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Effect of O_3 and NO_2 on Net Photosynthesis, Transpiration and Accumulation of Nitrite in Sunflower Leaves

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Photosynthesis and transpiration rates were simultaneously measured in attached sunflower leaves (*Helianthus annuus* L. cv. Russian Mammoth) during exposure to NO_2 and O_3 to determine the effect of mixed gas on photosynthesis and the stomatal aperture.

The application of O_3 alone reduced both the net photosynthetic and transpiration rates. An analysis of the CO_2 diffusive resistances indicated that the main cause affecting photosynthesis reduction during O_3 exposure was not the internal gas phase of the leaf (rCO_2^{liq}) but rather the liquid phase or mesophyll diffusive resistance (rCO_2^{liq}), suggesting that there is a very concomitant relation between photosynthetic reduction and rCO_2^{liq}. The application of NO_2 alone caused a marked reduction of the net photosynthesis yet no significant reduction of transpiration, indicating that NO_2 affects the CO_2 fixation processes with no influence on the stomatal aperture. A greater reduction in the photosynthesis of sunflower plants was caused by the application of NO_2 alone as compared to a combination of NO_2 and O_3. NO_2 alone reduced the photosynthetic rate by 90%, whereas a mixture of NO_2 and O_3 reduced it by 50%.

Key words : gas mixture, nitrite, NO_2, O_3, sunflower

1. Introduction

Recently, increasing attention has been given to the effects of air pollutant mixtures on plants due to the presence of numerous gaseous and particulate compounds in the lower atmosphere. Despite numerous studies dealing with the singular effects of sulphur dioxide(SO_2), nitrogen dioxide (NO_2), and ozone(O_3), there are still insufficient reports on the combined effects of air pollutants on plant growth[6,11.13,25)]. The responses of plants to air pollutant mixtures are frequently categorized for convenience into three types: additive, greater than additive, and less than additive[7)]. Numerous studies have been conducted on visible injuries and growth reductions caused by phytotoxic air pollutant mixtures[8,20)]. Air pollutants are the cause of a number of different biochemical effects on exposed leaves, which often result in changes in the cellular structure. Angela *et al.*[1)] reported that fumigation with SO_2 or NO_2 led to swollen thylakoids and a reduction in the number of grana stacks, when compared with the control. Ambient concentrations of O_3 in many areas can alter the permeability of plant cell membranes, disrupt the metabolism, decrease the foliar chlorophyll and photosynthesis, change the photosynthate allocation, and suppress the growth and yield[12,15,17)]. Unfortunately, however, the results are often contradictory, probably due to the influence of various factors such as light, temperature, humidity, duration of exposure, and the concentration ratio of the air pollutants in the mixtures. The antagonistic effects of SO_2 towards O_3 were reported on by Ashmore and Oenal[3)] for six cultivars of barley, and indicated that leaf injury was higher after exposure to O_3 alone than after exposure to both pollutants. Treatment with a mixture of O_3 and SO_2 induced a higher stomatal resistance compared to a single action[5], thereby suggesting antagonistic effects, i.e. the pollutant flux into the leaves was reduced.

These reported results have focused on the effects of SO_2 and O_3 or NO_2 and SO_2 mixtures. Furukawa[9] indicated that the most ubiquitous air pollutants contaminated in the atmosphere of urban and suburban areas have changed from SO_2 into NO_2 and O_3. However, there is little information concerning the effects of NO_2 and O_3 mixtures on the photosynthesis of higher plants. The present paper demonstrates the possible effects of NO_2 and O_3 and their mixtures on net photosynthesis and transpiration, even though the concentration of NO_2 applied was relatively higher than that found in the field.

2. Materials and Methods

2.1. Plant materials

Sunflower seedlings (*Helianthus annuus* L. cv. Russian Mammoth) were grown at 25℃ with 70% relative humidity in a growth chamber. The plants were cultivated for 4 weeks in plastic pots (11 x 15 cm) filled with a mixture of vermiculite, perlit, and gravel (2:2:1, v/v/v). Each pot contained 5 g of Magamp-K and 15 g of magnesia lime. No additional nutrients were supplied to increase the susceptibility to NO_2[21].

2.2. Fumigation system

The plants were exposed to NO_2 and/or O_3 using an acrylic assimilation chamber (125 liter, cubic) which was placed in a controlled environment room (1.7 x 2.3 x 2.0 m). The field air was passed in succession through activated charcoal and a catalyst containing MnOx and CuO filters to remove any ambient air pollutants and then fed into the controlled environment room. This filtration system was able to remove O_3 and SO_2 almost perfectly, however a trace amount of NO_2 (below 5 nl l^{-1}) remained in the room. NO_2 gas from a compressed cylinder containing 2 ml l^{-1} NO_2 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 consisting of a chemiluminescent NO-NO_2-NOx analyzer (Thermo Electron, Model 14). Ozone was generated by a silent electrical discharge in dry oxygen and regulated by a system similar to that described for NO_2 equipped with a controlling system consisting of a chemiluminescent O_3 analyzer (Kimoto, Model 806). Recordings of the pollutant concentrations in the room indicated that on starting the fumigation, the concentration reached 90 % of the fixed level within 5 min. Pollutant concentrations were regulated within ± 1% of the desired levels.

2.3. Measurement of gas exchange

Fully expanded leaves were accomodated in the assimilation chamber. The stem was led through a port at the bottom of the chamber, so that the leaves were inside whereas the roots and pot were outside. Measurements was performed at 28 ± 0.5 °C with 75% relative humidity. Two small fans (10 cm diameter) placed on the inner wall stirred the chamber air. The air was continuously sucked by a pump through a suction pipe on the upper side of the chamber. The air flow rate was measured by a rotameter and was adjusted to 55l min^{-1}. The wind speed inside the chamber was 0.4 m s^{-1}. This magnitude of wind speed minimized the diffusive resistance of the boundary layer to water vapor and a CO_2 transfer. Using wet filter paper of a similar size and orientation to the leaves, the resistance of the boundary layer to water vapor transfer was determined as 0.06 to 0.1 s cm^{-1}.

The illumination system consisted of 24 metal halide lamps (Yoko Lamp, Toshiba, 400 W). The light was filtered through a heat-absorbing glass filter, which removed any radiation above 800 nm. The quantum flux density in the assimilation chamber was 500 mmol m^{-2} s^{-1}. The transpiration rate was determined by the gravimetric method using an electronic top-loading balance (Mettler, Model PL-3000). The transpirational water loss was continuously recorded using a thermal data acquisition system. The pots were enclosed in plastic bags to prevent the evaporation of water from the pot surfaces. The net photosynthetic rate was determined in an open circuit system by measuring the CO_2 concentrations in the inlet and outlet of the chamber using an infra-red CO_2 analyzer (Shimazu, Model URA-2S).

2.4. Estimation of diffusive resistance

The photosynthesis and transpiration in the assimilation chamber were measured simultaneously, and the diffusive resistances to the CO_2 transfer from the bulk air to the CO_2 fixation site were also determined. The resistances to CO_2 diffusion through the boundary layer and internal gas-phase of the leaf (rCO_2^{gas}) and from the surface of the mesophyll cells to the site of CO_2 fixation (rCO_2^{liq}) were calculated from the net photosynthesis (μmol m^{-2} s^{-1}) and transpiration (mol m^{-2} s^{-1}) according to the method developed by Gaastra[10]. The degree of stomatal aperture was designated as the stomatal diffusive conductance. The diffusion coefficient of CO_2 was related to that of water vapor, and a conversion factor of 1.56[18] was applied to convert the gas-phase diffusive resistance for the flux of water vapor into stomatal diffusive conductance.

2.5. Nitrite assay

The nitrite content was determined using a modified version of the method developed by Yoneyama *et al*[26]. After measuring the fresh weight of the leaves, they were frozen and stored at -80℃ until used. The frozen leaves were ground in a cold mortar with liquid nitrogen and then with a 0.1 M potassium phosphate buffer (pH 7.5). The homogenate was squeezed through a nylon cloth and the filtrate was centrifuged at 18,000 x g for 20 min. To 0.1 ml of the supernatant, 1.5 ml of 1.0% (w/v) sulfamide in 1.5 N HCl and 1.5 ml of 0.02% (w/v) N-(1-naphtyl) ethylenediamine dihydrochloride were added and the mixture was left for 20 min. Thereafter, the optical density was measured at 540 nm.

2.6. Statistical analysis

To test if there was a significant difference between the two pollutant combinations, F statistics were applied. If the difference was insignificant at a 95% confidence interval, the effect was designated as additive. In contrast, if the difference was significant at the same confidence interval, the effect was noted as greater or less than additive.

3. Results

3.1. Effect of O_3 on photosynthesis, transpiration, and diffusive resistance

Treatment for 2 h with 10 nmol O_3 l^{-1} caused no significant changes in either net photosynthesis or transpiration in the sunflower leaves (Figs. 1A and 1B). The exposure to 20 nmol O_3 l^{-1} resulted in a progressive decline of photosynthesis and transpiration during the exposure periods. A 2 h exposure to 20 nmol O_3 l^{-1} reduced the net rates of photosynthesis and transpiration to 65 and 75 % of the pre-exposure rates, respectively (Figs. 1A and 1B). Fig. 1C shows the effect of O_3 on rCO_2^{gas} and rCO_2^{liq}. The net photosynthesis response to O_3 was largely reflected by changes in the rCO_2^{liq}.

If the inhibion of net photosynthesis resulted solely from the stomatal closure, the rCO_2^{liq} should remain constant during exposure to O_3. However, 20 nmol O_3 l^{-1} caused a gradual increase of rCO_2^{liq} immediately after exposure and finally the increase reached 1.5 times the initial value.

3.2. Effect of NO_2 on photosynthesis, transpiration, and diffusive resistance

Net photosynthesis was found to be more sensitive to NO_2 than transpiration (Figs. 2A and 2B). Exposure to NO_2 resulted in a rapid decline of the net photosynthetic rate. The inhibition of transpiration by NO_2 was much smaller than that of O_3. Exposure to 97 or 195 nmol of NO_2 for 2 h reduced the net photosynthetic rate by 20 and 90%, respectively, however, no significant reduction in transpiration was detected. Even when the net photosynthetic rate was reduced to 10% of the initial rate by exposure to 195 nmol of NO_2 l^{-1} for 2 h, no significant increase in rCO_2^{gas} was detected

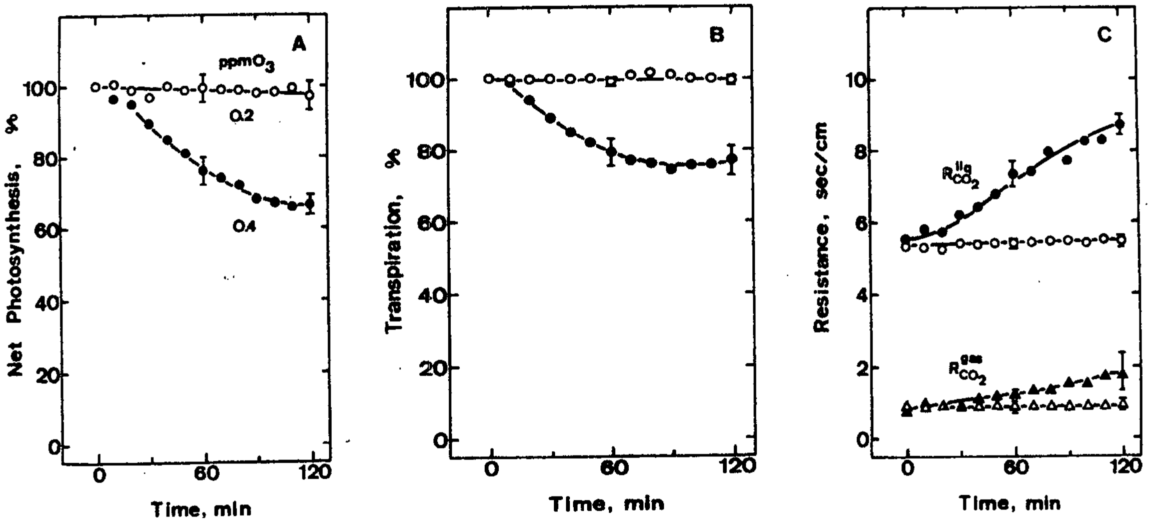

Fig. 1. Effect of O_3 on net photosynthesis(A), transpiration(B), and CO_2 diffusive resistances(C)(○△, 0.2; ●▲, 0.4) in sunflower leaves. Rates of net photosynthesis and transpiration are expressed as percentages of the pre-exposure rates. Gas-phase (rCO_2^{gas}) and liquid-phase (rCO_2^{gas}) diffusive resistances were estimated using the data of the net photosynthesis and transpiration rates. O_3 concentrations: 10 nmol l^{-1} (0.2, ○), 20 nmol l^{-1} (0.4, ●). Each point is the mean of at least three replicates. Error bars indicate representative standard deviations.

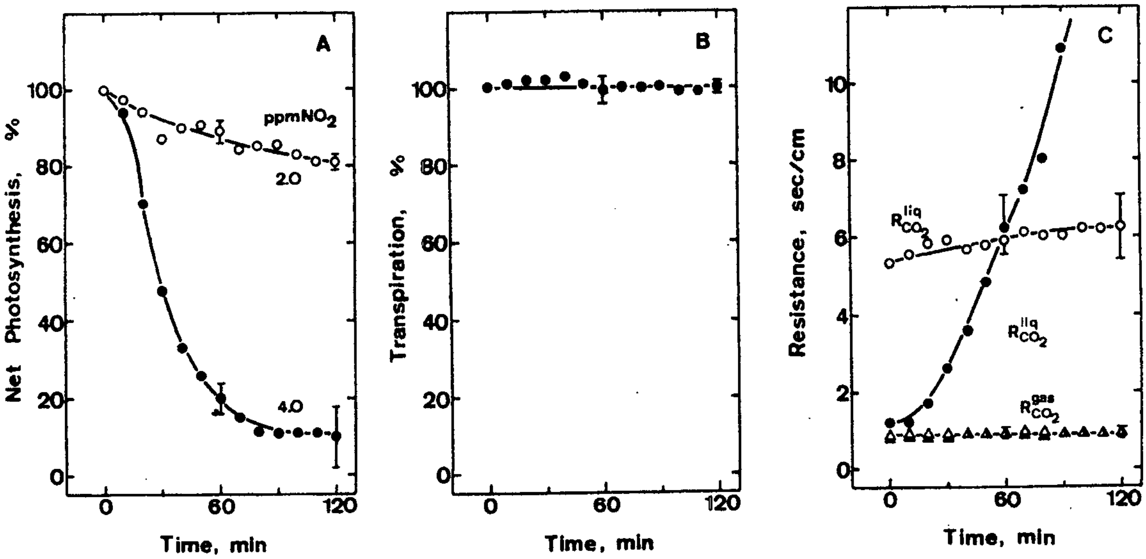

Fig. 2. Effect of NO_2 on net photosynthesis(A), transpiration(B), and CO_2 diffusive resistances(C)(○△, 2.0; ●▲, 4.0) in sunflower leaves. NO_2 concentrations: 97 nmol l^{-1} (2.0, ○), 195 nmol l^{-1} (4.0, ●). Each point is the mean of at least three replicates. Error bars indicate representative standard deviations.

(Fig. 2C). These results suggest that the threshold concentration required to cause stom-atal closure with a 2 h treatment of NO_2 isabove 195 nmol of NO_2 l^{-1} in sunflower leaves.

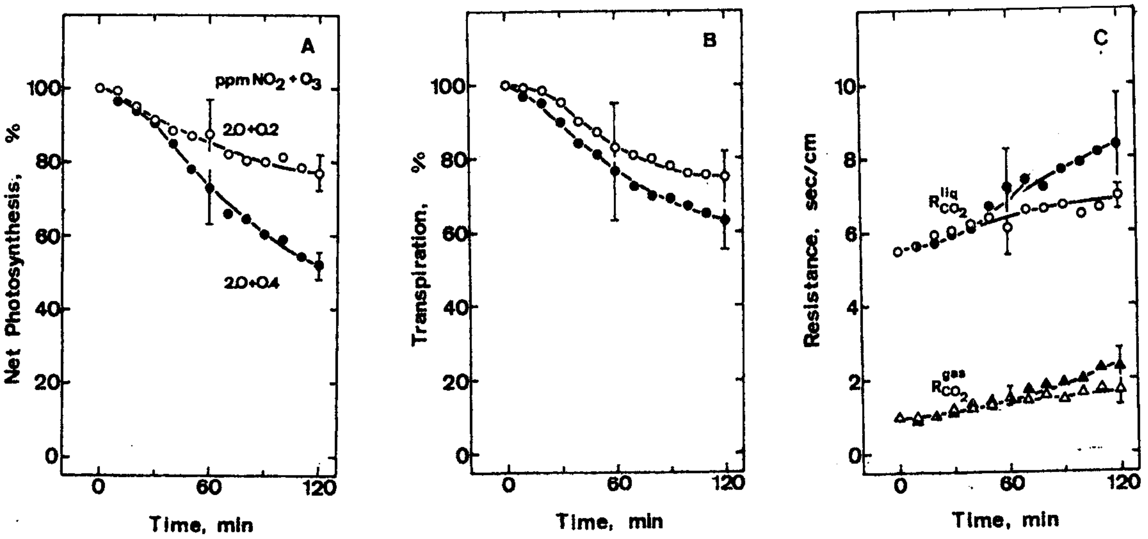

Fig. 3. Effect of O_3 and NO_2 mixtures on net photosynthesis(A), transpiration(B), and CO_2 diffusive resistances(C)(○△, 2.0+0.2; ●▲, 2.0+0.4) in sunflower leaves. The NO_2 concentration was 97 nmol l^{-1} and the O_3 concentration was 10 (2.0+0.2, ○) or 20 nmol l^{-1} (2.0+0.4, ●). Each point is the mean of at least three replicates. Error bars indicate representative standard deviations.

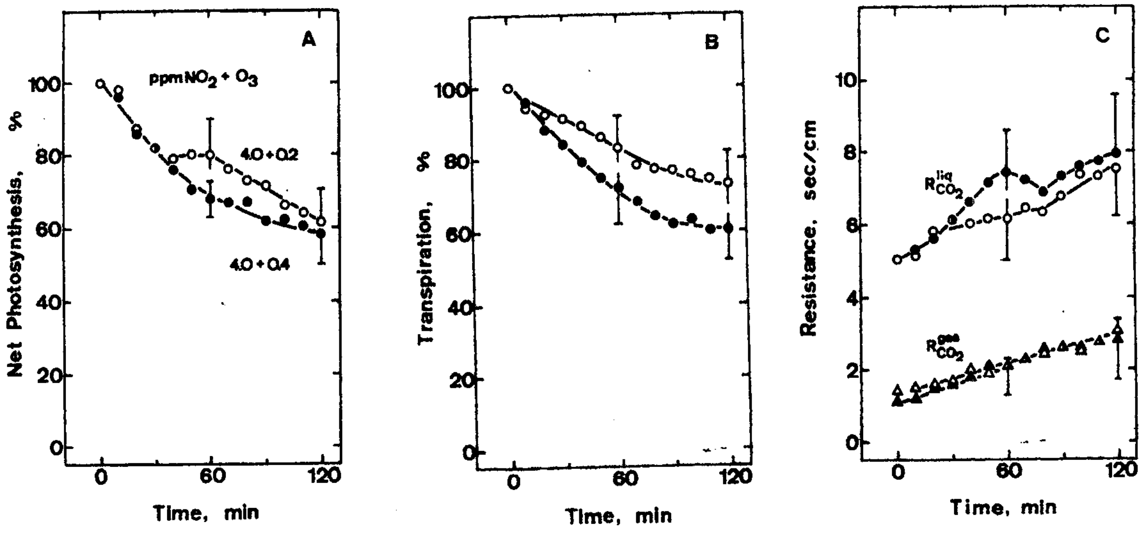

Fig. 4. Effect of O_3 and NO_2 mixtures on net photosynthesis(A), transpiration(B), and CO_2 diffusive resistances(C)(○△, 4.0+0.2; ●▲, 4.0+0.4) in sunflower leaves. The NO_2 concentration was 195 nmol l^{-1} and the O_3 concentration was 10 (4.0+0.2, ○) or 20 nmol l^{-1} (4.0+0.4, ●). Each point is the mean of at least three replicates. Error bars indicate representative standard deviations.

3.3. Effect of NO_2 and O_3 mixture on photosynthesis, transpiration, and diffusive resistance

The reduction of net photosynthesis induced by a mixture of NO_2 and O_3 was additive when the concentration of NO_2 in the mixture was low (Fig. 3A). Treatment with a mixture of 97 nmol of NO_2 l^{-1} plus 10 nmol of O_3 l^{-1} for 2 h reduced the net photosynthesis to 77 % of the initial rate, which was not significantly different from the inhibition caused by NO_2 or O_3 gas alone. Exposure to a mixture of 97 nmol of NO_2 l^{-1} plus 20 nmol of O_3 l^{-1} also caused an additive reduction of net photosynthesis. In contrast to these additive effects, when the concentration of NO_2 in the mixture was high enough (195 nmol of NO_2 l^{-1}) to almost perfectly inhibit net photosynthesis by NO_2 alone, the inhibition induced by 195 nmol of NO_2 l^{-1} plus 10 nmol of O_3 l^{-1} was less than additive (Fig. 3A). The behavior of transpiration differed considerably from that of photosynthesis during exposure to a mixture. A mixture of NO_2 and O_3 at any combination of concentrations of each gas produced a significantly greater reduction of transpiration than would be anticipated from summing the effect due to each gas alone (Figs. 3B and 4B). Since the reduction of transpiration and net photosynthesis occurred nearly simultaneously with the duration of the exposure, the degree to which net photosynthesis is affected by stomatal closure can be estimated. Figs. 3C and 4C show that rCO_2^{gas} and rCO_2^{liq} both increased just after the initiation of the mixed treatment. Exposure to a NO_2 and O_3 mixture caused a greater increase in rCO_2^{gas} than that caused by O_3 or NO_2 alone. The most significant increase in rCO_2^{gas} was observed when the leaves were treated with a mixture of 195 nmol of NO_2 l^{-1} plus 10 or 20 nmol of O_3 l^{-1}.

3.4. Accumulation of nitrite

To examine the nitrate accumulation in the NO_2-treated leaves, the nitrite content in the sunflower leaves treated with NO_2. was measured. Fig. 5 represents the relationship between the accumulated amount of nitrite and the relative rate of net photosynthesis. On a semilogarithmic semilogarithmic scale, a linear relationship between these two factors was observed in the NO_2-treated leaves.

4. Discussion

The present results suggest that the response of photosynthesis to a mixture of NO_2 and O_3 is quite different from that of transpiration. Bender *et al.*[6] have shown that the growth suppression of beans caused by O_3 alone did not occur when the plants were treated with NO_2, probably the stimulatory effect of NO_2 counteracts the negative effects of O_3. They indicated that the growth stimulation during vegetative growth by NO_2 and O_3+NO_2 was accompanied by corresponding changes in the plant nitrogen metabolism. Similar results were shown in the present study when the concentration of NO_2 was high enough to almost perfectly inhibit net photosynthesis (Fig. 2). The effect of a mixture was less than additive despite a significant inhibition of net photosynthesis by higher concentrations of O_3 (Fig. 3). When the concentration of NO_2 was low and the inhibition of net photosynthesis was moderate, the effect of a mixture was always additive, whether the concentration of O_3 was high or low. In contrast, the transpiration inhibition induced by a mixture was always greater than additive irrespective of the O_3 concentration. Even when the transpiration was reduced by half by 20 nmol of O_3 l^{-1} alone, the effect of a mixture was greater than additive. These results indicate that when the concentration of NO_2 is below the threshold to inhibit transpiration, the effect of a mixture is greater than additive even when treatment with O_3 inhibits transpiration.

The analysis of the CO_2 diffusion processes exhibited markedly different photosynthetic responses to individual and mixed gas treatments with NO_2 and O_3. The photosynthetic decline caused by NO_2 or O_3 alone was mainly attributed to the increase in rCO_2^{liq}, although rCO_2^{gas} increased slightly during treatment with O_3 alone (Figs. 1 and 2). In contrast, mixed gas treatments affected rCO_2^{gas} and rCO_2^{liq} simultaneously, suggesting that the contribution of the increase in rCO_2^{gas} to the decrease in net photosynthesis was comparable to that of rCO_2^{liq} in a mixed treatment (Figs. 3 and 4). The increase in rCO_2^{liq} reflects alterations in the available enzyme levels, since rCO_2^{liq} is influenced by enzymatic activity. Accordingly, these results indicate that both stomatal closure and photosynthesis inhibition occur concurrently during mixed treatments. This is also indicated by the observation that the increase in rCO_2^{gas} was roughly parallel to the increase in rCO_2^{liq} over the exposure period applied in the present experiment. These observations suggest that the less than additive inhibition of net photosynthesis induced by a NO_2 and O_3 mixture is caused by stomatal closure, resulting in the reduced incorporation of phytotoxic gas into leaves.

NO_2 has been shown to increase the nitrate reductase activity of conifer needles[23,24]. The flux of NO_2 into a spruce branch is linearly correlated with the stomatal conductance of the needles[23] and the nitrate reductase activity responds linearly to the uptake of NO_2-N[24]. According to a recently revised model[1,4], NO_2 is converted to NO_3- in the apoplast of the leaves and subsequently incorporated in amino acids and proteins[19]. Bender *et al.*[6] indicated that NO_2 treatments stimulate growth until anthesis, suggesting that plants are able to detoxify and utilize the additional nitrogen source.

The physiological data of Angela *et. al.*[2] from NO_2-exposed trees is not consistant with the current results. They reported that after NO_2-exposure at a concentration relevant to ambient conditions, the photosynthetic capacity increases together with a slight increase in pigmentation, however, the current results show a marked inhibition of photosynthesis. These results indicate that the effect of NO_2 fumigation depends greatly on the duration of the NO_2 exposure, NO_2 concentration, plant species, and ability of plant to detoxify NO_2. The linearlity between the accumulated amount of nitrite and the magnitude of the inhibition of net photosynthesis caused by the stomatal opening and absorption of NO_2 during treatment with NO_2 results in an increased nitrite concentration in leaves (Fig. 5). Yoneyama and Sasakawa[26] also identified a nitrite accumulation in NO_2-treated leaves. The exposure of plant leaves to NO_2 would seem to result in the leaves absorbing NO_2 through stomata and converting it into nitrite in the leaf tissues. Nitrite, which is accumulated in plant cells, can penetrate into chloroplasts and affect the photosynthetic processes. The inhibition of carbonic anhydrase by nitrite may be one of the mechanisms that inhibit CO_2 fixation, since CO_2 transfer conductance initially depends on carbonic anhydrase activity and this activity may be a limiting factor to photosynthesis under stress conditions[14,22]. Mohammad *et al.*[16] showed that a decreased carbonic anhydrase activity of about 18% can affect CO_2 diffusion towards carboxylation sites in the chloroplast. As a result, the CO_2 concentration in the chloroplast in the ribulose-1,5-bisphosphate carbosylase/oxygenase vicinity is reduced. Accordingly, further investigations are necessary to understand plant responses to the correlation between the accumulated amount of nitrite, stomatal opening, and photosynthesis.

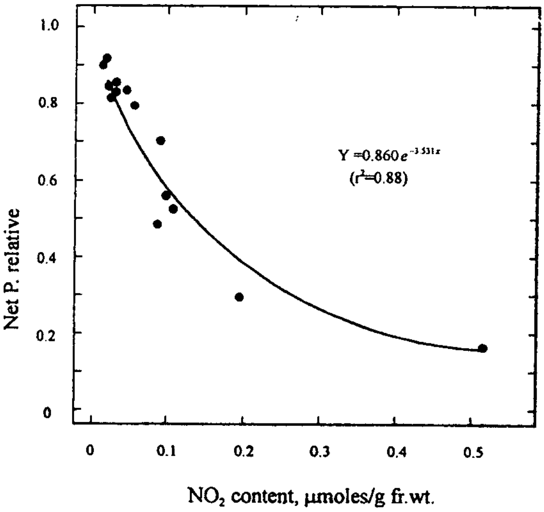

Fig. 5. Relationship between accumulated amount of nitrite in leaf and net photo- synthesis (Net P). 195 nmol NO_2 l^{-1} was treated for one hour then the accumulated amount of nitrite and Net P was measured in the leaves. The Net P shows the relative values to a control (1.0 means 100 %), which was not treated with NO_2.

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