Metabolic Basis for Injury to Plants from Combinations of O3 and SO2

STUDIES WITH MODIFIERS OF POLLUTANT TOXICITY

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ABSTRACT

Pisum sativum L. cv Alsweet (garden pea) and Lycopersicon esculentum Mill. flaccas (mutant tomato) were chosen to evaluate the metabolic basis for plant injury from combinations of O3 + SO2. The plants were exposed under conditions reported to specifically alter O3 or SO2 toxicity; light versus dark exposures, and treatment with the fungal metabolite fusococin (FC), the O3 injury inhibitor N-[2(2-oxo-1-imidazolidinyl)ethyl]-N'-phenylurea (EDU), and the SO2 injury stimulator diethylthiocarbamate (DDTC). Plants were grown in controlled environment chambers and exposed to combinations of O3 (0.05–0.2 micrometers per liter) and SO2 (0.1–0.3 micrometers per liter) for 2 hours. Peas treated with FC had the same greater injury (quantified by visual rating) with O3 + SO2 exposures compared to plants not treated with FC. For plants with open stomata in the dark as well as light, i.e. FC-treated peas and tomatoes, there was no change or an increase in foliar necrosis with O3 + SO2 exposures in the dark versus light. Peas treated with EDU had an almost complete absence of O3 injury, no change in SO2 injury, and moderate decreases in injury from combinations of O3 + SO2 compared to plants not treated with EDU. Tomatoes treated with DDTC showed the same or less injury compared to plants not treated with DDTC and exposed to O3 or SO2. The plant responses to the experimental treatments and O3 + SO2 resembled O3 responses more than SO2 responses. The evidence for O3-like responses are: no change or increase in injury in the light versus dark, and EDU-induced decreases in injury. Evidences for SO2-like responses are: incomplete protection from injury with EDU, and no change or increased injury to FC-treated versus untreated plants. Thus, a metabolic mechanism affected by both pollutants may be associated with the combination injury, e.g. effects the plasma membrane.

Combinations of the air pollutants O3 and SO2 can induce synergistic effects on foliar injury, growth, and yield in many plant species (10, 19). Apparently, these responses do not result from increased pollutant uptake. During combination exposures stomata generally close, decreasing gas-phase conductance (1, 5) and thus reducing the internal fluxes of O3 and SO2 to lower levels than for the single individual pollutants (6, 7). Consequently, within the leaf O3 and SO2 may be undergoing the following types of reactions to induce synergistic responses: O3 and SO2 may produce similar metabolites and the resultant injury primarily reflects the greater total amount of O3 + SO2 molecules within the leaf for the combined pollutants rather than each one alone; or the metabolites of one pollutant may increase the cellular sensitivity to the second pollutant (13). Evaluation of these alternative processes depends on the biochemical reactivity of O3 and SO2, and the interactions of their metabolites (13, 21).

SO2 toxicity involves either sulfite or free radicals formed during the photooxidation of sulfite to sulfate (25). Several lines of evidence support the involvement of sulfate in toxicity; (a) the greater toxicity of SO2 in the dark than light (17), which was associated with accumulation of sulfate (16); (b) the correlation between the rate of sulfate oxidation and intraspecific differences in SO2 injury (15); and (c) the greater toxicity of SO2 in plants treated with FC (18), which may increase plasma membrane permeability to SO2 metabolites (14). Evidence for the role of free radicals in toxicity is based on (a) the increased SO2 injury in plants treated with DDTC2, an inhibitor of SOD, an enzyme that decomposes free radicals (23), and (b) reports that chemicals that scavenge free radicals reduce SO2 or bisulfite-induced Chl destruction and leaf injury (20, 21).

Ozone toxicity involves either free radical metabolites and/or O3 molecules themselves (26). The association between decreased foliar injury and increased SOD activity in plants treated with EDU especially suggests involvement of free radicals (12). However, recent evidence indicates that the increased SOD activity may be a secondary event associated with the generation of free radicals following injury (4); this suggests that other nonradical metabolites are more important in determining O3 toxicity.

Thus, O3 + SO2 synergism could result from interaction of O3 and SO2 toxic metabolites, especially a metabolite common to both pollutants, i.e. free radicals, as suggested by Shuwen et al. (21). In the experiments discussed in this paper, we evaluated the metabolic basis for synergistic injury to plants from combinations of O3 or SO2 toxicity: light/dark exposures and chemical treatment with FC, DDTC, and EDU.

MATERIALS AND METHODS

Plant Culture. Peas (Pisum sativum L.) cv Alsweet and the tomato mutant flaca (Lycopersicon esculentum Mill. var. flaca) with permanently open stomata (11) were used in the studies as described by Olszyk and Tingey (17). Plants were grown from

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2 Abbreviations: DDTC, diethylthiocarbamate; EDU, N-[2(2-oxo-1-imidazolidinyl)ethyl]-N'-phenylurea; FC, fusicocin; PPFd, photosynthetic photon flux density; SOD, superoxide dismutase.
untreated seed in an artificial medium (Promix BX)³ and watered with North Carolina State University nutrient solution routinely checked for pH changes. Peas were cultured in controlled environmental chambers (Conviron model E15) at light/dark air temperatures and relative humidities of (20.7 ± 0.5 °C)/(19.8 ± 1.0°C) and (76 ± 14%)/(90 ± 14%), respectively, and PPFD of 301 ± 18 μmol m⁻² s⁻¹, 16 h/d. In foliar injury studies, the tomatoes were cultured in greenhouses flushed with charcoal-filtered air, for the first 14 d before transfer to the controlled environmental chambers with the same environmental conditions as the peas. Peas were exposed to pollutants when they had six fully expanded leaves at 19 to 21 d after seeding, and tomatoes when they had six to eight leaves greater than 20 mm in length at 26 to 30 d after seeding.

**Chemical Treatment.** All chemicals were sprayed on the plants until run-off using an air brush with nitrogen propellant. Fusco-cin (10 μm) was applied 24 h before exposure (17). The DDTC solution (12 mm) was prepared by dissolving Na-DDTC salt into 20 mm phosphate buffer solution (20 mm each of KH₂PO₄ and K₂HPO₄) adjusted to pH 7.8 with KOH followed by addition of two to three drops Triton X-100 surfactant per 50 ml of solution. The DDTC solution was applied 2 h before exposure. Initial tests using the concentration (120 mm) previously reported not to cause injury to plants (23), produced severe injury (about 80% leaf necrosis) in our studies. The EDU solution (4 mm) was prepared by dissolving EDU (50% wettable powder) in distilled H₂O and then adding two to three drops of Triton X-100. The EDU was applied to plants 24 h before exposure. Previous studies showed that this concentration protected plants from O₃ injury without the spray injuring plants (3). Control plants were sprayed with the same solutions minus the chemicals (FC, DDTC, or EDU).

**Pollutant Exposures.** For injury studies, plants were exposed in small exposure chambers (9) housed in growth chambers. Environmental conditions were the same as the pre-exposure plant growth conditions except that the air temperature was adjusted to maintain the same leaf temperature (measured with a fine wire thermocouple on the abaxial leaf surface) in the light and dark. SO₂ (1% in N₂) was metered into the chambers and monitored with fluorescent analyzers (Thermo Electron Series 43 and Monitor Labs model 8850). Ozone was generated by passing air over a UV light source and monitored with UV absorption analyzers (Dasibi model 1003AH). SO₂ and O₃ analyzers were calibrated on a daily basis with standards traceable to the National Bureau of Standards. The O₃ and SO₂ analyzers received periodic zero and span checks and standards were routinely audited to ensure accuracy of measurements.

The treatments used in the combination studies are shown in Table I. Plants were moved into the chambers approximately 4 h after initiation of the dark period or 1.5 h after initiation of the light period and equilibrated in the chambers for 1 to 2 h before and after each 2 h exposure. Light/dark exposures were made sequentially on the same date with plants of the same age from the same growth chamber. Corresponding control plants received identical treatment but were exposed to clean air.

Foliar injury (necrosis) on the adaxial surface was visually evaluated in 5% increments 3 d after exposure according to the procedure of Gumpertz et al. (8). Estimates of injury were averaged for all leaves present on the plant at the time of exposure.

**Gas Flux Measurements.** Fluxes of O₃ and SO₂ were measured using a whole-plant gas-exchange chamber (17, 24). SO₂ from a permeation tube, housed in a constant-temperature oven, was metered into the incoming air stream and monitored with flame photometric analyzers (Meloy models 160 and 185) equipped with H₂S and SO₂ (Meloy Sampleron model SS 20) scrubbers. O₃ generated by irradiating the incoming air stream with UV light was monitored with UV absorption analyzers (Dasibi model 1003AH). Both monitors were calibrated just prior to use with transfer standards. Pollutant concentrations were measured at the chamber inlet and outlet. The change in gas concentration, as it passed through the chamber, was corrected for the sorption of SO₂ and O₃ to the chamber surfaces determined under approximately the same conditions of light (320 μm m⁻² s⁻¹), air temperature (21°C), dewpoint (15.5°C), and CO₂ concentration (372 μl l⁻¹) as when a plant was present. The fluxes of SO₂ and O₃ were derived using an analog model (25, 26).

Flux measurements were made after a 2-h exposure to O₃ + SO₂ during hours 6 to 8 of the 8-dark period and hours 2 to 4 of the following 16-h light period. Each exposure was preceded by a 2-h acclimation period without pollutants in the chambers. The pollutant concentrations used were: 0.06 (tomato) or 0.11 (pea) μl l⁻¹ O₃, or 0.12 μl l⁻¹ SO₂ (both species).

**Experimental Design.** The injury studies were designed and analyzed according to analysis of variance procedures (22). Specific designs for the different treatments are given in Table I. For the gas-exchange studies, sequential dark and light measurements were taken from four single plants exposed on separate days, and analyzed using a paired t test design with four observations.

### RESULTS AND DISCUSSION

Plants exposed to combinations of O₃ + SO₂ responded most like O₃-exposed plants when treated with environmental conditions or chemical compounds previously reported to alter plant injury from O₃ or SO₂ alone (Table II). However, some similarities to SO₂ responses were found.

**FC Treatment.** Following exposure to O₃ + SO₂, peas treated with FC had similar or more foliar injury than untreated plants (Fig. 1A). This was similar to the increased injury from SO₂ alone for FC treated versus untreated plants, and in contrast to the lack of effect of FC on O₃ injury (Table II).

<table>
<thead>
<tr>
<th>Species</th>
<th>O₃</th>
<th>SO₂ Period</th>
<th>Chemical</th>
<th>Statistical Design</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pea</td>
<td>0.05</td>
<td>0.16 Light ± FC</td>
<td>Randomized block split plot with eight observations</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.12</td>
<td>0.11 Light ± FC</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.27</td>
<td>Light ± EDU</td>
<td>Randomized block split plot with eight observations</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.56</td>
<td>Light ± EDU</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.84</td>
<td>Light ± EDU</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.17</td>
<td>Light ± EDU</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.60</td>
<td>Light ± EDU</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.10</td>
<td>0.06 Light ± EDU</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.10</td>
<td>0.18 Light ± EDU</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.12</td>
<td>0.31 Light ± EDU</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tomato</td>
<td>0.20</td>
<td>Light ± DDTC</td>
<td>Randomized block split plot with twelve observations</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.65</td>
<td>Light ± DDTC</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.11</td>
<td>0.09 Light ± DDTC</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.12</td>
<td>0.21 Light Dark</td>
<td>Randomized block with 12 observations</td>
<td></td>
</tr>
</tbody>
</table>

³ Mention of trade names or commercial products does not constitute endorsement or recommendation for use.
Table II. Summary of Effects of Different Factors on Foliar Injury from O3, SO2, and O3 + SO2

Adapted from data reported in this paper and in Olzsyk and Tingey (17, 18).

<table>
<thead>
<tr>
<th>Factor</th>
<th>Effect on Foliar Injury from</th>
<th>O3</th>
<th>SO2</th>
<th>O3 + SO2</th>
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<tbody>
<tr>
<td>FC</td>
<td>none</td>
<td>increase</td>
<td>increase</td>
<td></td>
</tr>
<tr>
<td>EDU</td>
<td>decrease</td>
<td>none</td>
<td>decrease</td>
<td></td>
</tr>
<tr>
<td>Light</td>
<td>increase</td>
<td>decrease</td>
<td>increase</td>
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</table>

Table III. Total O3 and SO2 Flux in the Light and Dark for + and −FC Peas and Tomato

Values are averages of four observations measured at the end of 2-h exposures. Peas were exposed to 0.11 μl l⁻¹ O3 + 0.12 μl l⁻¹ SO2, tomato to 0.06 μl l⁻¹ O3 + 0.12 μl l⁻¹ SO2. For dark versus light and + versus −FC comparisons, * indicates a statistically significant difference at P < 0.05 level and one-tailed t test.

<table>
<thead>
<tr>
<th>Total Flux</th>
<th>Pea</th>
<th>Tomato</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dark Light</td>
<td>SE</td>
<td>SE</td>
</tr>
<tr>
<td>O3 (nmol m⁻² s⁻¹)</td>
<td>−FC</td>
<td>4.0</td>
</tr>
<tr>
<td></td>
<td>+FC</td>
<td>6.7</td>
</tr>
<tr>
<td></td>
<td>SE</td>
<td>1.1*</td>
</tr>
<tr>
<td>SO2 (nmol m⁻² s⁻¹)</td>
<td>−FC</td>
<td>3.9</td>
</tr>
<tr>
<td></td>
<td>+FC</td>
<td>5.7</td>
</tr>
<tr>
<td></td>
<td>SE</td>
<td>1.4 NS</td>
</tr>
</tbody>
</table>

In the light there was a large (5×) increase in injury to FC-treated compared to untreated plants exposed to a lower concentration of O3 (0.05 μl l⁻¹) plus SO2 (0.16 μl l⁻¹). There was no difference in injury between FC-treated and untreated plants exposed to a higher concentration of O3 (0.12 μl l⁻¹) plus SO2 (0.12 μl l⁻¹) in the light. In the dark, there was no difference in injury between FC-treated and untreated plants exposed to 0.05 μl l⁻¹ O3 + 0.16 μl l⁻¹ SO2. However, there was a large (7X) increase in injury to FC-treated versus untreated plants to 0.12 μl l⁻¹ O3 + 0.11 μl l⁻¹ SO2 in the dark.

Fluxes of O3 were significantly higher in −FC versus +FC peas in the light, and significantly lower in the dark (Table III). Fluxes of SO2 followed the same pattern as for O3; however, the differences in fluxes with FC treatment were not statistically significant. The effect of FC in the light was probably not caused by a difference in pollutant uptake since the fluxes of O3 or SO2 were actually lower in FC-treated than untreated plants. Thus, the results suggested that the increased O3 + SO2 injury with FC may be due to FC's effects on the metabolism of SO2 (e.g., an increase in sulfate transport across the plasma membrane and into cells [18]). In the dark, FC's effects on plant metabolism of pollutants could not be determined apart from FC's inducement of stomatal opening and coincident increase in pollutant flux.

Light/Dark Exposures. In general plants with open stomata, i.e. peas treated with FC (Fig. 1A) and tomato (Fig. 1B), had greater injury with exposures in the light versus dark. The only statistically significant light/dark effect was with FC-treated peas exposed to 0.05 μl l⁻¹ O3 + 0.16 μl l⁻¹ SO2. However, there was a trend toward greater light than dark for all exposures. This result was similar to the greater light than dark injury found with exposure to O3 alone (Table II). The O3 + SO2 light/dark response was opposite to the greater dark than light injury response with SO2 alone. This suggests that the detoxification of sulfate to sulfur in cells does not play a predominant role with O3 + SO2 combination exposures. There were no light versus dark differences in O3 or SO2 flux for either tomato or FC-treated peas (Table III).

Peas not treated with FC displayed approximately 7-fold more foliar injury than tomatoes when exposed in the light to similar concentrations of O3 plus SO2 (0.09–0.12 μl l⁻¹ of each pollutant) (Fig. 1A). However, the total flux of the two pollutants actually was greater in tomato (24.8 μmol m⁻² s⁻¹) than in pea (17.3 μmol m⁻² s⁻¹) under similar FC and light conditions (Table III), implying that peas were receiving a lower cellular exposure to the pollutants. This suggested that internal biochemical factors were associated with the greater toxicity of O3 + SO2 in pea than tomato.

EDU Treatment. The results of EDU treatment for O3 or SO2 exposures for peas were the same as in earlier studies on other species: a large decrease in O3 injury (Fig. 2A) but no consistent effect on SO2 injury compared to untreated plants (Fig. 2B). The EDU-treated plants had more injury than untreated plants at 1.17 μl l⁻¹ SO2 but less injury than untreated plants at 1.60 μl l⁻¹ SO2. Peas treated with EDU and exposed to O3 + SO2 exhibited

![Figure 1](https://academic.oup.com/plphys/article/77/4/935/6079795)

**Fig. 1.** Leaf injury to pea + and −FC (A) and tomato (B) plants exposed to O3 + SO2 for 2 h in the light and dark. Values are averages of 8 and 12 observations for pea and tomato, respectively. Standard errors for comparing light versus dark or + versus −FC values are given below pollutant concentrations. The + versus −FC difference in injury for the light for peas at 0.05 μl l⁻¹ O3 + 0.16 μl l⁻¹ SO2; the light versus dark differences for −FC peas at 0.12 μl l⁻¹ O3 + 0.11 μl l⁻¹ SO2 and +FC peas at 0.05 μl l⁻¹ O3 + 0.16 μl l⁻¹ SO2 are significant at P < 0.05 level.
60 to 93% less injury than untreated plants (Fig. 2C). In general, O3 + SO2 EDU response was the same as for O3 alone; however, the extent of injury prevention induced by EDU treatment was lower as the concentration of SO2 in the combination increased from 0.06 to 0.18 and 0.31 μl l\(^{-1}\).

**DDTC Treatment.** Tomato plants treated with DDTC and exposed to SO2 in the light or dark showed no statistically significant differences in injury compared to untreated plants (Fig. 3). There was no evidence for increased SO2 injury with DDTC treatment, as reported earlier (23). O3 injury in the light apparently was decreased in DDTC treated versus untreated plants. However, no statistical analysis of the effect could be made as the DDTC-treated and untreated plants were exposed to O3 on different days. The DDTC treatment had no effect on O3 injury in the dark. DDTC itself caused a small amount (<5% of area) of necrosis and induced epinasty, especially on the expanding leaves.

The lack of DDTC injury enhancement with either pollutant may be attributed to one of several metabolic causes, e.g., the SOD in tomato is different from the DDTC-sensitive SOD in spinach, and/or the free radicals produced by O3 and/or SO2 are not affected by the SOD induced by DDTC in these exposures. Lack of penetration of DDTC to the pollutant-active sites within leaves was not a problem; DDTC apparently did enter leaves and reach metabolically active sites, resulting in epinasty within several hours of treatment. Because of the lack of definite O3 or SO2 alone effects with DDTC, no O3 + SO2 exposures were conducted.

**Mode of Action for O3 + SO2 Injury.** These results suggest that the mode of action for O3 predominates in O3 + SO2 exposures. Initial steps in O3 toxicity are believed to involve interactions between the plasma membrane, and either O3 molecules themselves or oxyradicals produced as decomposition products of O3 in the gas-liquid interface surrounding cells (17, 26). Combination exposures with SO2 could produce increased injury from an increased plasma membrane load of oxyradicals produced during SO2 metabolism. An O3-dominated mode of action for O3 + SO2 responses would account for the O3 injury like diffuse necrotic symptoms found on many plants exposed to combinations of O3 + SO2 (13). SO2 also could be increasing O3 toxicity solely due to concurrent effects on the plasma membrane. However, direct interactions between SO2 and the plasma membrane have not been reported.

Plasma membrane permeability has in fact been shown to be specifically affected with O3 + SO2 compared to O3 or SO2 alone exposures, as indicated by increased total electrolyte leakage (2). Further research is required to evaluate changes in actual membrane constituents with exposure to O3 + SO2.

The plant responses reported here are for acute simultaneous exposures of only two species; other physiological mechanisms may be involved with other exposure conditions. Chronic injury and growth effects with long term exposure to O3 + SO2 may involve free radicals to an extent not present with acute exposures. For example, in chronic exposures SO2 increased SOD activity in young plants, which is indicative of increased free radical content (23).

In conclusion, exposure to O3 + SO2 combinations with chemical treatments and under light/dark conditions produced foliar injury responses primarily characteristic of O3, but with some evidence of SO2 response. This suggested that metabolites of both O3 and SO2 may be involved with synergism, and that one type...
of metabolite produced by both pollutants is not solely responsible. Additional experiments are needed to further define the effects of O₃ and SO₂ on the plasma membrane, a likely site of joint action. Studies also are needed to clarify the role of SOD and DDTC in plant detoxification of O₃ and SO₂ metabolites.

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