Induced Resistance to Ozone Injury of Soybean by Tobacco Ringspot Virus

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ABSTRACT

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Soybean plants, 12-18 days after sowing, were inoculated with tobacco ringspot virus (TRSV) on one primary leaf at 4, 6, 8, or 10 days before a 4-hr exposure to ozone at 697 or 797 $\mu g/m$ (0.35 or 0.40 ppm). Primary leaves of virus-inoculated plants became more resistant to ozone injury with increasing time after inoculation. Ozone resistance was more closely correlated to extent of TRSV-induced apical necrosis than to time after inoculation. The TRSV-induced apical necrosis required for a significant reduction in ozone sensitivity occurred prior to the detection of virus in the noninoculated primary leaves. Leaf conductances were similar in primary leaves of virus-infected and noninfected plants before an ozone exposure; during the exposure, conductances decreased more rapidly in infected plants, but conductances of noninfected plants were similar at the end of the 2-hr exposure. Although TRSV influenced stomatal response to ozone, this effect did not appear to be the primary factor associated with the induced resistance to ozone.

Fungi, bacteria, viruses, and abiotic stresses (including light, temperature, and humidity) affect plant sensitivity to the air pollutant, ozone (8, 9). Plant-virus infections, whether systemic or localized, have been reported to increase plant resistance to ozone injury. Brennan and Leone (2) reported an increase in ozone resistance of tobacco systemically infected with tobacco mosaic virus (TMV). Using primary leaves of pinto bean, Davis and Smith (4) observed rings of ozone-resistant tissue around local lesions induced by TMV, tomato ringspot virus (TOMRSV), tobacco ringspot virus (TRSV), and alfalfa mosaic virus (AMV); they reported (4) that half-leaf inoculations with TMV imparted ozone resistance over the entire leaf and, to some extent, on the opposite primary leaf. Other host-virus combinations that impart resistance to ozone have also been investigated (3, 11).

It was possible that virus infection reduced stomatal conductance and caused exclusion of ozone from the leaf. Brennan (1) reported that leaf diffusive resistance of TMV-infected and healthy tobacco plants were similar before, during, and after ozone exposure (E. Brennan, *personal communication*). There have been numerous reports that stomata must be open for ozone injury to occur (5, 6, 7). However, stomatal behavior appeared in most cases to be only one of the factors that influence ozone susceptibility (5, 10, 15, 18).

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The objectives of this study were to utilize a host-virus system in which ozone resistance would be induced in leaves other than those inoculated with virus and to test the hypothesis that altered stomatal behavior was responsible for the virus-induced ozone resistance.

MATERIALS AND METHODS

All experiments were conducted with soybean, *Glycine max* (L.) Merrill, 'Chippewa 64' (Minnesota Crop Improvement Association, St. Paul, MN 55101) and TRSV was selected as the infecting virus. Four seeds were sown in a potassium nitrate- and superphosphate-amended sand-composted soil (1:1:1 silty clay loam: horse manure and leaves: sand) mixture, pH 6.7-7.0 for each 589 cm^3 plastic pot. Plants were maintained in a greenhouse with auxiliary fluorescent lighting that provided a 14-hr photoperiod and an additional 4 Klux. Seedlings were thinned to two uniform plants per pot prior to inoculation. A single primary leaf of one plant per pot was mechanically inoculated with TRSV and the companion plant was maintained as a control. Inoculum consisted of TRSV-infected cotyledons of *Cucumis sativa* L. ground in 0.05 M phosphate buffer, pH 7.2, at 1:10 (w/v) and was applied with a brush to carborundum-dusted primary leaves.

Severity of TRSV-induced apical necrosis in soybean, commonly referred to as bud blight (12), was rated immediately prior to ozone exposure on a scale of 0 to 4: 0 = no symptoms, 1 = crooked non-necrotic apex, and 2, 3,

or 4 = <30%, 30-95%, or >95% necrosis, respectively, of the first trifoliolate leaf. The extent of apical necrosis appeared equivalent to the percentage necrosis on the first trifoliolate leaf for the plant age exposed to ozone.

Ozone exposures were conducted 22 days after seeding; at this time, primary leaves were from three-quarters to fully expanded. Ozone injury on primary leaves was subsequently evaluated as percentage leaf area injured with: 0 = no injury, 1 = 1-5% injury, 10 = 6-15% injury, 20 = 16-25%..., 90 = 86-95% injury, and 100 = 96-100% injury.

Response of TRSV-infected soybean to ozone.— In each of three experiments conducted in November and December 1975, 200 plants were divided into four groups and inoculated either 12, 14, 16, or 18 days after the seeds were sown, which corresponded to 10, 8, 6, or 4 days before ozone exposure. The earliest inoculation was performed when the primary leaves had separated completely from the main axis of the plant. Control leaves either were not inoculated or were rubbed on one leaf with a preparation from healthy cucumber cotyledons prepared for inoculum as described above. On the day preceding exposure to ozone, 20 plants were selected for uniformity from each of the four groups within the experiment and transferred to an M-2 Environmental Growth Chamber (Chagrin Falls, OH 44022) maintained at 24 C, 75% relative humidity, and 24 Klux. Severity of apical necrosis at the time of ozone exposure was rated as described previously. Beginning at 0900 hours, 22 days after sowing, plants were exposed in the growth chamber for 4 hr to ozone at 697 $\mu g/m^3$ (0.35 ppm) for experiment 1 and 797 $\mu g/m^3$ (0.40 ppm) for experiments 2 and 3. The higher ozone concentration in experiments 2 and 3 was necessary to achieve a similar level of foliar injury to that observed in experiment 1. Ozone concentrations were produced and monitored by methods previously described (3) and foliar injury was evaluated 72 hr after exposure.

The experiments were analyzed as a randomized complete-block design using the original percentages and arcsin transformations of the data (14). Means were separated using Waller and Duncan's modified (Bayesian) least significant difference test (20). Single comparison, unpaired *t*-tests (14) were conducted on the responses of the rubbed and nonrubbed controls which then were pooled for analysis. Single comparison, paired *t*-tests were performed to evaluate the responses of both primary leaves of the plants within individual day-virus or control treatment units.

Virus assays. One primary leaf per soybean plant per pot was inoculated with TRSV as described above. Ten days after inoculation, local lesion assays for the presence of virus were performed on the opposite, noninoculated primary leaves from five plants for each of the following stages of necrosis: 2, 3, 4 (24 hr after appearance), and 4 (24-48 hr after appearance). Each leaf was ground individually in 0.05 M phosphate buffer, pH 7.2, at a dilution of 1:2 (w/v) and the preparation was inoculated mechanically onto primary leaves of four cowpea plants, *Vigna senensis* (Torner) Savi 'Big Boy'. Controls, each assayed on three cowpea plants, consisted of buffer alone, preparation from healthy cucumber cotyledons, primary leaves from noninoculated control soybean plants, and threefold dilution series with two separate samples of TRSV-infected cucumber cotyledons previously quantified in similar assays. The experiment was conducted twice in May 1976.

Leaf conductance.— Soybeans were inoculated 12 days after sowing and 8-10 days prior to exposure to ozone. Noninoculated control plants were rubbed on one leaf with a preparation from healthy cucumber cotyledons. Plants exhibiting stage-3 apical necrosis and companion control plants were transferred to a growth chamber containing two similar Plexiglas inner chambers (13). One inner chamber was used for ozone exposure and the second for filtered-air controls.

An aspirated diffusion porometer (WREN Instrument Co., Hamden, CT 06514) (16, 17) was used to measure abaxial resistances of primary leaves before and at 30-min intervals during a 2-hr ozone exposure of 697 $\mu g/m^3$ (0.35 ppm). Resistances of noninoculated primary leaves were measured for TRSV-inoculated and rubbed control plants exposed to ozone or charcoal filtered-air. Four plants were evaluated for each treatment in each chamber per time interval. Plants were rated for ozone injury 3 days after exposure. Leaf resistances were converted to leaf conductances; the experiment was conducted three times in March 1977.

An analysis of variance was performed on conductance values for individual time intervals and subsequent ozone injury data for primary leaves of virus-blighted and control plants subjected to ozone or filtered air (14). Means were separated using Waller and Duncan's modified (Bayesian) least significant difference test (20).

RESULTS

Response of TRSV-infected soybean to ozone.— An increase of resistance to ozone injury in the primary leaves was observed with increasing time following inoculation (Table 1). Clear separation of means at the $P = 0.05$ level for virus-inoculated plants versus controls occurred at 10 days after inoculation.

Rearrangement of the data in Table 1 illustrated a relationship between ozone injury and severity of apical necrosis (Table 2). Apical necrosis increased with time, but an array of necrosis stages occurred on any given day after inoculation. Resistance of the plants to ozone increased as severity of virus induced apical necrosis increased; significant reductions in ozone injury occurred as early as 8 days after inoculation in experiments 2 and 3. Ozone response was not altered in plants with virus symptoms of stage 0 and stage 1 apices; with the onset of necrotic stages 2, 3, and 4, both strong trends and significant differences occurred.

Primary leaves on the same plant responded similarly to ozone. Ozone injury sustained by the virus-inoculated leaf versus that incurred by the opposite noninoculated leaf on the same plant were similar at the $P = 0.05$ level in nine out of 12 comparisons of 20 pairs of leaves each; responses to ozone of rubbed and nonrubbed primary leaves were statistically similar, $P = 0.05$ level. Mechanical inoculation appeared to have no effect on the response of the plants to ozone.

Local lesion assays for virus. Virus was not detected in the opposite noninoculated primary leaves of TRSV-inoculated soybean plants with apical necrosis of stages 2,

3, or 4 (24 hr after appearance). For severity stage 4 (24-48 hr after appearance), a single plant of the five sampled (one from each replicate) produced four infectious local lesions. Infectivity was determined by using these lesions to inoculate additional cowpea to ascertain that these few lesions were TRSV-induced.

Leaf conductance.—Based on preliminary experiments, abaxial conductances accounted for the majority of gas diffusion (19). Conductance rates for TRSV-infected and control soybean leaves were similar at the beginning and end of a 4-hr ozone exposure. Upon exposure to ozone, leaf conductances decreased more rapidly in virus-infected than in noninfected plants and remained lower than controls for as long as 2 hr; eventually, leaf conductances of noninoculated plants dropped to those of virus-infected plants. Small numbers of plants per time period did not permit a conclusive statistical test to determine whether this difference was significant. From these results, it was apparent that most of the injury was sustained within the first 2 hr of exposure to ozone.

The experiments reported herein were conducted with double the numbers of plants per time period during a 2-hr ozone exposure. The results from these experiments are illustrated in Fig. 1. In the absence of ozone, conductance of virus-infected plants was similar to noninfected plants at all time periods (Fig. 1-A). The conductance values of plants exposed to ozone yielded a significant virus-time interaction at the $P = 0.08$ level when all three experiments were pooled. There was a significant difference in conductances between experiments ($P = 0.01$) and when the error associated with

TABLE 1. Ozone injury to noninoculated primary leaves of soybean as a function of days between tobacco ringspot virus inoculation of opposite primary leaves and ozonization[a]

Days inoculated prior to ozonization	Experiment number 1		2		3	
	TRSV	Control	TRSV	Control	TRSV	Control
10	12.1[b]x[c]	19.0 yz	11.1 x	19.5 y	6.8 x	17.0 z
8	17.0 y	20.0 yz	14.0 x	19.5 y	12.1 y	13.5 yz
6	20.0 yz	22.5 z	21.0 y	21.5 y	15.6 yz	18.0 z
4	23.0 z	23.5 z	20.5 y	19.5 y	16.0 yz	17.5 z

[a]Plants were exposed to ozone at 697 $\mu g/m^3$ (0.35 ppm) for experiment 1 and 797 $\mu g/m^3$ (0.40 ppm) for experiments 2 and 3. Each leaf was evaluated for ozone injury on a scale of 0-100 where 0 = no visible injury, 10 = 1-10 percent injury, etc. Injury ratings for each leaf were summed and the mean calculated.

[b]Each value is the mean of 20 observations.

[c]Values followed by the same letter within experiment # are not significantly different. Means were separated by Wailer and Duncan's modified (Bayesian) least significant difference test at K = 100 (approximate equivalent is $P = 0.05$).

TABLE 2. Ozone injury to noninoculated primary leaves of soybean as a function of tobacco ringspot virus-induced apical necrosis at the time of ozonization[a]

Experiment number	Days inoculated prior to ozonization[c]	Extent of apical necrosis[b] 0	1	2	3	4
1	10	16.7(3)[d]vw[e]	20.0(1) vw	14.0(6) w	9.3(11) x	
	8	20.0(5) vw	20.0(3) vw	15.0(12) w		
	6	20.0(15) v	20.0(5) v			
	4	23.0(20) u				
2	10	18.6(7) vw		10.0(3) xy	6.9(9) y	0.0(1) z
	8	17.5(8) w	20.0(2) uvw	11.3(8) x	15.0(2) wx	
	6	21.6(9) u	10.0(1) xy			
	4	20.5(20) uv				
3	10	12.9(7) uv		10.0(1) vw	7.8(4) wx	0.5(8) y
	8	15.7(7) u	12.5(4) uv	10.0(8) vw	1.0(1) xy	
	6	15.4(17) u	15.0(2) uv			
	4	16.0(20) u				

[a]Each leaf was evaluated for ozone injury on a scale of 0-100 where 0 = no visible injury, 10 = 1-10 percent injury, etc. Injury ratings for each leaf were summed and the mean calculated.

[b]TRSV-induced apical necrosis was rated on first trifoliolate as: 0 = symptomless, 1 = crooked apex, 2 = <30% necrosis, 3 = 30-95% necrosis, and 4 = >95% necrosis. By stage 4, the apical structures were completely necrotic.

[c]Plants were exposed to ozone at 697 $\mu g/m^3$ (0.35 ppm) for experiment 1 and 797 $\mu g/m^3$ (0.40 ppm) for experiments 2 and 3.

[d]Number of observations per mean.

[e]Values followed by the same letter within an experiment are not significantly different. Means were separated by Waller and Duncan's modified (Bayesian) least significant difference test at K = 100 (approximate equivalent is $P = 0.05$).

repetition was considered, the virus-time interaction became significant at the $P = 0.01$ level. Pairwise mean separation tests were conducted on the pooled conductance data for each time interval. At time 0 there was no difference between conductance measurements of virus-infected or noninfected foliage. At 30, 60, and 90 min of ozone exposure, conductance in virus-infected plants was significantly lower than noninfected tissue. After 120 min of ozone exposure conductance was reduced to a similar level in both virus-infected and noninfected tissue. The pattern of response of soybean foliage to ozone over time was similar in each experiment, but the magnitude of conductance varied (Fig. 1-B). Ozone injury developed in all noninfected plants exposed to the gas for 60 min or longer (Fig. 1-C). Resistance to ozone-induced injury resulting from virus infection was significant ($P = 0.01$) when plants were exposed to ozone for 60, 90, or 120 min.

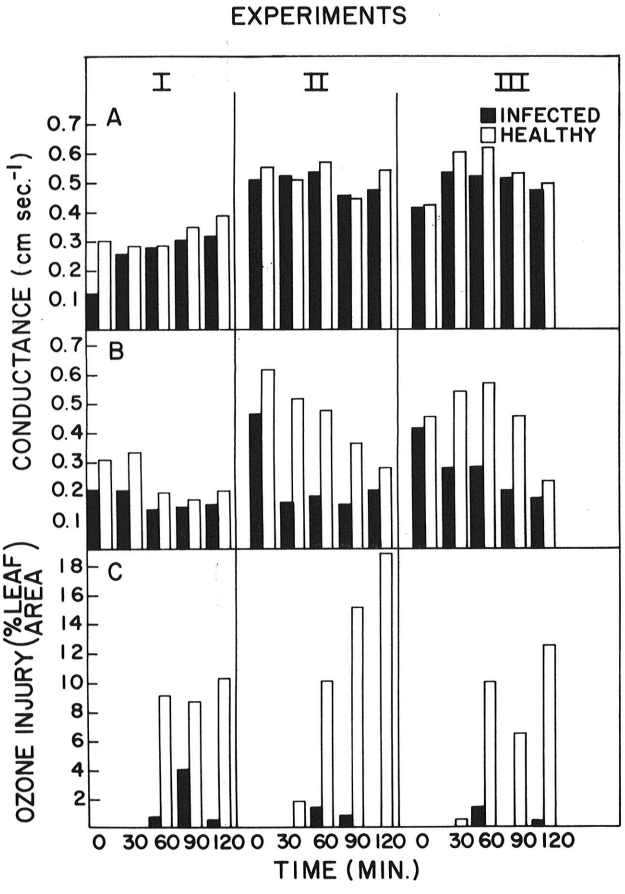

Fig. 1-(A to C). Effect of an exposure of 697 $\mu g/m^3$ (0.35 ppm) ozone for 2 hr to asymptomatic primary leaves of soybean plants inoculated with TRSV-infected or healthy cucumber cotyledons. **A)** Conductance rates at 30-min intervals in the absence of ozone. **B)** Conductance rates at 30-min intervals before, during, and after a 2-hr ozone exposure. **C)** Ozone injury based on percent leaf surface area affected 48 hr after exposure to ozone for indicated number of minutes.

DISCUSSION

Ozone sensitivity of the primary leaf opposite the leaf inoculated with TRSV was decreased with increasing time after inoculation. Reduction in ozone sensitivity was more closely correlated with increasing severity of TRSV-induced apical necrosis than it was to time after inoculation. In preliminary studies we obtained similar results on both primary leaves when cotyledons were inoculated instead of a single primary leaf.

Necrosis, per se, was probably not a requirement for the reduction in ozone response. Increased resistance may have resulted from physiological changes occurring simultaneously with the onset of necrosis, but not dependent upon the occurrence of necrosis. Brennan and Leone (2) observed a decrease in ozone sensitivity of TMV-infected tobacco when young leaves were mottled with no apparent necrosis. A reduction in ozone response occurred in tobacco exhibiting mild symptoms of tobacco etch virus infection (11). With bean common mosaic virus-infected pinto bean, Davis and Smith (*personal communication*) observed a decrease in ozone response in the absence of virus symptoms. Davis and Smith (4) also have shown virus-inoculated pinto bean to be more ozone resistant after the appearance of local lesions; in most instances, resistance was localized around the lesions.

Low virus concentrations were detected in only two of 10 assays of the noninoculated primary leaves of plants with apical necrosis of severity stage 4 (24-48 hr after appearance). Significant reduction in ozone response occurred in primary leaves of plants with apical severity values as low as stage 2 in which virus was assayed to be absent. Therefore, the presence of virus at detectable levels was not required in the leaf tissue for induction of ozone resistance. Similar results have been reported for systems utilizing virus-induced local lesions (4).

We considered the possibility that TRSV-infection might decrease leaf conductance which could account for the increased ozone resistance of the more severely blighted plants. Since the differences between leaf conductances of virus-infected and noninfected plants were not significant at anytime in the absence of ozone (Fig. 1), resistance was not explainable simply by a chronic reduction of leaf conductance in the infected soybeans. These results agreed with those reported by Brennan (1) for a TMV-tobacco interaction with ozone.

While TRSV did not decrease leaf conductance, it was decreased more rapidly in virus-infected than healthy tissue when exposed to ozone. The differences in conductance between leaves of virus-infected and noninfected plants were significant ($P = 0.05$) at 30, 60, and 90 min after ozone exposure but not at earlier or later times. We concluded that there was an interaction between virus infection, ozone exposure, and the conductance of soybean leaves. It seems probable that guard cells in leaves of TRSV-infected plants collapsed more readily in the presence of ozone than did guard cells of noninfected plants.

Significant differences in leaf conductance values between virus-infected and control plants do not insure that these are the cause of the observed differences in ozone injury. Stomatal aperture is not the sole factor regulating ozone-induced, foliar injury of plants. The absolute conductance values for experiment 1 are

significantly lower than those for the other two experiments (Fig. 1-B). In fact, the conductance values for the noninfected plants in experiment 1 are similar to values for virus-infected plants in the other two experiments. The levels of ozone injury in experiments 1 and 3 were nearly identical (Fig. 1-C). The more rapid drop in conductance of virus-infected plants does not appear to fully explain the resistance to ozone-induced necrosis. Within an experiment, differences in leaf conductance between virus-infected and control plants do not appear to coincide sufficiently with the differences in ozone injury. This is strongly supported by our preliminary experiments (19).

When conductance is resitricted, ozone injury is reduced but causality has not been established. However, as others have concluded (5, 10, 15, 18), stomatal behavior does not appear to be the primary factor influencing ozone induced foliar injury.

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