Plant Physiol. (1984) 76, 823–826
0032-0889/84/76/0823/04/$01.00/0

*Short Communication*

# Injury, Stomatal Conductance, and Abscisic Acid Levels of Pea Plants following Ozone plus Sulfur Dioxide Exposures at Different Times of the Day[1]

Received for publication February 15, 1984 and in revised form July 16, 1984

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## ABSTRACT

**Pea (*Pisum sativum* L. cv Alsweet) plants were exposed to mixtures of ozone plus sulfur dioxide at different times of the day. Injury, evaluated either as necrosis or chlorophyll, was greatest at midday when stomatal conductance was greatest. Abscisic acid levels were similar over the day, and showed no relation to stomatal conductance.**

Investigations of pollutant exposure at different times of the day have been undertaken with $O_3$ and $SO_2$ singly (5). As early as 1949, Katz (10) showed that barley and alfalfa grown in fields were less sensitive to $SO_2$ early and late than during the middle of the day. Such variations in diurnal injury responses to single pollutants have been confirmed in controlled environments with other crops (7, 22), including peas (18). No diurnal studies with mixtures of pollutants were found.

The reduced injury early and late in the day has been related closely to stomatal response during the period of exposure (18). Plants exposed early and late in the day exhibited greater stomatal closure than plants exposed at midday (18).

Mechanisms regulating the changes in stomatal conductance of plants exposed to pollutants at different times of the day are not understood. Olszyk and Tibbitts (18) suggested that ABA might be involved in controlling stomatal responses. This suggestion is supported by the fact that when ABA content of leaves was increased by foliar applications or by water stress, stomata closed, resulting in decreased $O_3$ uptake and hence less injury to plants (4, 9). Also, ABA concentrations were higher in $SO_2$-resistant plants which had greater stomatal closure during exposure than $SO_2$-sensitive plants (12, 13). Thus, ABA may have a role in controlling stomatal responses to pollutants. However, it is not known whether variations in ABA levels over the day are responsible for different stomatal responses, or whether pollutants may alter ABA levels which in turn produce particular stomatal responses. Fluctuations in ABA concentrations of plants at different times of the day have been reported, although patterns over the day have not been consistent (3, 8, 14, 17, 20, 23). No reported studies of fluctuations in ABA concentrations of plants exposed to pollutants at different times of the day were found.

Plants were exposed for 2 h to a mixture of $O_3$ plus $SO_2$, two pollutants commonly occurring together in the ambient environment. Exposures were undertaken for 2 h to simulate the length of ground level $SO_2$ exposures around coal-fired electrical generating stations (21). To evaluate the physiological basis for any diurnal variation in plant sensitivity to air pollutants, injury, stomatal conductance, and ABA concentrations of leaves were measured.

## MATERIALS AND METHODS

**Plant Culture.** Pea (*Pisum sativum* L. cv Alsweet) seeds treated with Captan were planted 1.25 cm deep in peat-vermiculite medium (W. R. Grace and Co.) in 10-cm plastic pots. Plants were grown from seed in a controlled environment room in the Biotron at the University of Wisconsin. Cool-white fluorescent plus incandescent lamps for 16 h/d provided a photosynthetic photon flux density (PPFD) of 276 ± 18 $\mu$mol $m^{-2}$ $s^{-1}$ ($\mu$E $m^{-2}$ $s^{-1}$) as measured with a Lambda quantum sensor. Temperature was 20.4 ± 1.1°C as measured with thermocouples. RH was 75 ± 3% as measured with an aspirated thermocouple psychrometer. Plants were watered to excess four times/d with approximately 35 ml of the ASHS baseline nutrient solution (6).

**Plant Exposure.** Plants were exposed 13 d after seeding when the fourth trifoliolate leaf was in the cup phase. Pollutant exposure and control treatments were in 0.3 $m^3$ Plexiglas chambers located in a room adjacent to the growing room. Environmental conditions were the same as in the growing room. Air was drawn from the room and passed through a dry $KMnO_4$ (Purafil) filter before entering the chambers. Air exchanges in chambers were nine/min. $CO_2$ was maintained at 325 to 350 $\mu$l/l. $CO_2$ buildup from humans was prevented by wearing a mask. Significant $CO_2$ depletion by plants was prevented by the rapid air exchange rate and the small amount of plant material in the chambers.

$O_3$ was provided by passing air over UV lamps and monitored with a Monitor Labs model 8410 chemiluminescent $O_3$ analyzer. $SO_2$ was provided from cylinders containing 0.1% $SO_2$ in $N_2$ and monitored with a ThermoElectron model 43 pulsed fluorescent $SO_2$ analyzer. This addition of $N_2$ was negligible in proportion to the amount of $N_2$ in air entering the chambers. Both analyzers were calibrated with a Bendix 8861DA gas phase titration calibrator through use of a UV lamp for $O_3$ and an EPA-certified tank for $SO_2$. Pollutant concentrations were 0.10 ± 0.01 $\mu$l $l^{-1}$

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$O_3$ plus 0.60 ± 0.01 $\mu l\ l^{-1}$ $SO_2$ as measured in the center of the chamber at plant height. These concentrations of pollutants were chosen because they produced moderate injury within a range that could be measured at all times of the day.

Seven separate groups of plants were exposed to $O_3$ plus $SO_2$ for 2 h during sequential periods equally spaced over the 16-h light period. Five plants were placed into each chamber for each exposure. For the ABA determinations, exposures were made only at intervals of 0 to 2 h, 7 to 9 h, and 14 to 16 h during the light period, with 24 plants maintained in each chamber per period. Exposures in all studies were repeated on 2 d, except that ABA studies were repeated on 3 d.

**Stomatal Conductance Measurements.** Stomatal conductance was measured with a LiCor model LI-65 automatic diffusive resistance meter equipped with a horizontal sensor, and calibrated at 20°C. Measurements were made on upper and lower surfaces of the second trifoliolate leaves. Measurements were taken in the control chamber between 1 and 1.5 h, and in the exposure chamber between 1.5 and 2 h after the start of each 2-h exposure.

**ABA Measurements.** For ABA determinations, two samples of leaves were harvested from plants in each chamber (control and exposed) immediately at the end of each exposure period on each day. Each sample consisted of first and second trifoliolate leaf blades pooled from 6 or 7 plants to provide 2 g fresh weight of tissue. Samples were frozen on dry ice and stored at −27°C until extracted.

Leaf tissue was extracted for ABA according to the methods of Schussler *et al.* (19). Residue from extracted leaf samples was then stored at −20°C for approximately 6 months. In previous studies, no losses in ABA were found when stored under these conditions.

ABA in the residue was analyzed using reverse phase HPLC for preparative separation, and GLC for analytical quantification (19). During analytical GLC, methyl ABA was eluted at 14.3 min with a peak width at half height of 0.13 min.

Quantification of methyl ABA in the sample was determined with standard solutions containing known ABA concentrations. Radioactivity ($^{14}C$) in the remaining ABA sample was determined with a liquid scintillation spectrometer (Beckman 9000) and used to estimate per cent recovery of [$^{14}C$]ABA. Average recovery rate was 22%, and data were corrected accordingly.

**Injury Evaluation.** Injury was evaluated both as necrosis and as Chl concentration of the second trifoliolate leaf blade 7 d after pollutant exposures. Necrosis was estimated visually as per cent of leaf surface area showing necrosis. Chl was extracted with ethanol using the method of Knudson *et al.* (11).

**Statistical Analyses.** Data were analyzed using one-way analysis of variance followed by a Duncan's new multiple range test.

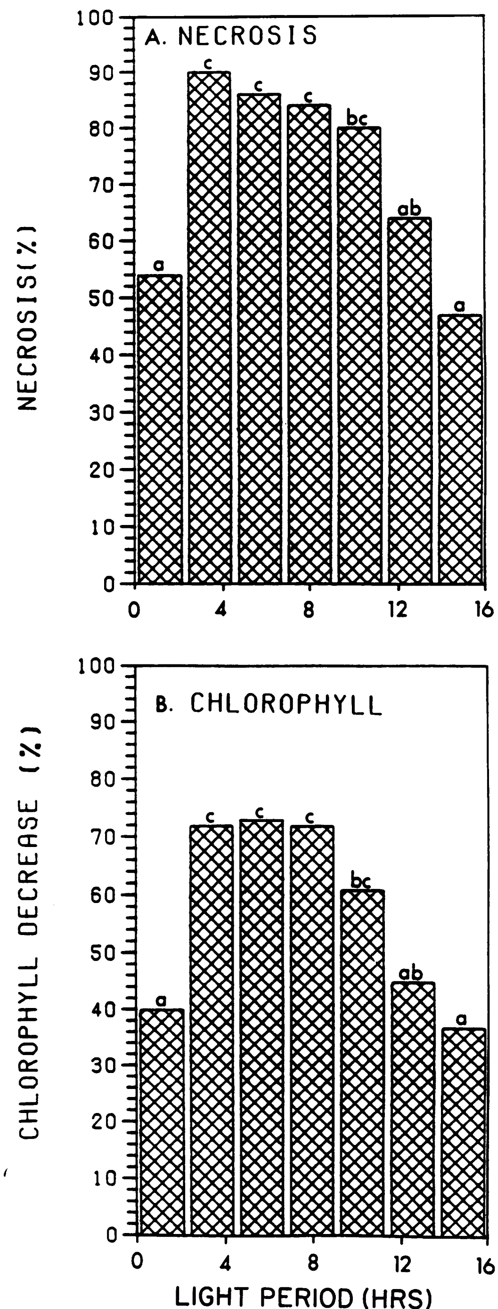


FIG. 1. Necrosis (A) and Chl decrease (B) of second trifoliolate leaf of peas exposed to $O_3$ (0.10 $\mu l\ l^{-1}$) plus $SO_2$ (0.60 $\mu l\ l^{-1}$) for 2 h at different times of the day. Chl decrease expressed as per cent from control. Chl concentration in control plants was 16.7 ± 0.4 mg $g^{-1}$ dry weight. Averages indicated by bars with different letters are significantly different at 1% level using Duncan's new multiple range test. Each average is from 10 observations.

## RESULTS AND DISCUSSION

Figure 1 shows the percentage injury measured as necrosis (A) and Chl decrease (B) to pea leaves on plants fumigated at different times of the day with the mixture of $O_3$ plus $SO_2$. Patterns of injury response over the day were similar whether expressed as necrosis or Chl decrease. Injury was low during the first 2 h of the light period, and greatest from approximately 2 to 9 h into the light period. Plants exposed after 9 h of light had decreasing injury until the end of the light period. Injury was similar during the first and last 2 h of the light period. The diurnal response to the mixture of $O_3$ plus $SO_2$ was similar to that reported for $O_3$ or $SO_2$ alone (7, 18, 22).

The reduced injury early and late in the day with $O_3$ plus $SO_2$ mixture was associated with greater stomatal closure particularly at the end of the day as shown by stomatal conductance measurements (Fig. 2). Conductance toward the end of each fumigation was 0.24 cm/s early in the light period (0–2 h), 0.35 cm/s during the middle of the light period, and only 0.15 cm/s late in the day (12–16 h). Conductances of control plants had a similar increase and decrease over the day, but the percentage differences were not as great as on the exposed plants. Conductances of control plants were statistically similar over the day, whereas those of exposed plants were not. These trends agree with previous studies undertaken with single pollutants where injury was greater at midday (7, 18, 22), and the greater injury was associated with higher stomatal conductance during exposure (18). However, stomatal conductance is apparently not the only controller

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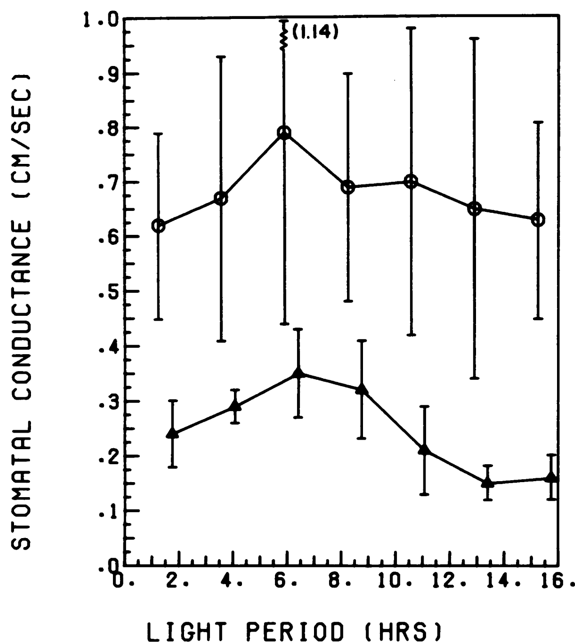


FIG. 2. Stomatal conductance (average of upper and lower surfaces) of second trifoliolate leaf of control plants (O) and plants exposed to 0.10 $\mu l\ l^{-1}$ $O_3$ plus 0.60 $\mu l\ l^{-1}$ $SO_2$ (Δ) at different times of the day. Conductance calculated as the inverse of the measured resistance values. Bars indicate standard deviation of averages. The large standard deviation of control plant averages results from the fact that the resistance measurements were very low and small variations made large variations in conductance.

Table I. *Effect of Exposures at Different Times of the Day with Ozone (0.10 $\mu l\ l^{-1}$) plus Sulfur Dioxide (0.60 $\mu l\ l^{-1}$) on ABA Concentration in First and Second Trifoliolate Leaves of Pea Plants*

The data are the average of six samples.

| Time after Start of Light Period | ABA | |
|---|---|---|
| | Control | Exposed to $O_3$ and $SO_2$ |
| *h* | *ng g⁻¹ fresh wt* | |
| 0–2 | 52.0 ± 5.8[a] | 55.3 ± 29.6[a] |
| 7–9 | 62.9 ± 24.4[a] | 56.6 ± 12.7[a] |
| 14–16 | 52.7 ± 12.2[a] | 54.1 ± 17.9[a] |

[a] Averages in a column followed by similar letters are not significantly different at the 5% level using Duncan's new multiple-range test.

of injury because conductances during the last two exposure periods (0.15 and 0.16 cm/s) were lower than during the first exposure period (0.24 cm/s), although injuries were similar. Perhaps a threshold conductance value exists, below which injury is the same, or perhaps other physiological or biochemical factors which vary over the day are also involved in determining injury.

Stomatal responses to pollutants did not relate to leaf ABA levels, for ABA did not decrease at midday when stomata had the least closure, nor did ABA levels increase with early and late exposures when stomates closed significantly (Table I). Instead, ABA concentrations were similar over the entire day and were not altered significantly by pollutant exposure. ABA in plants after the 2-h exposures to $0_3$ plus $SO_2$ was approximately 55 ng $g^{-1}$ fresh weight, and equal to the concentrations in control plants. ABA in control plants was somewhat higher near midday (7–9 h) than early (0–2 h) or late (14–16 h) in the day, but not significantly higher. This midday level in control plants was also not significantly different from the midday level of exposed plants, at the 5% confidence limit.

ABA concentrations were determined on fresh tissue which is a potential problem in data interpretation since per cent moisture of tissues varies over the light period. To evaluate this problem, an estimation of daily change in ABA concentrations on a dry weight basis was calculated with dry weight percentage data collected from plants grown in separate experiments under the same environmental conditions. Using this data, ABA concentrations of control plants in this experiment were calculated to be 508, 557, and 437 ng $g^{-1}$ dry weight compared to 52.0, 62.9, and 52.7 ng $g^{-1}$ fresh weight, at the sampling times of 2, 9, and 16 h, respectively. Thus, ABA in control plants tended to be higher at midday when expressed on both a fresh and a dry weight basis. ABA concentrations in exposed plants were calculated to be 544, 502, and 445 ng $g^{-1}$ dry weight compared to 55.3, 56.6, and 54.1 ng $g^{-1}$ fresh weight at the same sampling times, indicating a decrease in ABA on a dry weight basis as the day progressed. This decrease was also not correlated with stomatal closure patterns as shown in Figure 2. Thus, dry weight estimations of ABA for both control and fumigated plants, as with fresh weight estimations, provided no close relationship with stomatal conductances over the day.

The present analysis of concentrations of ABA for entire leaves cannot rule out the possibility that there may be compartmentalization of ABA to provide elevated concentrations at different times of the day in certain parts of the leaf tissue (1). Localized elevated levels of ABA in cells around the stomatal pores has been indicated by several workers to be the controlling factor for stomatal responses (2, 3, 15, 16). The entire leaf determinations used in this study would not have detected compartmentalization of ABA that could be controlling stomatal opening.

*Acknowledgments*—The authors thank Morrison Brothers Seed Company for donation of seed, Harold Frank for calibration of pollutant analyzers, and Dr. Gary Seckinger for his aid in extraction of ABA.

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