Mutual Antagonism of Sulfur Dioxide and Abscisic Acid in Their Effect on Stomatal Aperture in Broad Bean (Vicia faba L.) Epidermal Strips

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ABSTRACT

Abscisic acid (ABA) was found to counteract the stomatal opening in Vicia faba L. caused by SO2. The antagonism between SO2 and ABA was mutual, and their combined effect depended upon which compound was in the greatest concentration. Stomatal apertures were monitored in detached epidermal strips floated in the light on aqueous solutions of SO2 (sulfurous acid) and/or ABA in 0.01 molar sodium citrate buffer (pH 5.8). Low concentrations of sulfuric acid (10-10 to 10-7 molar) increased stomatal aperture, but concentrations greater than 10-5 molar decreased it. A progressive decrease in aperture size occurred as ABA was increased from 10-10 to 10-4 molar.

No evidence was found for a direct chemical reaction between the buffered sulfuric acid and ABA (exogenous or endogenous). Extractable, endogenous ABA in the strips remained relatively constant after exposure to several different concentrations of sulfuric acid. A technique for quantitating ABA from methanolic extracts of small samples of epidermis (20 milligrams dry weight) using reverse phase high performance liquid chromatography is described.

MATERIALS AND METHODS

Plant Material. Broad beans (V. faba L. cv. Exhibition Long Pod) were germinated and grown for 3 to 4 weeks (1 seedling per 12.5 cm pot) in a controlled environment with a 16-h photoperiod (150 W m-2) at 20°C during the day and at 16°C during the dark period. Prior to the removal of epidermal strips, plants were dark-acclimated for 4 h. Abaxial epidermal strips (about 9 mm2) were taken from the youngest fully expanded leaves and immediately floated on the buffered solutions.

Reagents. The dilution series of H2SO3 solutions was prepared from commercial sulfuric acid (i.e. a solution of SO2 in water; assay [as SO2] 6.0%; Fisher Scientific Co.) and was then buffered at a constant molarity and pH with sodium citrate buffer (10 m; pH 5.8) (15). To minimize oxidation, a fresh, sealed bottle was used to make each series, and, following buffering, solutions were stored in tightly closed bottles at 4 to 5°C until required for use. Similar series were prepared from H2SO4 and ABA.

Measurement of Stomatal Aperture. The strips were incubated for 4 h in open glass jars (4-cm diameter; 3 strips/jar) on 5 ml buffered test solution (i.e. ABA, H2SO3, or H2SO4). The jars were partially submerged in a water bath at 30°C under fluorescent lights (Sylvania Gro-Lux; intensity 46 w m-2 at the level of the floated strips). The strips were then examined on a projecting microscope (x 800), and the apertures of 20 stomates on each of the strips (3 strips/treatment) were measured. Each treatment was repeated three times so that mean stomatal apertures are based on measurements of 180 stomates.

Measurement of ABA in Buffered Solutions. Solutions of ABA (100 ml) containing H2SO3 and/or buffer were incubated as described above and then partitioned three times with equal volumes of ethyl acetate at pH 3.0. The ethyl acetate was then evaporated in vacuo at 35°C, and the residue was methylated with excess ethereal diazomethane prior to analysis of the samples by GLC-EC.

Measurement of Endogenous ABA in the Epidermal Strips. Extraction of Epidermal Strips. Fifty to 60 strips (about 20 mg dry weight) were incubated 4 h for each treatment and then plunged into liquid N2, lyophilized, weighed, and homogenized for 2 min with 10 ml methanol: H2O (80:20) in a motor-driven tube-and-pestle tissue grinder (40-ml capacity). At the time of homogenization, 8.86 ng (2,508 dpm) of [2-14C]ABA (New England Nuclear) were added to each tube. The extracts were filtered, dried in vacuo at 35°C, and dissolved in a small amount of methanol. The methanolic solution was filtered through a FH Millipore filter, pore size 0.5 μm, and then reduced to a suitable volume.

SO2 is a major atmospheric pollutant which significantly affects plant metabolism and function (6, 23, 25). Relatively low levels of SO2 may increase stomatal aperture or inhibit closure (2, 20), thus encouraging desiccation and increasing pollutant access to the leaf mesophyll (10). A decrease in stomatal aperture following SO2 fumigation has also been shown to occur (4, 13, 14, 24). This discrepancy appears to reflect differences in the concentration of SO2 and/or differences in the sensitivity of various species to SO2.

The natural plant hormone ABA is known to promote stomatal closure under conditions of water stress (12). Recently, Kondo and Sugahara (8) demonstrated a relationship between SO2 sensitivity and endogenous ABA levels in a number of species. Species which were resistant to SO2 damage had higher ABA levels than those which were more sensitive. Those species with high endogenous levels of ABA responded rapidly to SO2 fumigation by reducing stomatal aperture, thus decreasing the amount of SO2 absorption and subsequent damage.

In the present study, epidermal strips of Vicia faba L. were exposed to aqueous solutions of SO2 to define the range of SO2 concentrations which (a) increases stomatal aperture and (b) induces stomatal closure. The possibility of an interaction between SO2 and ABA (endogenously and exogenously applied) was then examined.


2 Abbreviations: GLC-EC, gas-liquid chromatography with electron capture detector; Me-ABA, abscisic acid methyl ester; t-ABA, trans-abscisic acid; MSA, mean stomatal aperture.
volume (50–100 µl) under N₂ prior to injection onto a HPLC column.

**Purification of Extracts by HPLC.** The extracts were chromatographed on a Waters Associates high performance liquid chromatograph, gradients being obtained from two solvent reservoirs containing, respectively, methanol:H₃OAcetic acid (10:89:1) (solvent A) and methanol (solvent B). Initially, the methanolic extract residues were injected onto a Radial-Pak C₁₈ (octadecylsilane) reverse phase cartridge (Waters Associates), which was eluted at 4 ml min⁻¹ with a linear solvent gradient beginning with 19% methanol and ending with 100% methanol. (Authentic ABA was eluted in this system at 610 to 680 s.) Fractions of 2 ml or 4 ml were collected between 510 and 750 s, and a ½ aliquot was removed and added to 10 ml Biofluor (New England Nuclear). ¹⁴C activity was counted on a liquid scintillation counter (Isocap/300 Liquid Scintillation System, Searle Analytic Inc., Des Plaines, IL). Active fractions were reduced to dryness in vacuo, methylated as noted above, then dissolved in 50 µl of methanol and injected onto a µBondapak C₁₈ reverse phase column (Waters Associates). The column was eluted at 2 ml min⁻¹ for 25 min with a linear solvent gradient beginning with 37% methanol and ending with 82% methanol. (Authentic ABA was eluted in this system from 540 to 590 s; Me-ABA was eluted from 800 to 870 s.) Active fractions in the region of the authentic Me-ABA were collected, reduced to dryness in vacuo at 35°C, and made up to 10 µl in methanol prior to injection on the GLC column.

**GLC-EC Analysis.** A Varian Series 3700 gas chromatograph with 1.83 mm × 2 mm i.d. glass columns packed with 5% SE-30 on Chromosorb W (80/100 mesh) or 2% Epon 1001 on Chromosorb W (60/80 mesh) at a gas-flow rate (90% argon:10% methane) of 60 ml min⁻¹ was used, Me-ABA being detected by electron capture (³²Ni). The retention times for authentic samples on the two columns were: 5% SE-30 (200°C) ABA, 5.2 min; t-ABA, 6.7 min; 2% Epon 1001 (200°C) ABA, 7.7 min; and t-ABA, 9.4 min.

Quantification of endogenous ABA was made by comparing peak areas of Me-ABA standards with those of the purified extracts occurring at the same retention time. Losses due to the purification procedure or to the removal of aliquots for counting were compensated for on the basis of [¹⁴C]ABA losses (final recovery ranged from 14.1 to 32.9%).

The presence of ABA in the extracts was further verified by exposing a solution of the derivatized material in a sealed spectrophotometric cuvette to UV radiation at 254 nm for 3 h followed by GLC-EC analysis. Due to isomerization, the peak which corresponded to ABA was reduced, and a new peak, corresponding to the retention time of t-ABA, could be observed.

**RESULTS**

Epidermal strips were incubated in concentrations of buffered sulfuric acid (aqueous SO₂) ranging from 10⁻₁² M to 10⁻² M, and the MSAs were then determined (Fig. 1). A biphasic pattern in stomatal aperture was observed: decreasing SO₂ concentration from 10⁻⁸ M to 10⁻⁶ M H₂SO₃ progressively increased MSA, while concentrations greater than 10⁻⁴ M H₂SO₃ caused a progressive reduction in MSA. The pH of these solutions was maintained at 5.8 by the buffer until sulfuric acid was increased to 10⁻³ M (pH 5.6) and 10⁻² M (pH 4.4). At pH 5.8, sulfuric acid exists predominantly as the bisulfite ion (H₂SO₃⁻ > 90%) [17].

When sulfuric acid was substituted for sulfuric acid (10⁻¹² M to 10⁻² M), the MSA did not deviate significantly from the controls (MSA = 10.1 µm) until the buffering capacity of the citrate buffer was destroyed at 10⁻⁵ M H₂SO₄ and the stomates remained closed (MSA = 2.25 µm). At 10⁻¹² M to 10⁻⁴ M H₂SO₄, the pH was maintained at 5.8; 10⁻³ M and 10⁻² M H₂SO₄ gave pH values of 5.4 and 2.7, respectively.

When epidermal strips were incubated in a range of ABA concentrations from 10⁻¹¹ M to 10⁻⁴ M, a progressive decline in

**DISCUSSION**

While numerous investigators (6, 25) have studied the effect of direct gaseous SO₂ fertilization on plant tissue, aqueous solutions...
of SO$_2$ (H$_2$SO$_3$) have been used much less frequently, presumably because they were not appropriate for most experimental systems. There is some justification, however, for assuming that the physiological effects produced by exposure to buffered H$_2$SO$_3$ are the same as those which occur following SO$_2$ fumigation. Gaseous SO$_2$ enters the leaves of higher plants mainly through the stomates (18); it is highly soluble in water and rapidly dissolves to form sulfurous acid in the intercellular spaces (19). The sulfurous acid is then buffered within the plant tissue (the predominant ionic species would be bisulfite [HSO$_3^-$] or sulfite [SO$_3^{2-}$] [25]). The use of direct applications of buffered sulfurous acid has several obvious experimental advantages over gaseous fumigation: ease of handling; reaction in a single (liquid) phase; and increased accuracy for monitoring and controlling concentration and pH.

In the present experiments, it is clear that stomatal response is highly dependent on SO$_2$ concentration, i.e. low levels of SO$_2$ induce stomatal opening in the isolated epidermal strips, while high concentrations promote closure. In general, stomatal aperture appears to depend on the turgor pressures of the guard cells relative to other epidermal cells (5). At least two theories have been proposed to account for the apparently opposite effects of SO$_2$ at high and low concentrations on these turgor pressures. Heath (6) suggests that exposure to low concentrations of SO$_2$ causes a build-up of sulfite ions at the guard cells, thereby increasing their osmotic pressure, which in turn causes increased turgor and induces stomatal opening. At higher concentrations of SO$_2$, guard cell photosynthesis would be inhibited, and osmotic pressure would drop. This theory assumes a preferential accumulation of sulfite in the guard cells; sulfate, for example, had no effect on stomatal aperture in our experiments. It has also been suggested by Willmer and Mansfield (22) that, for detached epidermal strips of V. faba, at least, solutes are taken up equally by the guard cells and the epidermal cells. This suggestion is based on the observed uptake of neutral red dye, which may or may not mimic the uptake of inorganic ions. Black and Black (3) account for the difference in stomatal reponse at high and low SO$_2$ levels by postulating differential impairment of membrane permeability or function. With low concentrations of SO$_2$, membrane damage of the adjacent cells would reduce their turgor, allowing passive opening to occur. At higher concentrations, the guard cells them-

**FIG. 2.** The effect of ABA in 10 mM sodium citrate buffer (pH 5.8) on stomatal aperture in isolated epidermal strips of Vicia faba. The strips were floated on the solutions and incubated for 4 h at 30°C with a light intensity of 46 w m$^{-2}$ prior to measurement of the stomates. The mean width ($\mu$m) of 180 stomates ± SE is given for each concentration of ABA.

**FIG. 3.** The effect of sulfurous acid in 10 mM sodium citrate buffer (pH 5.8) on stomatal aperture in Vicia faba epidermal strips in the presence of ABA. Incubation was as in Figure 1 legend. The mean width of 180 stomates ± SE is given for each concentration of sulfurous acid. The control lines represent stomatal response in buffer alone without ABA or sulfurous acid. A, Sulfurous acid plus 10$^{-6}$ M ABA; B, sulfurous acid plus 10$^{-8}$ M ABA.
Table 1. Endogenous ABA Levels in Epidermal Strips of Vicia faba

The epidermal strips were floated on buffered aqueous solutions of SO₂ (pH 5.8) for 4 h at 30°C with a light intensity of 46 w m⁻². Values are the mean of two replicate experiments.

<table>
<thead>
<tr>
<th>H₂SO₃ Concentration</th>
<th>ABA μg/g dry wt</th>
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<tbody>
<tr>
<td>0</td>
<td>2.16</td>
</tr>
<tr>
<td>10⁻¹⁰</td>
<td>2.28</td>
</tr>
<tr>
<td>10⁻⁷</td>
<td>1.70</td>
</tr>
<tr>
<td>10⁻⁴</td>
<td>2.63</td>
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selves would be damaged, preventing opening.

Turgor pressure changes in the guard cells which normally control stomatal aperture are caused by the movement of monovalent ions, particularly K⁺ (16), and the transport and synthesis of certain organic anions, such as malate (1). Stomatal closure is apparently induced by ABA through its effect on these processes (7, 11, 21). Loveys (9) has suggested that ABA is not synthesized in the stomatal complex itself but is produced in mesophyll chloroplasts and transported to the epidermis in times of stress. He found that detached epidermal strips of V. faba, stressed by exposure to mannitol solutions, did not accumulate ABA, whereas the epidermis of intact tissue, under the same conditions, did. Our finding that ABA levels were essentially the same in epidermal strips which were exposed to a range of SO₂ concentrations, and displayed wide differences in stomatal aperture, may be the result of this lack of synthetic ability in the isolated epidermis. It is clear, however, that ABA does function in the presence of SO₂, since stomatal opening induced by SO₂ could be prevented with sufficiently high levels of exogenous ABA. Equally, sufficiently high levels of SO₂ destroyed the effectiveness of exogenous ABA and, most probably, endogenous ABA. (The lowest concentration of H₂SO₃ which is effective in stimulating stomatal opening is that concentration [μM] which is equivalent to the concentration [μM] of applied exogenous ABA [Fig. 3], and we would expect that, in the absence of exogenous ABA [Fig. 1], the lowest concentration of H₂SO₃ which is effective must be that concentration which can counteract the endogenous ABA in the strips [i.e. 10⁻¹⁰ M H₂SO₃]. If the SO₂ effect on aperture results from membrane damage, first to the cells adjacent to the guard cells and finally to the guard cells themselves, as Black and Black (3) have suggested, then ABA's ability to control solute flux, in spite of this, requires explanation.

Further experiments involving the fumigation of whole plants will be necessary to resolve some of these difficulties. Our present results confirm the opposite effects of SO₂ at high and low concentrations in a simple in vitro system and demonstrate the ability of ABA to function effectively as an antagonist to SO₂ in controlling stomatal opening.

LITERATURE CITED

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