.1 and 0.2 ppm ozone. Destruction of chlorophyll *a* was significant at 0.3 ppm ozone. Changes of carotenoid content were similar to that of chlorophyll *a* content (data not shown). MDA formation was observed even at 0.1 and 0.2 ppm ozone and especially prominent at 0.3 ppm. SOD activity and L-ascorbate content showed little change at 0.1 and 0.2 ppm ozone but a noticeable decrease at 0.3 ppm.

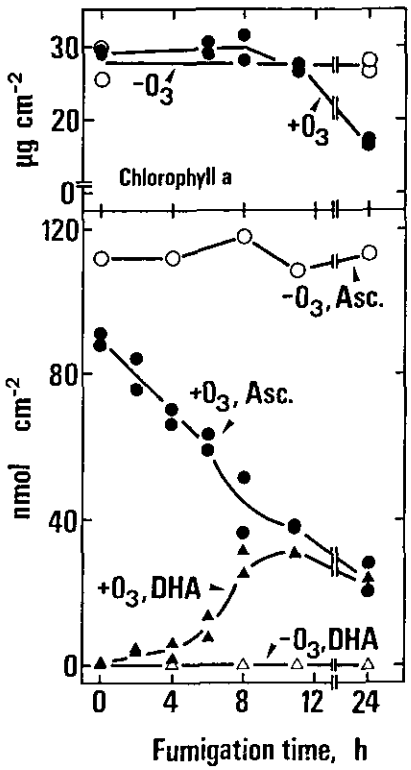

Fig. 3 Effect of ozone fumigation on the endogenous L-ascorbate and dehydro-L-ascorbate contents

Plants were placed in each two growth cabinets controlled to the same environmental conditions. After about 2 h of preconditioning, 0.5 ppm ozone was introduced into one cabinet. Fifteen leaf discs were used to obtain one point of the contents of L-ascorbate and dehydro-L-ascorbate. Asc, L-ascorbate; DHA, dehydro-L-ascorbate.

Table 3 Polyphenol oxidase activity in osmotically shocked chloroplasts from spinach leaves fumigated with ozone

Treatment	Polyphenol oxidase	
	μmol (mgchl^{-1}) h^{-1}	%
None	0.96 ± 0.12	100
Ozone (0.5 ppm, 4 h)	2.33 ± 0.16	243

Means ± SD of 4 differently prepared chloroplast samples are presented.

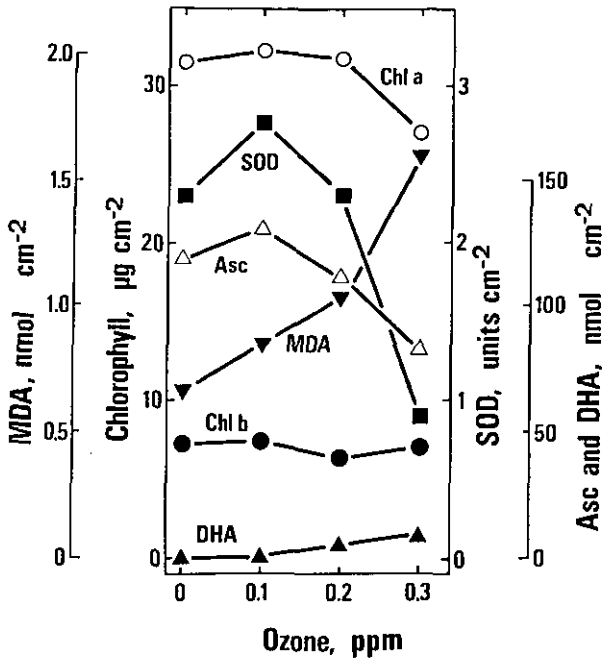

Fig. 4 Effect of various concentration of ozone on the contents of chlorophyll, MDA, and ascorbates, and the activity of SOD

Fumigation with ozone was perfomed at the same time using four growth cabinets controlled to the same environmental conditions except for ozone concentrations. Plants in each cabinet were fumigated with ozone for 24 h in the light. Each point is the average of the two samples.

Discussion

Injuries of membrane lipids may be the essential factor for foliar damage caused by ozone. Pauls and Thompson (1980) have reported the alteration of the physical properties of isolated microsomal membranes and a significant formation of MDA after exposure to ozone. In the present work, the content of MDA was considerably increased in spinach leaves by ozone from the beginning of the fumigation (Fig. 1). The MDA formation seems to be divided into at least two phases. The first phase with a low accumulation rate continued for about 8 h from the beginning of the fumigation and thereafter the second phase with a high accumulation rate started (Fig. 1).

Polyphenol oxidase activity increased by ozone in chloroplasts during the first phase of MDA accumulation (Table 3). Elevation of polyphenol oxidase activity has also been observed in soybean plant after fumigation with ozone (Tingey *et al.*, 1975). This enzyme is bound to the thylakoid membranes in the latent state and is activated by the various treatments which lead to the loss of structural integrity of the membranes. Therefore, the increased activity of polyphenol oxidase as well as the gradual accumulation of MDA strongly suggest that the integrity of the thylakoid membranes may be substantially affected in the early stage of ozone fumigation.

In the second phase of MDA accumulation, even when the ozone fumigation was stopped, the content of MDA in the leaves increased strongly in the light but not in darkness (Table 1). Ozone fumigation reduced the levels of SOD and L-ascorbate, endogenous scavengers of O_2^-, in spinach leaves before the onset of the second phase of MDA formation (Figs 2 and 3). Besides, MDA formation caused by ozone required the presence of O_2 (Table 2). These results lead us to suppose that active oxygens might participate in the lipid peroxidation process induced by ozone. This supposition is supported by the finding that the application on EDU to snap bean leaves caused the enhancement of ozone tolerance as well as the increase of SOD levels (Lee & Bennett, 1982). Effects of active oxygen scavengers suggested that O_2^- and possibly $^1O_2$ may participate in this process (Table 2). We have already shown that active oxygens take part in the lipid peroxidation in SO_2-fumigated spinach leaves (Shimazaki *et al.*, 1980). Active oxygen participation in the damage of plant cells under several environmental stresses has recently been reviewed by Elstner (1982).

As shown in Fig. 4, MDA content increased to 125 and 156% by 0.1 and 0.2 ppm ozone, respectively, while neither SOD activity nor L-ascorbate content was reduced. MDA would be formed from the ozonization of unsaturated fatty acid (Mudd *et al.*, 1971). Also in the present experiment, MDA may be formed, at least in part, from the ozonization of unsaturated lipids with ozone fumigation.

We observed that chlorophyll *a* and carotenoids were destroyed with 0.3 and 0.5 ppm ozone fumigation (Fig. 1 and 4). However, the pigments were not destroyed during 8 h of 0.5 ppm ozone fumigation in spite of the significant increase of MDA (Fig. 1). The breakdown of the pigments started only after the thylakoid membranes had been substantially disintegrated as described above. It is known that the pigment bound to the thylakoids are stable while free pigments in organic solvents are labile and sensitive to oxidative degradation. Thus the membrane disintegration and the resultant loosening of pigment binding to the membranes may be the major causes of massive pigment destruction. The breakdown of pigments required the presence of O_2 and was effectively inhibited by the application of O_2^- scavengers (Table 2). The levels of SOD and L-ascorbate were reduced with 0.3 and 0.5 ppm ozone fumigation (Figs 2, 3, and 4). These results suggest that the pigment destruction was caused by O_2^- but not by direct reaction of ozone.

Besides our observation of the increase in polyphenol oxidase activity, the accumulation of substrates of this enzyme has been also observed in ozone-fumigated leaf tissues (Howell, 1970). According to the work by Elstner *et al.* (1976), the activation of polyphenol oxidase in chloroplast thylakoids amplified the formation of O_2^- upon illumination in the presence of the enzyme substrate, dopamine, in spite of the marked inactivation of electron transport activity. Thus the increased activity of polyphenol oxidase may participate, at least partly, in the formation of O_2^-.

Ozone induces the changes in membrane permeability to water and various solutes, resulting in the net water loss from plant cells (Heath, 1975). In our experimental condition, the ratio of fresh weight to dry weight in spinach leaves fumigated with 0.5 ppm ozone for 0, 4, and 8 h were 9.5, 7.8, and 4.1, respectively (results not shown). Thus the plants were subjected to severe drought stress in the early stage of ozone fumigation. Dhindsa and Matowe (1981) have shown that SOD and catalase activities in drouhgt-sensitive moss were significantly inactivated and not restored during dehydration and rehydration processes. These results suggest that ozone-induced drought stress may be a cause of the inactivation of SOD. L-Ascorbate is oxidized by ozone and active oxygens. Probably, regeneration system of L-ascorbate from dehydro-L-ascorbate would also be damaged by ozone.

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オゾン接触によるホウレンソウ葉の光合成色素と脂質の破壊：活性酸素の役割について

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鉢植えのホウレンソウ（*Spinacia oleracea* L. cv. New Asia）を明所でオゾンに接触させたところ，クロロフィルとカロチノイドの破壊，及び脂質の過酸化分解の指標であるマロンジアルデヒド（MDA）の生成が起こった。葉内のクロロフィル*a*とカロチノイドは，ホウレンソウを0.5 ppmのオゾンに接触させ始めてから6－8時間経過した後に分解が始まった。一方MDAはオゾン接触後8時間目まで直線的に増加し，その後更に急激に増加した。オゾンに6時間接触させた葉からディスクを打ち抜き，種々の条件下で色素破壊及びMDAの生成を調べた。クロロフィル*a*とカロチノイドの破壊，及びMDAの生成は明所で，かつ好気条件下でのみ進行した。活性酸素の消去剤をディスクに添加した実験結果から，クロロフィル*a*とカロチノイドの破壊，及びMDAの生成に活性酸素，特にスーパーオキシド　アニオン（O_2^-）が関与していることが示唆された。内生のO_2^-消去物質であるスーパーオキシド　ディスムターゼ（SOD）活性，及びアスコルビン酸含量は，オゾン接触後それぞれ3.5及び8時間目に半分に低下していた。以上の結果は，オゾン接触によって活性酸素に対する内生の防御機構が破壊され，蓄積した活性酸素が光合成色素及び脂質を破壊したことを示している。

またオゾンに4時間接触させた葉の葉緑体膜におけるポリフェノールオキシダーゼ活性は対照の240％に増加していた。このことは，オゾン接触によって光合成色素が破壊される以前に，葉緑体のチラコイド膜が強く損傷を受けていることを示している。

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Res. Rep. Natl. Inst. Environ. Stud., Jpn., No. 65, 1984.

Enhancement of Damages in Sunflower Plants by Probable Involvement of Factors Generated in the Mixing of NO_2 and O_3

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In the definition commonly used for synergistic effect of two mixed pollutants, it was found that it was hard to say whether the increased damages were due to the factors generated in the mixing of two pollutants or simply due to the increase of the concentration of pollutants. An experimental procedure is proposed to solve this problem.

When pollutant A has a highly specific effect on plants and this effect is known to be impossible by pollutant B, we can say that combination of pollutants A and B has a mixture effect if the specific effect by pollutant A is enhanced by coexistence of pollutant B. When the effects caused by pollutant A and B cannot be separately distinguished by the method used, the mixture effect can be evaluated by the procedure as described below if the experimental conditions were those under which two pollutants show damages on plants as expressed by an exponential function and the degree of damage is below the inflection point. Firstly, damages by pollutant A at the concentration C_A and by pollutant B at the concentration C_B should be measured: the damage degrees are termed D_A and D_B, respectively. Secondly, the damage (D_M) of combination of pollutant A at the concentration of aC_A and pollutant B at the concentration of bC_B should be measured: where $0<a$, $b<1$ and $a+b<1$. On evaluation if D_M is more than D_A or D_B, it can be said that the factors which cause enhanced damages are generated in the mixing of the two pollutants, but if not it is hard to say so.

Following the proposed procedure the effects of mixed gas of NO_2 and O_3 on visible injury and inhibitions of photosynthetic and transpiratory activities of sunflower leaves were evaluated using the data obtained in this institute. The results suggest that the mixing of NO_2 and O_3 generated some factors which enhanced visible injury and transpiration inhibition. However, the nature and mechanisms of the factors could not be characterized so far.

Keywords: Mixed gas, NO_2, O_3, synergistic effects, visible injury, sunflower plant

The finding by Menser and Heggestad (1966) that mixture of air pollutants brought about more severe damages to plants than individual of air pollutants is important, because this indicates the possible occurrence of air pollutant damages at the concentrations as low as individual pollutants do not bring about any damages.

The effects of mixture of two air pollutants were classified into three categories as follows

by Tingey and Reinert (1975) and Reinert *et al.* (1975).

1) Additive effect: The extent of damage caused by a mixture of two pollutants equals to the sum of the damages caused by two individual pollutants.
2) Synergistic effect: The damage caused by a mixture is greater than additive of individual effects.
3) Antagonistic effect: The damage caused by a mixture is less than additive of individual effects.

Following this definition, it was believed that there were synergistic effects in the mixture fumigation with NO_2 and SO_2 on the photosynthetic CO_2 fixation (Ashenden and Mansfield, 1978) and on the transpiration (Ashenden, 1979), in the mixture fumigation with O_3 and SO_2 on the visible injury (Applegate & Durrant, 1969; Inose, 1980), and on the plant growth (Dochinger *et al.*, 1970; Tingey *et al.*, 1971), and in the mixture fumigation with O_3 and NO_2 on the photosynthetic CO_2 fixation (Furukawa & Totsuka, 1979) and visible injury (Fujiware, 1973). However, our experiences in the course of studies on the effects of air pollutants on plants have led us to find some unclearness in the definition of synergistic effect.

A procedure to check the generation of the factors leading to enhancement of damages in the mixture of two pollutants

Generally two cases may occur on the evaluation of effects of the treatments by mixture of air pollutants.

Case 1 is as follows. Pollutant A causes a specific damage which can not be caused by pollutant B only. On the fumigation of two pollutants A and B in combination the specific damage might be enhanced by coexistence of pollutant B as if pollutant B is a catalyzer of the pollutant A reactions in plants.

Case 2 is as follows. Both pollutants A and B may cause same damage, and the extent of contribution on it by individual pollutants can not be evaluated separately in the treatment in combination. Most problems encountered in the research on air pollutant effect are grouped in case 2, because simply determinable indices, such as visible injury and inhibitions of photosynthetic CO_2 fixation and transpiration, have been commonly used. If visible injury greater than additive appeared by mixture of pollutants, it has been grouped as synergistic effect in the usual definition. However, from our opinion it is also possible to say simply that the increased effects may be caused by the increase of the concentrations of pollutants.

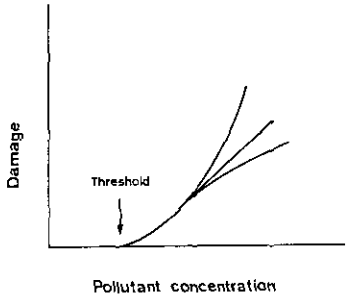

Fig. 1 A relationship between pollutant concentration and damage

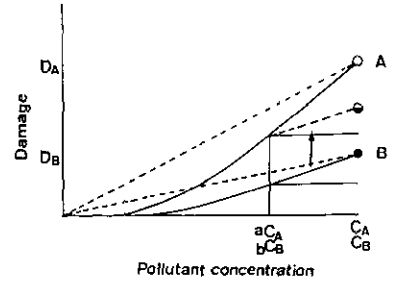

Fig. 2 Schematic expression of effects of two pollutants to evaluate the "mixture effect"

The damage usually does not appear below a certain threshold concentration but this damage increases exponentially with the increase of the concentration of pollutant as schematized in Fig. 1. This is true for NO_2 effect on the photosynthesis of alfalfa and oat (Hill & Bennett, 1970) and on the visible injury on sunflower leaves as shown in Table 1. How can we evaluate the mixture effect on plants which respond exponentially to the increase of pollutant concentration. We will try to propose a procedure to evaluate the mixture effect in Case 2.

Table 1 Visible injury in sunflower leaves caused by NO_2 and/or O_3 fumigation

Fumigated gas concentration (ppm)		Visible injury* (%)	Evaluation by	
NO_2	O_3		Old definition	Proposed definition
0	0	0		
1.0	0	0		
2.0	0	0		
4.0	0	2.2 ± 1.1		
8.0	0	7.8 ± 5.5		
0	0.2	0		
0	0.4	1.9 ± 0.5		
0	0.8	22.5 ± 1.4		
1.0	0.2	11.0 ± 6.3	Synergism	Enhanced damage
2.0	0.2	9.2 ± 2.7	Synergism	Enhanced damage
4.0	0.2	19.5 ± 6.8	Synergism	Enhanced damage
8.0	0.2	44.8 ± 11.3	Synergism	Impossible to be evaluated

* Visible injury appeared on the fourth, fifth, sixth, seventh and eighth leaves of ten plants are indicated as averages with standard deviations. (Redrawn from the data by Inose, 1980)

Under the conditions where the injurious effects of air pollutants may appear exponentially, pollutants A and B show the damages D_A and D_B (note: this values should be below the inflection points) at the concentrations of C_A and C_B respectively (See Fig. 2). If the two pollutants were mixed at the concentrations of C_A and C_B, we cannot infer the estimated degree of damages and evaluate the experimental results whether effects appeared truely by mixture of two pollutants or simply due to the increase of poisonous pollutant concentrations, because we do not know what will occur at the concentrations beyond C_A and C_B. If two pollutants were employed at the concentrations below C_A and C_B, the expected damages may be below the straight lines connecting between origin and A (C_A, D_A) or B (C_B, D_B). When the concentration of pollutant A is aC_A ($0<a<1$) and that of pollutant B is bC_B ($0<b<1$, and $a+b \leqq 1$), the effect of mixtures of the two pollutants could be inferred as if the damage by pollutant B was added to the damage by pollutant A, or *vice versa*, and the expected degree of damage could be between D_A and D_B. Therefore, if the degree of damage by mixture of two pollutants at the concentrations mentioned above was below the larger one of D_A and D_B, it is hard to find any factors leading to enhancement of damages. In contrast to this, if the damages are more than the larger one, some factors could be surely generated in the mixing of two pollutants.

In the light of the proposed definition, the data by Tingy *et al.* (1971) that the leaf injury

was caused by mixture of NO_2 and SO_2 at 5 to 25pphm suggest the involvement of some factors generated in the mixing of two pollutants, because 50pphm SO_2 or 200pphm NO_2 was required to induce the leaf injury by single pollutant. We described the damages caused by NO_2 and O_3 mixture from the view of new definition below.

NO_2 and O_3 effects on sunflower leaves

NO_2 inhibited photosynthetic CO_2 fixation (Bennet & Hill, 1974; Hill & Bennett, 1970; Srivastava *et al.*, 1975) and caused visible injury on plant leaves (Srivastava *et al.*, 1975). O_3 also inhibited the photosynthetic activity (Furukawa & Kadota, 1974). Inose (1980) examined the effects of NO_2 and O_3 mixture gas on the visible injury on the leaf surface of sunflower (*Helianthus annuus* L. cv. Russian Mammoth), and damages were counted by naked eyes as the percentages in the leaf after 24-hrs' store in the dark after gas fumigation. The results are shown in Table 1, and evaluation based on the old or newly proposed definitions was carried out by present authors as indicated in that table. Effect of NO_2 and O_3 fumigation in combination or in individuals on CO_2 fixing and transpiratory activities of sunflower leaves were examined as follows in the similar manner with Furukawa and Totsuka (1979). Four-week old sunflower plants were fumigated with NO_2 and/or O_3 in a controlled environment room (1.7 X 2.3 X 2.0 m). The light source was consisted of twentv-four 400 w halide lamps (Toshiba Co., Ltd.), and the light intensity was around 30 Klx at the plant height. An air velocity in this room was 0.2–0.4 m/s, and a ventilation rate was around 200 m^3/h. NO_2 was supplied from a cylinder containing 500 ppm NO_2 in N_2, and the concentration of NO_2 in the room was monitored by a Thermo Electron NO_x analyzer (Model 14). O_3 was produced by a silent electrical discharge in dry oxygen, and regulated by a controlling system of a Kimoto chemiluminescent O_3 analyzer (Model 806). To measure the photosynthetic CO_2 fixing activity of leaves, a single attached mature leaf was sealed into a plexiglass assimilation chamber (30 X 22 X 1 cm). Prior to gas fumigation the leaf was preilluminated for more than one hour to get the steady state of photosynthetic uptake, then treatment by NO_2 and/or O_3 was started. The rate of net photosynthesis was determined by measuring the CO_2 concentrations at the inlet and outlet of the assimilation chamber using a Shimazu infrared CO_2 analyzer (URA-2S). To measure the transpiratory water loss, the decrease of the weight from the potted plant was chased on a Mettler balance (PE 11): the loss of water from the soil surface was prevented covering the pot with a vinyl sheet.

All data on the changes in photosynthetic and transpiratory activities by one and two hours' fumigation with NO_2 and/or O_3 are summarized in Table 2. NO_2 at 2ppm or O_3 at 0.2ppm did affect so much on the photosynthetic activity. Mixture gas treatment by 1ppm NO_2 and 0.1ppm O_3 seemingly decrease that activity compared with single treatment by 2ppm NO_2 or 0.2ppm O_3, though this was not significant. Treatment by mixture of 2ppm NO_2 and 0.2ppm O_3 greatly decreased the photosynthetic CO_2 fixing activity. Transpiratory water loss was slightly affected by 0.2ppm O_3 treatment, whereas 2ppm NO_2 did not show any significant decrease during 2 hours. Mixture treatment by 1ppm NO_2 and 0.1ppm O_3 caused remarkable decrease of transpiration rates, and the treatment by 2ppm NO_2 and 0.2ppm O_3 caused more severe effect.

If we follow the old definition, results of the treatment by mixture of 2ppm NO_2 and 0.2ppm O_3 in comparison with the single treatment by each gas shows that NO_2 and O_3 have syneristic inhibitory effects on photosynthetic and transpiratory activities in sunflower. However, judging from the conception of proposed new definition, mixture effects of 1ppm NO_2 and 0.1ppm O_3 should be compared with the effects by 2ppm NO_2 and 0.2ppm O_3. The evaluation in the lignt of new definition indicates that there is significant mixture effect on the

Table 2 Effect of NO_2 and O_3 fumigation alone or in combination on the photosynthetic and transpiratory rates of sunflower leaves[a]

Treatment	Date of Experiment	Photosynthesis		Transpiration	
		1-hour	2-hour	1-hour	2-hour
NO_2 2 ppm	800321	+4		0	
	428	-1	+11	-1	-2
	503	+3	+2	-1	+2
	507	0	-5	0	-3
	508	+2(+2)[b]	+3(+3)	+3(0)	+7(+1)
O_3 0.2 ppm	800324	+4		-2	
	325	+1		-9	
	426	-1	+1	-8	-6
	502	+2	+2	-6	-11
	507	-8	-11	-12	-13
	508	-4(-1)	-7(-4)	-5(-7)	-11(-10)
NO_2 1 ppm	800403	-4	-6	-29	-39
O_3 0.1 ppm	424	-6	-24	-15	-26
	425	-4	-2	-22	-25
	429	-3	-8	-21	-36
	430	-1(-4)	-8(-10)	-26(-23)	-28(-31)
NO_2 2 ppm	800321	-27	-42	-51	-65
O_3 0.2 ppm	324	-24	-31	-76	-45
	328	-15(-22)	-30(-34)	-43(-57)	-57(-56)

a, All data are expressed as percentage increase (+) or decrease (-) compared with the values just before gas treatments. Each experiment consisted of 4 replicates.

b, The numerals in the parentheses are the averages of experiments.

transpiratory activity but not on the photosynthetic activity. In the similar manner we can say that NO_2 and O_3 have a mixture effect on the appearance of visible injury (Table 1). Some inhibitory factors could be generated in the mixing of the two pollutants.

Mechanisms of NO_2/O_3 mixing effects

There have been few attempts to explain the interactive effects not only of NO_2 and O_3 but also of other combinations of air pollutants. Three mechanisms of interactive effects were considered in the combination of NO_2 and O_3. (i) The two pollutants may react with each other in the atmosphere before contact with foliage, and produce some toxic substances which may enhance the damage. Mixing NO_2 and O_3 gases is known to induce the decrease of the concentrations of individual gases, and produce NO_3 and N_2O_5 gases and unidentified compounds. The products even at low concentrations might be very toxic to plants when they are absorbed. (ii) One pollutant may promote foliar uptake of the another pollutant by altering stomatal resistance to diffusion of gases. (iii) One pollutant may change the reactivities of another gas in plant cells or destroy the protecting mechanisms against to the attack by another pollutant. Both NO_2 and O_3 gases have oxidative activities, and these activities in plant cells might be enhanced by coexistence of the two gases. Ozone is reportedly a destroying agent of NAD(P)H *in vitro* (Menser & Heggestad, 1966), therefor, the reduction of nitrite came from NO_2 might be inhibited through the decrease of the NADPH supply.

We conducted some investigations as indicated below to characterize the possible mechanisms of synergistic effects of NO_2 and O_3 using 3-week old sunflower plants. Under a controlled environmental condition previously described in the present report, we fumigated 0.2–0.3ppm O_3 for 4 hours to the plants which had already treated with 2ppm NO_2

continuosly for 2 hours in the light.

Check 1: Nitrite was not accumulated in the leaves fumigated with 2ppm NO_2, and additional O_3 treatment did not cause an increase of nitrite in leaves.

Check 2: *In vitro* nitrite reductase activity (Yoneyama *et al.*, 1978) in the leaves did not decrease by addition of O_3. Reduction processes were not impaired.

Check 3: Malondialdehyde test, an indicator of cell lipid peroxidation (Heath & Packer, 1968), showed that the activities did not differ in the leaves fumigated with NO_2 alone or NO_2-O_3 mixture.

Check 4: Changing the ventilation rates from ordinary one (200 m^3/h) to larger (1,200 m^3/h) in order to reduce the toxic products which were induced by the mixing of NO_2 and O_3, did not bring about any affection to the inhibition extent of NO_2-O_3 in the transpiration of sunflower plants.

So far, we could not characterize any possible reasons of the damage enhanced by fumigation of NO_2-O_3 mixture. Other methods should be tried to find out the reasons.

What should be done first of all in the research on the mixture effect of air pollutants is to clarify whether the phenomena caused by fumigation with mixture of pollutants is truely due to enhancement by the factors generated in the mixing or simply due to increase of pollutant concentrations. To make this clear, new experimental systems such as proposed here should be devised. We did not discuss additive or antagonistic effects in the present communication. Further effort is necessary to know what is caused by air pollutant mixture and how it happens under complex environments.

Acknowledgment

Authors thank Mr. M. Yokoyama for his help in measurements of photosynthetic and transpiratory activities and Dr. H. Sasakawa for his assistance in the determination of nitrite reductase activities.

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NO_2 と O_3 の混合により生じた要因によるヒマワリでの障害の増加—相乗作用の評価をめぐって—

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一般に使われている大気汚染ガス二成分混合による相乗効果の定義からは増加した障害が二つの汚染質の混合により生じた要因によるのか，単純な汚染質の濃度の上昇によるのかどちらとも言えないと考えられた。この問題に対する実験上の一解法を提案する。

もし汚染質Aが植物に非常に特異的な障害をもち，この障害は汚染質Bで起こらないことがわかっている場合に，汚染質Aの障害が汚染質Bの共存のもとで増加すれば，混合効果があったといえる。次に汚染質A，Bの効果が使われた方法では区別できない場合次の方法で混合効果が評価されうる。

まず汚染質A（濃度C_A），汚染質B（濃度C_B）による障害（D_A，D_B）を測定する。次に汚染質A（濃度aC_A）と汚染質B（濃度bC_B）の混合による障害D_Mを測定する。（但し$0<a$，$b<1$，$a+b\leqq 1$）。ここでもしD_MがD_AやD_Bより大であれば，混合により生じた要因が障害を高めたといえる。

この定義に従って，ヒマワリ葉でのNO_2とO_3混合による光合成活性，蒸散活性の阻害を評価したところ，可視障害や蒸散障害に汚染ガス混合により生じた要因の関与が推定されたが，その要因の性質，メカニズムはまだ明らかではない。

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Res. Rep. Natl. Inst. Environ. Stud., Jpn., No. 65 1984.

The Change in Leaf Proteinase and Proteinase Inhibitor Activities by Air Pollutant*

I. Participation of Proteinases in Cellular and Molecular Damages of Plant Leaves by SO_3^{2-} and H_2O_2

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Proteinase activity increased in Spinacia oleracea leaves but not in roots when sodium sulfite, hydrogen peroxide and sodium azide were injected through the petiole under light condition, but the activity was not affected by sulfuric acid. On the other hand, proteinase-inhibitory activity in both leaves and roots were decreased by the injection. Proteinase activity in *Ricinus communis* leaves increased when hydrogen peroxide and sodium sulfite were injected through the petiole and kept for 4 h under light condition. No visible injuries were caused to the leaf during 4-h light illumination. On the other hand, proteinase-inhibitory activity in leaves was decreased by the injection of hydrogen peroxide. Changes in the activity of proteinase probably caused the injury of leaves such as chlorosis and necrosis, which were observed on leaves one week after the injection with hydrogen peroxide. Proteinase extracted from leaves of sodium sulfite or hydrogen peroxide-treated *S. oleracea* was inhibited by proteinase inhibitor from *S. oleracea* roots. Proteinase from *R. Communis* leaves was inhibited by proteinase inhibitor from *R. Communis* leaves themselves.

These results suggest that in the healthy leaf the proteinase inhibitor protects the cellular components from the proteinase.

Key words: *Hydrogen peroxide, Proteinase, Proteinase inhibitor, Ricinus communis-* Sodium sulfite, *Spinacea oleracea.*

Sulfur dioxide is one of the major air pollutants which cause chlorophyll destruction in plant leaves (Peiser & Yang, 1977) being the main symptom and some damages to the mesophyll (Soikkeli & Tuovinen, 1979). SO_2-fumigation increased active oxygen, O_2^- and hydrogen peroxide, in the chloroplast and the increase was greater under illumination than in darkness. H_2O_2 formation was dependent on light (Tanaka *et al.*, 1982). O_2^- was formed on

* A part of this study had been published in Bilo. Plant. (Praha), 25, 100-109 (1983).

thylakoid membranes under illumination and initiated the aerobic chain oxidation of sulfite to yield a large amount of active oxygen, O_2^-, $^1O_2$, H_2O_2 and OH• (Asada & Kiso, 1973; Asada *et al.*, 1974).

Superoxide dismutase (SOD) in chloroplasts decreases the steady state concentration of O_2^- to about 0.01%. However, SO_2 fumigation significantly inactivated SOD (Shimazaki *et al.*, 1980), and therefore, the concentration of O_2^- should increase greatly in chloroplasts. In addition, O_2^- production would be amplified via aerobic chain oxidation of sulfite, initiated by O_2^- (Asada & Kiso, 1973; Asada *et al.*, 1974).

H_2O_2 was produced from O_2^- in the process of SO_3^{2-} oxidation in leaves (Tanaka *et al.*, 1982). Chlorophyll bound to protein is relatively stable to light and oxygen, while free chlorophyll in an organic solvent is extremely labile to superoxide radicals, which are produced during the aerobic oxidation of bisulfite (Peiser & Yang, 1977). These signs of leaf injury could be explained by increased proteolysis in SO_3^{2-} or H_2O_2 treated-leaves, and it might be reasonable to assume that the protein degradation is due to increased activities of proteinases present in leaf. However, relatively few neutral proteinases have been defined which have the property degrading intracellular protein. In the present study, effects of H_2O_2 and SO_3^{2-} on proteinase and proteinase inhibitor were examined with special reference to active oxygen participation.

Materials and Methods

Plant material

Spinach (*Spinacia oleracea* L. cv. New Asia) and castor bean (*Ricinus communis*) plants were grown in pots containing vermiculite, peat moss, perlite and fine gravels (2:2:1:1. v/v). These plants were grown in a glasshouse under sunlight. The temperature was 20°C in the day time and 15°C at night for *S. Oleracea* and 25°C in the day time and 20°C at night for *R. communis* with a relative humidity of 70 ± 5%. The test plants grown in the glasshouse were transferred to a growth cabinet for injection. *S. oleracea* and *R. communis* were preconditioned for 2 weeks in the cabinet at 20°C and 25°C, respectively, under illuminance of 30 klx at leaf level. Plants used for the experiment were 7–8 weeks old.

Injection of chemicals

One-tenths ml/petiole of solution of 1 mM sodium sulfite with 1 mM EDTA, 1 mM sodium azide, 29.4 mM hydrogen peroxide, 1 mM sulfuric acid and distilled water each was injected into the petiole of *S. oleracea* by means of a syringe. Then test plants were kept at 20°C under 30 klx at leaf level for 1h. *R. communis* plants were preconditioned before injection for 2 weeks in the cabinet at 25°C under 20–30 klx at leaf level. A half ml/petiole of solution of 1 mM sodium sulfite containing 1 mM EDTA was injected into the ten petioles of *R. communis* by means of a syringe. A half ml/petiole of 1 mM sodium azide, 29.4 mM hydrogen peroxide and distilled water each was injected into ten petioles. Then test plants were kept at regular intervals under 20–30 klx at leaf level for 1.5, 4 h and 1 week.

Prot einase extraction

Approximately 100 g of leaves of 21 test spinach plants were homogenized in a polytron homogenizer at 4°C in 100 ml of 100 mM Tris-HCl buffer, pH 7.4, containing 500 mM NaCl and 1 mM dithiothreitol. The homogenate was centrifuged at 10000 x g for 15 min, and the

supernatant was filtered through filter paper. The fraction of 30–70% saturated ammonium sulfate (i.e. crude fractions) was subjected to gel filtration on Ultrogel AcA 44, followed by affinity chromatography of proteinase using amino porous glass reacted with the serine proteinase inhibitor, aprotinin, and 1-ethyl-3- (3-dimethyl-aminopropyl) carbodiimide (Watanabe *et al.*, 1982).

Procedures for homogenization of the *R. communis* leaves and purification of the extracts have been as described above. The buffer was 50 mM Tris-HCl, pH 7.4, containing 500 mM NaCl and 1% glycerin. The fraction of 30–70% saturated ammonium sulfate was subjected to gel filtration on Ultrogel AcA 44, followed by affinity chromatography on aprotinin-porous glass (Watanabe *et al.*, 1982)

Proteinase inhibitor extraction

Proteinase inhibitor was extracted from *S. oleracea* roots or *R. communis* leaves according to the method described by Watanabe and Watanabe (1981). Approximately 90g of *S. oleracea* roots were homogenized at 4°C in 100 ml of 6% perchloric acid. The homogenate was centrifuged at 10000 x g for 30 min, and the supernatant was filtered through five layers of gauze. Solid ammonium sulfate was added to the crude extract to 30% saturation, and the resulting precipitate was collected by centrifugation and discarded. The supernatant of 30% saturated ammonium sulfate was fractionated by adding solid ammonium sulfate to 70% saturation and was again centrifuged at 10000 x g for 30 min. The precipitate was dissolved in 10 ml of 100 mM Tris-HCl buffer, pH 7.4, containing 500 mM NaCl. The 30–70% saturated ammonium sulfate fraction was applied on Ultrogel AcA 44 column (2.64 x 90 cm) equilibrated with 50 mM Tris-HCl buffer, pH 7.4. Then gel filtered fraction was subjected to DEAE-Sephacel ion exchange chromatography (2.64 x 60 cm) and to affinity chromatography on immobilized tryspin. Harvest and homogenization of *R. communis* leaves were done as described previously (Watanabe *et al.*, 1982).

Proteinase assay

a) Peptidase Activity: The reaction was initiated by addition of 0.8 ml of the proteinase fraction and 0.5 ml of 50 mM Tris-HCl buffer, pH 7.4, containing 150 mM NaCl and 0.5% glycerin to 0.2 ml of 3.5 mM H-D-valyl-L-leucyl-L-lysine-p-nitroanilide (S-2251) in an assay microcuvette and ΔA/min (405 nm) was determined. The increase in absorbance at 405 nm was recorded for 5 min. To obtain pH profile of tripeptide (S-2251)-hydrolyzing activity present in enzyme purified by affinity chromatography, the enzyme reaction was carried out in 50 mM Tris-acetate buffer, containing 150 mM NaCl, 5 mM $CaCl_2$ and 0.5% glycerin, of different pH values from 4.0 to 9.0.

b) Proteinase Activity: The activity of proteinase (protein-hydrolyzing activity) was measured by hydrolysis of 0.2 ml of 0.5% α-casein added to 0.2 ml of 50 mM Tris-HCl buffer, pH 7.4, or 50 mM Tris-acetate buffer, pH 5.0, containing enzyme sample at 37°C for 1h.

Proteinase inhibitor assay

Proteinase-inhibitory activity was assayed on the basis of the method described by Watanabe and Watanabe (1981). The reaction was carried out in the microcell of a spectrophotometer in a reaction mixture containing 0.4 ml of 50 mM Tris-HCl buffer, pH 7.4, 0.05 ml of plasmin (0.6 casein units ml^{-1}), 0.35 ml of proteinase inhibitor fraction in the same buffer and 0.2 ml of 2 mM S-2251 as substrate. The inhibitory activity toward plasmin was measured by decrease of absorbance at 405 nm at 37°C for 5 min (Naito & Aoki, 1978). As a blank test the activity of 0.6 casein unit ml^{-1} plasmin without inhibitor was measured under the

same assay condition.

Protein determination

Protein contents were determined by the method of Bradford (1976) using lyophilized preparation of bovine plasma gamma-globulin as standard. These experimental results, shown, are the average of three measurements. All spectrophotometric determinations were carried out using a Shimazdu UV 200S recording spectrophotometer.

Chemicals and substrates

Plasmin and S-2251 were purchased from Kabi Diagnostica, Sweden. Coomassie brilliant blue G-250 (Bio-Rad protein assay kits) was a product of Bio-Rad Laboratories, U.S.A. H_2O_2 was obtained from Mitsubishi Gas Kagaku Co., Tokyo. DEAE-Sephacel was purchased from Pharmacia Fine Chemicals, Sweden and Ultrogel AcA 44 from LKB, France. Other chemical reagents were purchased from Nakarai Chemical Co., Kyoto.

Results

Proteinase activity was detected in the leaf of *S. oleracea* grown in a greenhouse under sunlight. When various chemicals were injected into the petioles and the plant then kept for 1 h under light conditions, a significant increase in proteinase activity of crude fraction from the leaves was detected (Table 1). The induction of the activity by light illumination was stimulated by injection of sodium azide, hydrogen peroxide and sodium sulfite but not by distilled water and sulphuric acid (Table 1). Most marked was the stimulation by sodium azide-injection, the specific activity of a crude proteinase being increased about 3 times. Such an induced appearance of proteinase activity was not observed when injected plants were kept under dark condition (Table 1). *S. oleracea* leaf extracts inhibited the blood proteinase plasmin. Table 2 demonstrates that the inhibitory activity in the leaves toward this serine proteinase was decreased by injection of hydrogen peroxide, sodium azide and sodium sulfite by 57, 68 or 57%

Table 1 Induction of proteinases in *S. oleracea* leaves by injection of chemicals into the petiole

Plants were treated by injection into petiole with 0.1 ml/petiole × ten petioles of solution indicated. After injection, plants were transferred to a growth cabinet under illuminance of 30 klx at leaf level or under dark condition at 20°C for 1 h.

Treatment	Light				Dark	
	Protein content [mg ml^{-1} of extract]	Proteinase activity [ΔA min^{-1}]	Specific activity [$\Delta A \times 10^{-3}$ min^{-1} mg^{-1} of protein]	Relative activity	Protein content [mg ml^{-1} of extract]	Proteinase activity [ΔA min^{-1}]
H_2O	0.759	0.022	29	1.00	0.438	0.001
Na_2SO_3 (1 mM)	0.572	0.033	58	1.99	0.538	0.001
H_2O_2 (29.4 mM)	0.530	0.038	72	2.47	0.713	0.004
NaN_3 (1 mM)	0.568	0.049	86	2.98	0.491	0.002
H_2SO_4 (1 mM)	0.684	0.018	26	0.91	–	0.002
None	0.771	0.020	–	–	–	–

after 1 h under light condition, respectively. Proteinase activity was detected in the root, even when *S. oleracea* was injected with distilled water, hydrogen peroxide, sodium azide, sodium sulfite or sulfuric acid through the petiole and then kept for 1 h under light condition (Table 3).

The inhibitory activity of root toward plasmin was detected and decreased by the injection of sodium azide and hydrogen peroxide (Table 3). As shown in Table 4, a significant activity of proteinase was detected in leaves of *R. communis*. When distilled water was injected into the petiole and then the plant kept for 1.5 and 4 h under light condition, no increase in proteinase activity and no visible injuries were observed in any leaves. Injection of sodium sulfite and hydrogen peroxide into the petiole increased proteinase activity in the leaf. Hydrogen peroxide-treated leaves were notably injured, i.e., chlorosis and necrosis observed on the surface of leaves after 1 week under light condition.

As shown in Fig. 1, the activity of proteinase in the gel-filtered fraction of extract from sodium sulfite and hydrogen peroxide-treated *S. oleracea* leaves was inhibited by proteinase inhibitor (PI-1) from *S. oleracea* roots. Gel-filtrated proteinase from *R. communis* leaves was inhibited by the proteinase inhibitors from hydrogen peroxide-treated (PI-PL (H_2O_2)) and distilled water-treated (PI-RL (D.W.)) *R. communis* leaves by 15.2 and 52.2%, respectively (Fig. 2). Proteinase extracted from *R. communis* leaves and purified by affinity chromatography had a pH optimum of 7.4, whether the substrate was α-casein (Table 5) or val-leu-lys-pNA (Fig. 3). The unbound enzyme had a pH optimum of 5.0 (Table 5), being an acid proteinase.

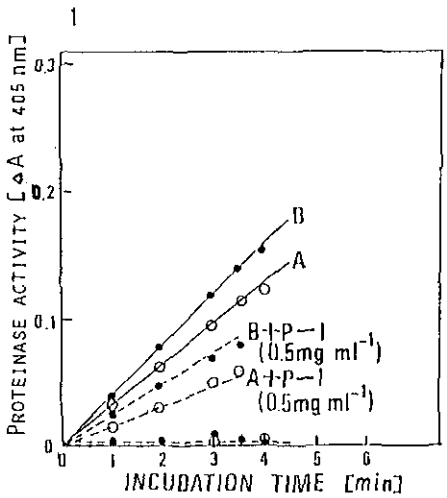

Fig. 1 Effect of proteinase inhibitor from *S. oleracea* root on proteinase from *S. oleracea* leaf

–A: 1 mM Na_2SO_3 injection into petiole. 0.1 ml/petiole × 10. B: 29.4 mM H_2O_2 injection into petiole, 0.1 ml/petiole × 10. P – I: 0.967 mg ml^{-1} of proteinase inhibitor from *S. oleracea* root; 0.502 mg ml^{-1} of proteinase inhibitor from *S. oleracea* root. – The enzyme reaction was carried out in the microcuvette of spectrophotometer in a reaction mixture containing 0.6 ml of 50 mM Tris-HCl buffer, pH 7.4, 0.1 ml of enzyme solution, 0.1 ml of inhibitor solution (0.967 mg of protein/ml or 0.502 mg of protein/ml) and 0.2 ml of S-2251 as substrate.

Table 2 Effect of extracts from *S. oleracea* leaves on plasmin activity
Experimental procedure as in Table 1.

Injection	Protein content [mg ml^{-1} of extract]	Residual plasmin activity [C.U. x 10^{-2} min^{-1}]	Activity C.U. x 10^{-3} [min^{-1} mg^{-1} of portein]	Relative inhibition
H_2O	0.759	3.8	50	1.00
Na_2SO_3 (1 mM)	0.572	6.7	117	0.43
H_2O_2 (29.4 mM)	0.530	6.2	118	0.43
NaN_3 (1 mM)	0.568	8.8	155	0.32
H_2SO_4 (1 mM)	0.684	4.2	61	0.82

Table 3 Proteinase activity and inhibitory activity for plasmin of extracts from roots of *S. oleracea* injected with sodium sulfite and hydrogen peroxide through petioles

After the injection, the plant was transferred to a growth cabinet under light intensity of 30 klx at leaf at 20°C for 1 h.

Injection	Specific Proteinase activity [ΔA x 10^{-3} min^{-1} mg^{-1} of protein]	Protein [mg ml^{-1} of extract]	Residual activity plasmin [C.U. x 10^{-2}]	Activity [C.U. c 10^{-3} min^{-1} mg^{-1} of protein]	Relative inhibition
H_2O	0.1	0.192	5.2	271	1.00
Na_2SO_3 (1 mM)	0.1	0.157	5.6	357	0.76
(1 M)	0.1	0.161	7.1	441	0.62
H_2O_2 (29.4 mM)	0.4	0.113	6.6	584	0.46
NaN_3 (1 mM)	1.1	0.083	6.2	747	0.36
H_2SO_4 (1 mM)	0.1	0.197	6.6	335	0.81
None treated	0.1	–	–	–	–

Table 4 Proteinase activity of extracts from *R. communis* leaves

Petioles injected as described in the Materials and methods. After the injection, the plant was transferred to a growth cabinet under illuminance of 20-30 klx at leaf level at 25°C for 1.5, 4 and 168 h.

Injection	Proteinase activity [ΔA min^{-1} mg^{-1} of protein]				Visible damage on leaf surface after			
	0	1.5 h	4 h	168 h	0 [%]	1.5 h [%]	4 h [%]	168 h [%]
H_2O	13.1	14.2	11.4	42.0	0	0	0	10
Na_2SO_3 (1 mM)	12.9	18.1	18.8	–	0	0	0	–
H_2O_2 (29.4 mM)	13.7	17.3	46.1	418.0	0	0	0	66

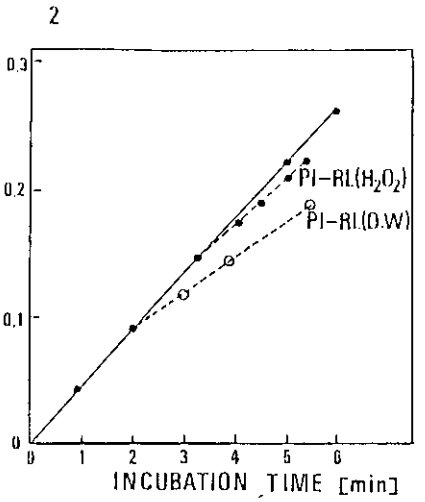

Fig. 2 Effect of proteinase inhibitors from *R. communis* leaf on proteinase activity of *R. communis* leaf. – P: 3.5 μg protein/0.1 ml, proteinase fraction of gel filtration on Ultrogel AcA 44 of 30–70% saturated $(NH_4)_2SO_4$ fraction from H_2O_2-treated *R. communis* leaf. (30 klx for 4 h). PI – RL (H_2O_2): 0.1 ml, proteinase inhibitor (PI), fraction from H_2O_2 treated *R. communis* leaf, (30 klx for 4 h). PI – RL (D.W.): 0.1 ml, proteinase inhibitor (PI), fraction from D.W. treated *R. communis* leaf. (30 klx for 4 h).

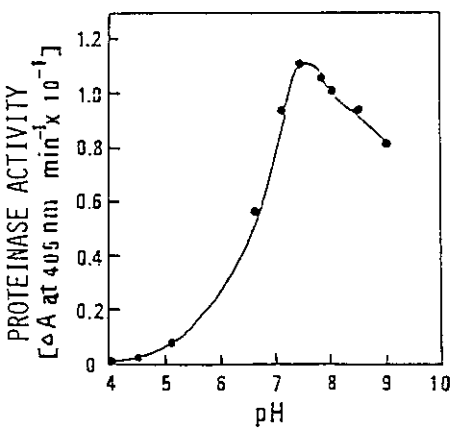

Fig. 3 pH profile of Val-leu-lys-pNA (S-2251)-hydrolyzing activities present in the purified proteinase from *R. communis* leaves

Table 5 The hydrolysis of alpha casein of proteinase purified by affinity chromatography

Treatment of enzyme solution	Proteinase activity [ΔA at 280 nmh^{-1}]	
	bound enzyme[a]	unbound enzyme[b]
None	0.073	0.040
80°C for 15 min	0	0.011

[a] The activity of neutral proteinase was measured by hydrolysis of 0.5% α-casein in 50 mM Tris-HCl buffer, pH 7.4.
[b] The activity of acid proteinase was measured by hydrolysis of 0.5% α-casein in 50 mM Tris-acetate buffer, pH 5.0.

Discussion

Protein-like proteinase inhibitors are widely distributed in the plant king-dom (Birk, 1976; Watanabe & Watanabe, 1981). Most of these inhibitors have been shown to be present in the seed of various plants, but they are not necessarily restricted to this part of the plant (Birk, 1976).

Recently, in animal cells attention has been directed to the coexistence of a proteinase and its specific inhibitor in a cell or organelle and the involvement of such an inhibitor considered in the regulation of intracellular proteinase activities as well as proteinase-mediated proteinases (Nishiura *et al.*, 1978; Waxman & Krebs, 1978). However, little information is available concerning the physiological significance of proteinase inhibitors in plant cells (Ryan, 1973; Gustafson & Ryan, 1976; Richardson, 1977; Salmia, 1980).

We found in the present study the change in activities of proteinases and their inhibitors in *S. oleracea* and *R. communis* leaves after treatment with hydrogen peroxide and sodium sulfite under light condition. Secondly present results suggested that leaves of *R. communis* contain inhibitors of endogenous proteinases. It was also shown that *R. communis* leaf injury caused by hydrogen peroxide or sulfite was associated with increase of proteinase activity. It seems likely that, in general, hydrogen peroxide induces an increase in proteinase activity and a decrease in proteinase inhibitor activity in the leaf of higher plants. Although the preparation of proteinases and their inhibitors were not high in purity in the present work, the simplest explanation for these results is that the increase of proteinase activity in the leaf of *S. oleracea* and *R. communis* might have been caused by activation of the proteinase by active oxygen, which was produced in the process of SO_3^{2-}oxidation. Illuminated chloroplasts isolated from SO_2-fumigated *S. oleracea* leaves accumulated more H_2O_2 than those from non-fumigated ones did, accompanying O_2 uptake. The H_2O_2 formation, 260 mM H_2O_2/h in SO_2-fumigated chloroplast, was dependent on light and inhibited by 3-(3,4-dichlorophenyl)-1, 1-dimethylurea (DCMU) (Tanake *et al.*, 1982). Probably, the decrease of proteinase-inhibitory activity in leaves and roots may also be caused by inactivation of the inhibitor due to active oxygen.

We propose that such changes in the activity of proteinase cause the senescence of leaves resulting in chlorosis and necrosis which were observed on *R. communis* leaves injected with hydrogen peroxide after about one week. We would speculate that in the healthy leaf the proteinase inhibitor.protects the cellular components from the proteinase (Fig. 4) (Watanabe, 1982; Baumguartner & Chrispeels, 1976). Such protection may also operate during the

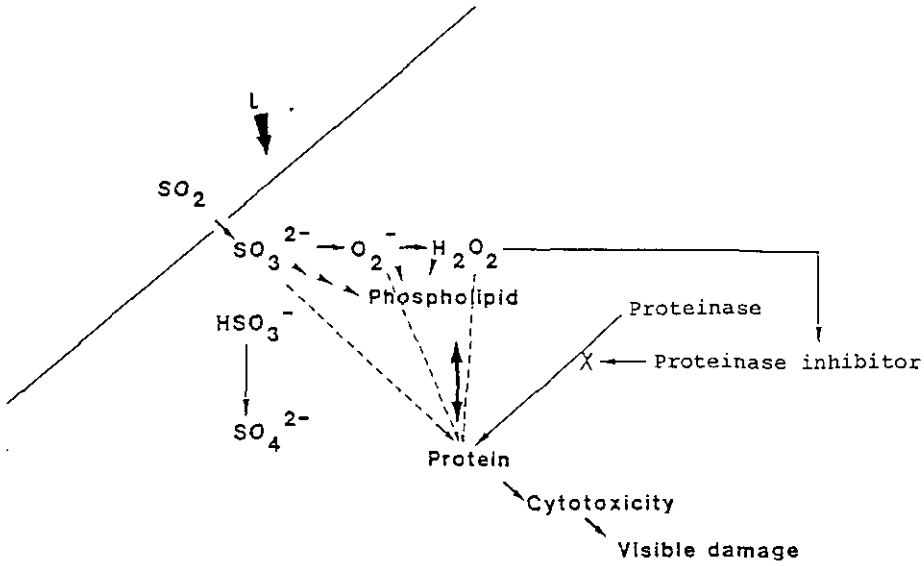

Fig. 4 Scheme for the process from SO_2 fumigation to the appearance of visible damage on plant leaves (Watanabe, 1982, Watanabe & Kondo, 1983; Watanabe *et al.*, 1983)

germination (Salmia, 1980). Proteinases and their inhibitors have been studied in a large number of microorganisms (Holzer *et al.*, 1975; Fischer & Thompson, 1979). Proteinases and their inhibitors have often been found to be located in separate compartments, the enzymes in vacuoles or lysosomes and the inhibitors in the cytosol. It has been suggested that the role of these inhibitors is to protect the cytoplasm against the accidental rupture of the proteinase-containing vesicles. It is not clear, however, whether the inhibition of leaf proteinase by proteinase inhibitor from root is due to the irreversible inactivation by inhibitor or due to competitive inhibition by inhibitor. Furthermore, mechanisms of transport of the inhibitor from *S. oleracea* root to leaf are still obscure *in vivo*. Further investigation to clarify these mechanisms and physiological roles of proteinase and its inhibitor are now in progress.

Acknowledgments

We wish to thank Dr. T. Fujii of University of Tsukuba for his helpful discussions and advice and Dr. K. Sugahara of the National Institute for Environmental Studies (NIES) for his encouragement and support. Authors are indebted to members in the Engineering Division of NIES for their adept cultivation and provision of plants.

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大気汚染物質によるプロティナーゼと プロティナーゼインヒビター活性の変化 I　亜硫酸や活性酸素による細胞傷害発現時 におけるプロティナーゼの関与

渡辺恒雄[1,2]・近藤矩朗[1]

ホウレンソウやトウゴマの茎の中空部に亜硫酸や活性酸素の一種，過酸化水素水を注射器で注入処理した後，光照射した。4時間の光照射条件下では，葉の表面の可視害は観察されなかったが，葉のプロティナーゼ活性が増加することが見いだされた。7日後には葉面積の60％に可視害が認められプロティナーゼ活性は約10倍高くなった。過酸化水素水処理したホウレンソウやトウゴマ葉から得られたプロティナーゼインヒビターは，無処理のインヒビター活性と比較すると，阻害活性が低下することが見いだされたことから，大気汚染物質による植物の傷害発現にプロティナーゼとインヒビター系のバランスの乱れが関与している可能性が示唆された。

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The Change in Leaf Proteinase and Proteinase Inhibitor Activities by Air Pollutant
II. Purification and Some Properties of Proteinase and Its Inhibitor in the Leaf of *Ricinus communis*

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Neutral proteinase was found in the leaves of *Ricinus communis* as assayed with α-casein and H-D-Val-Leu-Lys-pNA as substrates. The enzyme is maximally active at pH around 7.4. A selective adsorbent for serine proteinase was prepared by attaching aprotinin to aminoalkyl-porous glass. When partially purified leaf proteinase was passed through a column containing the adsorbent, the proteinase activity present was bound to the porous glass. The proteinase eluted at 1M NaCl was inhibited by aprotinin, leupeptin, DFP, phenylmethylsulfonyl fluoride (PMSF) and serine proteinase inhibitor from *R. communis* leaves, whereas pepstatin, EDTA, EGTA, and DTT had no effect on the enzyme. This inhibition profile suggests that the leaf proteinase is a neutral proteinase, such as a serine proteinase.
Key words: *Ricinus communis*, Serine proteinase, Serine proteinase inhibitor.

Intracellular proteinases are thought to play an important role in continuous turnover of cellular proteins. In the case of higher plant tissues, several endoproteinases such as acid (Dalling *et al.*, 1976; Feller *et al.*, 1977; Frith *et al.*, 1978), thiol (Wittenbach, 1978; Miller & Huffacker, 1981) and serine proteinases (Martin & Thimann, 1972; Drivdahl & Thimann, 1978) have been isolated and characterized. However, little is known concerning the nature of leaf serine proteinases, in spite of their possible importance, owing to difficulty in their extraction and purification. Due to their self-digesting nature, proteinases are difficult to be isolated.

Recently, we have partially purified and characterized a neutral serine proteinase and its inhibitor from leaves of *Spinacia oleracea* (Watanabe & Kondo, 1983). The tissue distribution of this type of proteinases is not yet known, but an enzyme with plasmin-like activity has also been isolated from *R. communis* leaves treated with hydrogen peroxide (Watanabe, 1982). Increase in activity of serine proteinase and decrease in activity of serine proteinase-inhibitor

Abbreviations: DTT, dithiothreitol; DFP, diisopropylfluorophosphate; PEG-6000, polyethylene glycol-6000; PMSF, phenylmethylsulfonylfluoride; SBTI, soybeam trypsin inhibitor.

have been observed in homogenates of hydrogen peroxide-treated leaves before occurrence of necrosis and senescence (Watanabe, 1982). The concentration of the serine proteinase in the leaf is very low (T. Watanabe, unpublished work), so that it is difficult to prepare the enzyme from leaves in a homogeneous form in sufficient quantities for its purification and characterization.

This report presents some information on the endoproteolytic system of *R. communis* leaves. Our results indicate the presence of serine proteinase in hydrogen peroxide-treated leaves. The serine proteinase which is not readily detected in normal untreated leaves was purified by affinity chromatography on immobilized aprotinin-porous glass beads, and has 3,000-fold activity of the ammonium sulfate fraction previously described (Watanabe & Kondo, 1983). A serine proteinase inhibitor which inhibits the serine proteinase from hydrogen peroxide-treated leaves was also isolated from normal leaves of *R. communis*.

Materials and Methods

Plant material

Ricinus communis was grown in a phytotron as described previously (Watanabe & Kondo, 1983).

Reagents

Commercial materials were purchased as follows: plasmin, H-D-valyl-L-leucyl-L-lysine-p-nitroanilide dihydrochloride (S-2251), H-D-valyl-L-leucyl-L-arginine-p-nitroanilide dihydrochloride (S-2266), H-D-prolyl-L-phenylalanyl-L-arginine-p-nitroanilide dihydrochloride (S-2302) and L-pyroglutamyl-glycyl-L-arginine-p-nitroanilide hydrochloride (S-2444) from Kabi Diagnostica; α-casein from Sigma; Ultrogel AcA 44 from LKB; long-chain amino porous glass (Glass-Co-NH-$(CH_2)_6$ NH_2)) for affinity-support from Electro-Nucleonics, Inc., standard proteins from Bio-Rad; and all other chemicals from Boehringer Mannheim.

Proteinase extraction and purification

Procedures for homogenization of the leaf and purification of the extracts have been previously described (Watanabe & Kondo, 1983). The 30–70% ammonium sulfate fraction was subjected to gel filtration on Ultrogel AcA 44, followed by affinity chromatography of proteinase. The affinity adsorbent was prepared by reaction of 5 g of long chain amino porous glass with 100 ml of 5000 kallikrein-inhibitory units/ml of aprotinin and 0.05 ml of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide, in a similar manner to the procedure described by Larsson and Mosbach (1971).

Proteinase-inhibitor extraction and purification

Details of procedures for the extraction and purification were described previously (Watanabe & Kano, 1982; Watanabe & Kondo, 1983).

Assay

Proteinase activity was determined by the hydrolysis of α-casein (Watanabe & Kondo, 1983). Amidase activity was measured colorimetrically by the hydrolysis of S-2251 and S-2302 as substrate (Chrispeels & Boulter, 1975). Details of the incubation procedure and analysis of the reaction products were described in the previous report (Watanabe & Kondo, 1983). One

unit was defind as the amount of enzyme which liberates 1 μmole of p-nitroaniline equivalents for 1 min at 37°C.

Inhibitor assay

Plasmin-inhibitory activity was assayed by mixing an aliquot of the inhibitor solution with plasmin (Castellino & Powell, 1981) and determining the amidolytic activity of the mixture (Watanabe & Kondo, 1983). One unit of inhibitory activity was defined as the amount of inhibitor which inhibits 0.03 units of plasmin by 50%.

For the measurement of serine proteinase-inhibitory activity, 0.3 ml of the proteinase inhibitor from leaves were incubated with 0.4 ml of 50 mM Tris-HCl buffer, pH 7.4, containing 500 mM NaCl and 0.5% PEG, and 0.1 ml of enzyme solution from leaves. After 10 min incubation the aasay was started by adding 0.2 ml of 2 mM S-2251 to the reaction mixture, which was incubated for 20 min at 37°C. The absorbance was read at 405 nm.

Protein determination

Protein was measured according to the method of Bradford (1976) using bovine immunoglobulin as a standard.

Mol. Wt. determination

Determination of mol. wt. by gel filtration was done by chromatography on Ultrogel AcA 44, using rabbit muscle phosphorylase a (1: mol. wt. 94,000), human albumin (2: mol. wt. 67,000), trypsin (3: mol. wt. 24,000) and sperm whale myoglobin (4: mol. wt. 17,200) as standards.

Profiles of pH dependence of proteinase activity

pH profiles of α-casein-hydrolyzing activity were made with the enzyme purified by affinity chromatography. The assay buffers were 50 mM citrate-phosphate buffer, pH 4.5 to 6.5, and 50 mM Tris-HCl buffer, pH 5.0 to 8.7. Each buffer system contained 150 mM NaCl and 0.05% PEG-6000. For determination of the pH dependence of α-casein degradation, the activity of proteinase was estimated spectrophotometrically (A280) from changes in TCA-soluble amino acid content after hydrolyzation (10h, 37°C) of 0.2 ml of 0.5% α-casein by 0.2 ml of enzyme solution and 0.6 ml of the buffer.

Results

Serine proteinase and its inhibitors were purified from *R. communis* leaves. A combination of ammonium sulphate precipitation, gel filtration on Ultrogel AcA 44 and affinity chromatography on aprotinin-amino-porous glass (PG) achieved a 2,950-fold purification (Table 1). Chromatography on Ultrogel AcA 44 separated the proteinase activity into major I and II and a minor (III) fractions (Fig. 1). After gel filtration, III-a and III-b were separated readily by affinity chromatography on aprotinin-PG (Fig. 2). III-b was not bound to a column equilibrated with 50 mM Tris-HCl buffer, pH 7.4. III-b had a pH optimum of 5.0, in the case of substrate S-2302. III-a was bound to the column and eluted from the column with 1.0 M NaCl. III-a activity was unaffected by $CaCl_2$ and $MgCl_2$ and inhibited with $ZnCl_2$ by 63%. DTT, EDTA and pepstatin had no effect. III-a activity was inhibited with DFP by 95%. Addition of 1 mM PMSF to the reaction mixture reduced III-a activity by 42%. III-a activity was inhibited by aprotinin and SBTI by 58 and 31%, respectively (Table 2). III-a had pH optima of 7.4 to 8.4,

Table 1 Purification of serine proteinase from *R. communis* leaves

Fraction	Volume ml	Proteinase activity Protein mg/ml	Units $\times 10^3$	units/mg of protein $\times 10^3$	Purification fold	Yield %
30–70% saturated ammonium sulfate	100	5.89	0.22	0.004	1	100
Ultrogel AcA 44	13	0.03	0.62	2.07	516.5	36
Affinity chromatography	3	0.01	1.18	11.8	2950.0	16

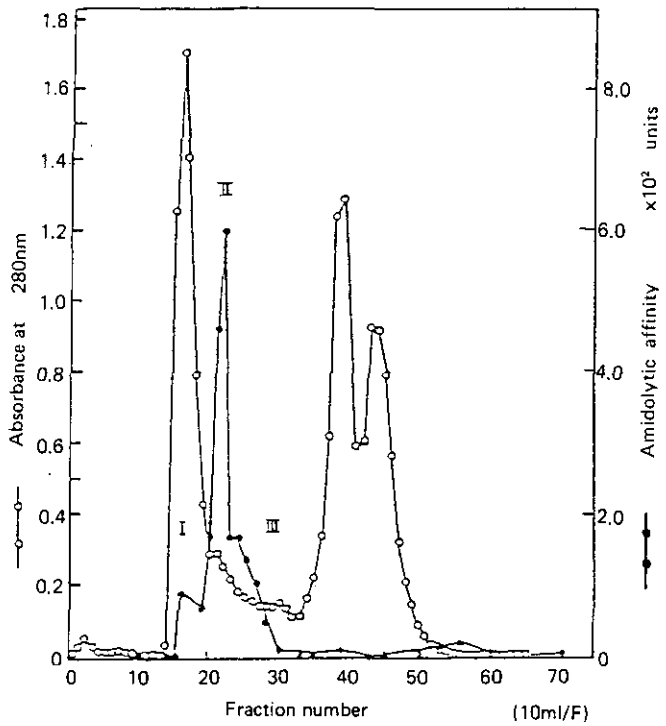


Fig. 1 Ultrogel AcA 44 column chromatography of proteinase from *R. communis* leaves (column size, 264 x 960 mm)

in the case of substrate S-2251 (Fig. 3).

Various artificial substrates were hydrolyzed by III-a (Table 3). Among tripeptide substrates for serine proteinase, Val-Leu-Lys-pNA was hydrolyzed at the highest rate by III-a. These results suggest that III-a may be a serine proteinase. The time course for hydrolysis by III-a of 0.35 to 3.5 mM Val-Leu-Lys-pNA was linear for 20 min (Fig. 4). The enzyme was unstable to heat and its activity was decreased by heating at 50°C for 15 min to almost a half (Table 4). The mol. wt. of III-a was 48,000 ± 2,000 as determined by gel filtration on Ultrogel AcA 44 (Fig. 5). The gel-filtrated fractions containing serine proteinase inhibitor were combined and fractionated on an affinity column consisting of trypsin covalently linked to

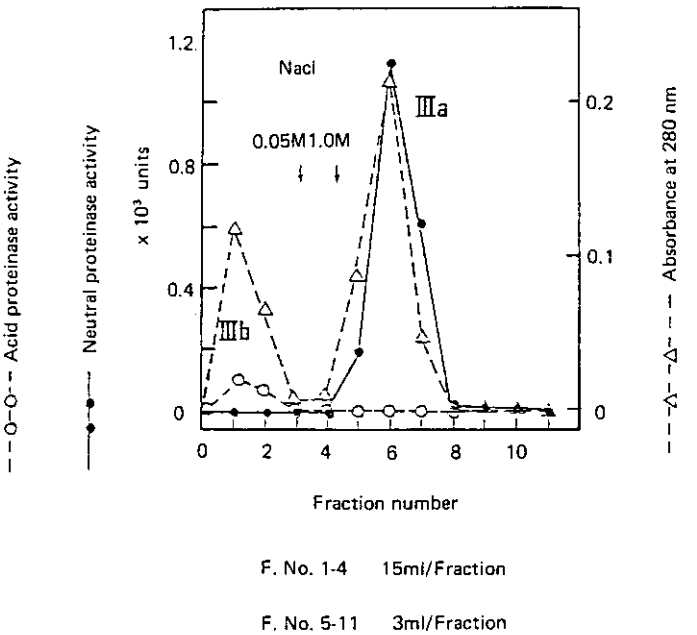


Fig. 2 Elution profile of the pooled Ultrogel AcA 44 fractions (III) on a column of immobilized aprotinin-amino-porous glass

Table 2 Influence of various chemicals on neutral proteinase activity

Treatment		Relative neutral proteinase activity (%)
None		100
$MgCl_2$	1 mM	101
$MnCl_2$	1 mM	80
$CaCl_2$	1 mM	97
$ZnCl_2$	1 mM	37
EDTA	1 mM	97
EGTA	1 mM	96
DTT	1 mM	104
PMSF	1 mM	58
DFP	1 mM	5
SBTI	100 μM	70
Aprotinin	100 μM	42
Leupeptin	1 μM	32
Pepstatin	10 μM	105
Urea	0.5 M	76
SDS	4.0%	0
PEG-6000	0.05%	121

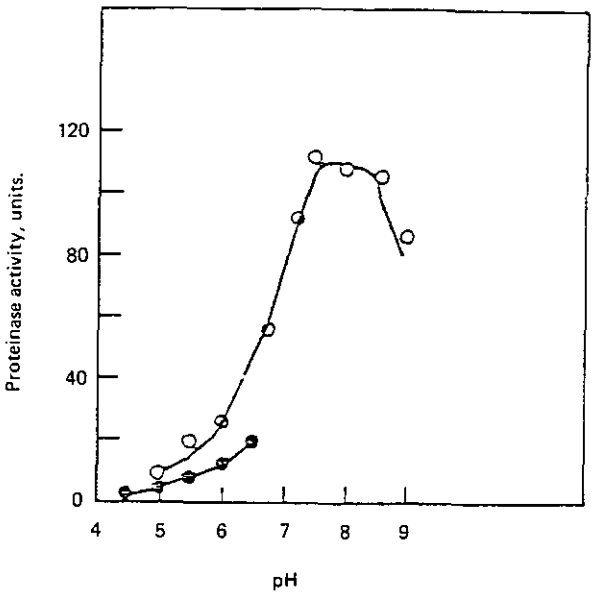


Fig. 3 pH profile for the hydrolysis of α-casein by purified proteinase III-a

Table 3 Substrate specificity of neutral proteinase from *R. communis* leaves

Substrate (3.0 mM)	Treatment of enzyme solution	Relative proteinase activity %
S-2266	none	36
S-2302	none	0
S-2444	none	28
S-2251	none	100
S-2251	80°C for 5 min	12
S-2251	65°C for 60 min	0
S-2251	50°C for 15 min	60
S-2251	45°C for 60 min	100

Proteinase activity was measured spectrophotometrically by means of the plasmin, glandulary kallikreins, plasma kallikrein and urokinase specific tripeptide substrate, 3.0 mM S-2251, S-2266, S-2302 and S-2444, respectively. (See Materials and Methods).

amino-porous glass. The elution pattern is shown in Fig. 6. The column was washed with the same buffer and then eluted with 10 mM HCl to remove the bound trypsin inhibitor. Table 4 shows that the activity of *R. communis* proteinase was inhibited by 50% by a 10-min preincubation with 0.02 μg/ml of proteinase inhibitor extracted from untreated leaves of *R. communis*.

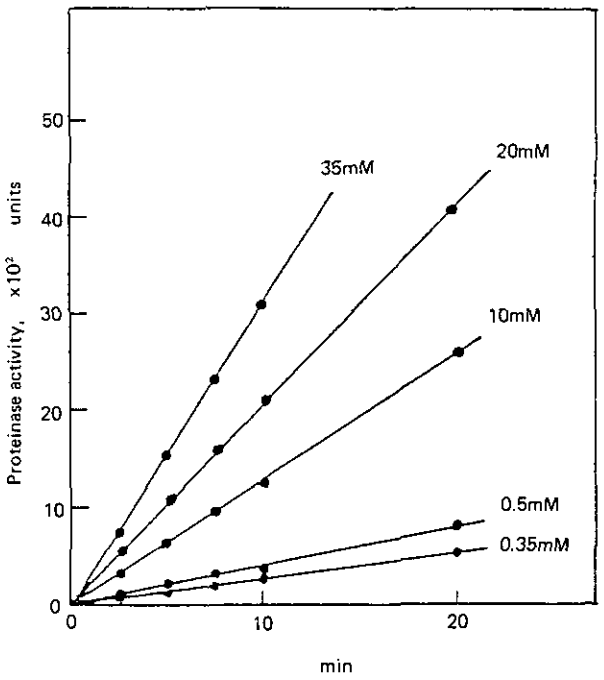


Fig. 4 Proteinase activities at different concentrations of S-2251

Table 4 Effect of proteinase inhibitor on serine proteinase from *R. communis*

Proteinase inhibitor	Proteinase activity units	Residual proteinase activity %
None	410	100
Proteinase inhibitor		
0.02 μg/ml	200	48.7
0.05 μg/ml	32	7.8

Discussion

In the leaves before the occurrence of visible damage caused by hydrogen peroxide, previous work has shown the presence of at least two proteinases (Watanabe & Kondo, 1983). One of these enzymes was active at neutral pH and showed a marked increase in activity before the appearance of visible symptom. The other enzyme was active at acid pH values and its activity was increased more slowly by hydrogen peroxide. The previous work has shown the presence of several kinds of neutral, alkaline and acid proteinases in leaves. Neutral serine

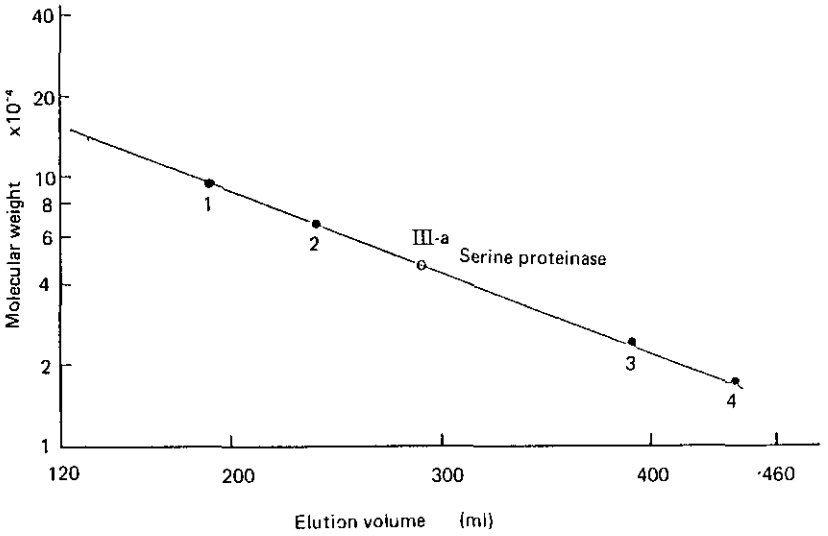
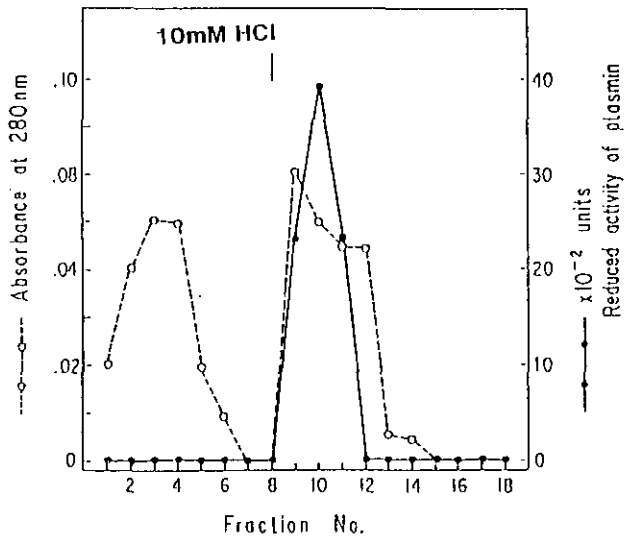


Fig. 5 Molecular weight determination by rechromatography of purified serine proteinase on a Ultrogel AcA 44 column
Fractions of 10 ml were collected at a flow rate of 1 ml/2 min. (column size; 27.0 × 950 mm).



F. No. 1-7 15ml/Fraction

F. No. 8-18 3ml/Fraction

Fig. 6 Elution profile of serine proteinase inhibitor fractions on a column of immobilized-trypsin-amino-porous glass
The fractions on Ultrogel AcA 44 column with the inhibitory activity were pooled and used.

proteinase may play a role of regulator in the protein metabolism. A serine proteinase inhibitor was purified from 7-week-old healthy leaves. This proteinaceous inhibitor was inhibited by hydrogen peroxide *in vivo* and *in vitro* (Watanabe, 1982; Watanabe & Kondo, 1983). Protein mobilization or senescence in leaves may be dependent on the increase in neutral endopeptidase (Chrispeels & Boulter, 1975) or neutral proteinase (Watanabe & Kondo, 1983) activities in the tissue. Whether this increase in enzyme activity is due to *de novo* synthesis of protein, the activation of inactive proenzyme, or the disappearance or inactivation of inhibitors remains to be demonstrated. Since healthy leaves of *R. communis* are a rich source of serine proteinase inhibitors, we decided to study the last possibility (Watanabe, 1982).

Relatively little is known about the role of plant proteinase inhibitors in the regulation of protein metabolism in plants (Ryan, 1973; Baumgartner & Chrispeels, 1976; Salmia & Mikola, 1980). The serine proteinase inhibitor had an approximate mol. wt. of about 10,000 daltons (unpublished data) and completely inhibited the activity of the proteinase if added in sufficient excess. Increases in proteolytic activity was found in leaves before visible injury has been observed in the hydrogen peroxide-treated leaves of *R. communis* and *S. oleracea* (Watanabe & Kondo, 1983). This increase in proteolytic activity was associated with a decrease in proteinase inhibitory activity. Increase in proteolytic activity during germination has been observed in the cotyledons of a variety of legumes. This increase in proteolytic activity was also associated with a decrease in trypsin-inhibitory activity (Pusziai, 1972; Hobday *et al.*, 1973), but there is no evidence that these two events are causally related. Royer *et al.* (1974) showed that the removal of trypsin inhibitor from extracts of cowpea cotyledons increased the caseolytic activity of these extracts, without affecting the amidase activity. Shain and Mayer (1968) measured proteinase activity and proteinase inhibitors in germinating lettuce seeds. They observed a 50-fold increase in trypsin-like activity during the 1st 3 days of germination and a complete disappearance of the trypsin-inhibitory activity during the 1st day of germination. The increase in enzyme activity may be causally related to the decrease in inhibitory activity.

The *in vivo* kinetics of the decrease in inhibitory activity and of enhancement of enzyme activity and the subcellular localization of the inhibitory activity are not clear. Proteinases and their inhibitors have been studied in a large number of microorganisms (Holzer *et al.*, 1975) and have often been found to be located in separate compartments: the enzymes in vacuoles or lysosomes, and the inhibitors in the cytosol. It has been suggested that the role of these inhibitors is to protect the cytoplasm against the accidental rupture (Watanabe, 1982) of the proteinase-containing vesicles. This may also be the function of the proteinase inhibitors in *R. communis* leaves (Watanabe, 1982). Further investigations to clarify these mechanisms are now in progress.

Acknowledgments

We wish to thank Dr. T. Fujii of University of Tsukuba for his helpful discussions and advice and Dr. K. Sugahara of the National Institute for Environmental Studies (NIES) for his encouragement and support. The authors are indebted to the members in the Engineering Division of NIES for their adept cultivation and provision of plants.

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大気汚染物質によるプロティナーゼと プロティナーゼインヒビター活性の変化 Ⅱ　トウゴマ葉のセリンプロティナーゼ とインヒビターの性質

渡辺恒雄[1,2]・近藤矩朗[1]

過酸化水素によって活性化されるプロティナーゼのうち，セリンプロティナーゼに関して得られた結果について報告する。過酸化水素水処理したトウゴマ葉から得られたプロティナーゼをセリンプロティナーゼインヒビターであるアプロチニンをアミノ化多孔質ガラスに結合させたカラムを用い，アフィニティクロマトで精製した。分子量約48,000±2,000で，アプロチニン，ロイペプチン，DFP，PMSF で阻害されペプスタチンで阻害されないことから，このプロティナーゼはセリンプロティナーゼであろうと推定した。

過酸化水素水処理したトウゴマ葉のプロティナーゼ活性はトウゴマ葉から得られたセリンプロティナーゼインヒビターによって阻害された。この結果もこのプロティナーゼがセリンプロティナーゼである可能性が示唆している。

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（改　称）

国立公害研究所研究報告

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第 13 号　Studies on chironomid midges of the Tama River. (1980)
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Part 2. Description of 20 species of Chironominae recovered from a tributary.
（多摩川に発生するユスリカの研究
― 第1報　その一支流に見出されたユスリカ各種の分布と下水による汚染度との関係 ―
― 第2報　その一支流に見出された Chironominae 亜科の20種について ―）

第 14 号　有機廃棄物，合成有機化合物，重金属等の土壌生態系に及ぼす影響と浄化に関する研究 ― 昭和53，54年度　特別研究報告．（1980）

※第 15 号　大気汚染物質の単一および複合汚染の生体に対する影響に関する実験的研究 ― 昭和54年度　特別研究報告．（1980）

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第 18 号　Preparation, analysis and certification of PEPPERBUSH standard reference material. (1980)
（環境標準試料「リョウブ」の調製，分析および保証値）

※第 19 号　陸水域の富栄養化に関する総合研究（Ⅲ）── 霞ヶ浦（西浦）の湖流 ── 昭和53，54年度．（1981）

第 20 号　陸水域の富栄養化に関する総合研究（Ⅳ）── 霞ヶ浦流域の地形，気象水文特性およびその湖水環境に及ぼす影響 ── 昭和53，54年度．（1981）

第 21 号　陸水域の富栄養化に関する総合研究（Ⅴ）── 霞ヶ浦流入河川の流出負荷量変化とその評価 ── 昭和53，54年度．（1981）

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第 29 号　Studies on chironomid midges of the Tama River. (1981)
Part 3. Species of the subfamily Orthocladiinae recorded at the summer survey and their distribution in relation to the pollution with sewage waters.
Part 4. Chironomidae recorded at a winter survey.
（多摩川に発生するユスリカ類の研究
── 第３報　夏期の調査で見出されたエリユスリカ亜科 Orthocladiinae 各種の記載と，その分布の下水汚染度との関係について ──
── 第４報　南浅川の冬期の調査で見出された各種の分布と記載 ──）

※第 30 号　海域における富栄養化と赤潮の発生機構に関する基礎的研究 ── 昭和54，55年度　特別研究報告．（1982）

第 31 号　大気汚染物質の単一および複合汚染の生体に対する影響に関する実験的研究 ── 昭和55年度　特別研究報告．（1981）

第 32 号　スモッグチャンバーによる炭化水素－窒素酸化物系光化学反応の研究 ── 環境大気中における光化学二次汚染物質生成機構の研究（フィールド研究１）── 昭和54年度　特別研究報告．（1982）

第 33 号　臨海地域の気象特性と大気拡散現象の研究 ── 大気運動と大気拡散過程のシミュレーション ── 昭和55年度　特別研究報告．（1982）

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（環境標準試料「池底質」の調製，分析及び保証値）

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第 40 号　大気汚染物質の単一及び複合汚染の生体に対する影響に関する実験的研究 ── 昭和56年度　特別研究報告．（1983）

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※第 42 号　底泥の物性及び流送特性に関する実験的研究．（1983）

※第 43 号　Studies on chironomid midges of the Tama River. (1983)

Part 5. An observation on the distribution of Chironominae along the main stream in June with description of 15 new species.

Part 6. Description of species of the subfamily Orthocladiinae recovered from the main stream in the June survey.

Part 7. Additional species collected in winter from the main stream.

（多摩川に発生するユスリカ類の研究

── 第５報　本流に発生するユスリカ類の分布に関する６月の調査成績とユスリカ亜科に属する15新種等の記録 ──

── 第６報　多摩本流より６月に採集されたエリユスリカ亜科の各種について ──

── 第７報　多摩本流より３月に採集されたユスリカ科の各種について ──）

第 44 号　スモッグチャンバーによる炭化水素－窒素酸化物系光化学反応の研究　── 環境大気中における光化学二次汚染物質生成機構の研究（フィールド研究２）── 昭和54年度　特別研究中間報告．（1984）

第 45 号　有機廃棄物，合成有機化合物，重金属等の土壌生態系に及ぼす影響と浄化に関する研究 ── 昭和53年～55年度　特別研究報告．（1983）

第 46 号　有機廃棄物，合成有機化合物，重金属等の土壌生態系に及ぼす影響と浄化に関する研究 ── 昭和54，55年度　特別研究報告　第１分冊．（1983）

第 47 号　有機廃棄物，合成有機化合物，重金属等の土壌生態系に及ぼす影響と浄化に関する研究 ── 昭和54，55年度　特別研究報告　第２分冊．（1983）

第 48 号　水質観測点の適正配置に関するシステム解析．（1983）

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第 50 号　陸水域の富栄養化防止に関する総合研究（Ⅰ）── 霞ヶ浦の流入負荷量の算定と評価 ── 昭和55～57年度　特別研究報告．（1984）

第 51 号　陸水域の富栄養化防止に関する総合研究（Ⅱ）── 霞ヶ浦の湖内物質循環とそれを支配する因子 ── 昭和55～57年度　特別研究報告．（1984）

第 52 号　陸水域の富栄養化防止に関する総合研究（Ⅲ）── 霞ヶ浦高浜入における隔離水界を利用した富栄養化防止手法の研究 ── 昭和55～57年度　特別研究報告．（1984）

第 53 号　陸水域の富栄養化防止に関する総合研究（Ⅳ）── 霞ヶ浦の魚類及び甲殻類現存量の季節変化と富栄養化 ── 昭和55～57年度　特別研究報告．（1984）

第 54 号　陸水域の富栄養化防止に関する総合研究（Ⅴ）── 霞ヶ浦の富栄養化現象のモデル化 ── 昭和55～57年度　特別研究報告．（1984）

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RESEARCH REPORT FROM
THE NATIONAL INSTITUTE FOR ENVIRONMENTAL STUDIES, JAPAN

No. 65
国立公害研究所研究報告　第65号
(R—65 —'84)

昭和59年12月20日発行

編集　国立公害研究所編集委員会
発行　環境庁国立公害研究所
茨城県筑波郡谷田部町小野川16番2

印刷　株式会社イセブ
茨城県新治郡桜村天久保2—11—20

Published by the National Institute for Environmental Studies
Yatabe-machi, Tsukuba, Ibaraki 305, Japan.
December 1984