Effects of Ozone on Infection of Soybean by Pseudomonas glycinea

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ABSTRACT

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Ozone inhibited infection by *Pseudomonas glycinea* in primary and trifoliolate leaves of soybean inoculated 1 day prior to, or several days after fumigation with 490 $\mu g/m^3$ (0.25 ppm) O_3 for 4 hr. The protective effect persisted over the period that primary leaves were susceptible to the bacterium. Trifoliolate leaves that were beginning to expand at the time of exposure also were protected when inoculated subsequently. Similar effects were observed when primary and young trifoliolate leaves were exposed to 157 $\mu g/m^3$ (0.08 ppm) for 4 hr, but not when fully expanded trifoliolate leaves were exposed.

Additional key words: pollutant-parasite interaction, air pollution, bacterial diseases.

Ozone (O_3) interacts with plant parasites and thus influences plant diseases. It has been demonstrated that exposure of plants to ozone generally reduces infection, invasion, and sporulation by fungal pathogens (5). In some instances, however, diseases caused by facultative parasites may be enhanced by O_3 exposure (14, 15, 16) as in the case of *Botrytis cinerea* on field-grown potatoes.

Interactions of bacterial pathogens and O_3 have been investigated to a limited extent. Kerr and Reinert (7) reported inhibition of O_3 injury surrounding bacterial lesions on *Phaseolus vulgaris*. Also, it has been reported that O_3 explosure decreased nodule formation by *Rhizobium* sp. on soybean, and nodule number, size, and weight on pinto bean (13, 21). Pell et al. (18) examined the interaction of O_3 and a *Pseudomonas* sp. which caused a hypersensitive reaction on soybean. The plant's response to O_3 and *Pseudomonas* sp. combined was more severe than either treatment alone (10, 18).

In many states, including Minnesota, substantial cropping of soybeans is done within 150 km of metropolitan areas and those fields may be exposed to elevated O_3 concentrations. Ozone-type symptoms have been observed on field-grown soybeans within 120 km of St. Paul (S. Krupa, *personal communication*).

The response of soybean to O_3 has been described and genetically controlled sensitivity of cultivars has been documented (22). Symptoms of O_3 injury include purple stippling and flecking of the upper leaf surface, and bifacial necrosis when high O_3 concentrations are used (19, 20, 22).

Bacterial blight of soybean, a disease caused by *Pseudomonas glycinea* Coerper (syn. *P. syringae* van Hall), is widespread in the USA and is a common leafspot disease throughout the upper midwest. Typical symptoms include small, water-soaked angular leafspots, sometimes surrounded by a yellow halo.

Owing to the importance of soybean as a crop, the increased periods of elevated O_3 concentrations in rural areas (17, 23), and the lack of information on interactions between O_3 and bacterial pathogens, studies were undertaken with the objectives of: (i) identifying the relationship of O_3 exposure to *P. glycinea* infection of soybean, and (ii) evaluating the pollutant-parasite interaction as a basis for air quality standards. A summary of a portion of this research has been published (9).

MATERIALS AND METHODS

Culture of soybean.—Chippewa 64 soybean seeds were sown, seven per 10-cm square pot, in a layer of sand underlain by steamed greenhouse soil mix. Pots were maintained in a greenhouse at approximately 21 C with a 16-hr photoperiod. Sixteen days after planting, seedlings were thinned to three per pot and inspected to assure that neither O_3 nor bacterial blight symptoms were present. Twenty-three days after planting, plants were fertilized uniformly with 50 ml of an all-purpose liquid fertilizer (N:P:K: 20-20-20).

Culture of Pseudomonas glycinea.—An isolate of *P. glycinea* obtained from field-grown soybean plants and identified as race 2 after inoculation of standard differential cultivars (1), was used in all experiments. The stock culture was maintained on nutrient agar at 5 C, and was re-isolated periodically from infected plants to maintain pathogenicity.

Inoculum was prepared by suspending 48-hr nutrient

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agar cultures, grown at 27 C in sterile distilled water, and adjusting the suspension with a colorimeter to 10^7 cells/ml.

Ozone exposure procedure.—Two days prior to treatment, pots were transferred to and randomly placed in a conditioning growth chamber receiving charcoal-filtered air (Environmental Growth Chambers, Model M-2, Integrated Development and Manufacturing Co., Chagrin Falls, OH 44022). The plants were maintained at 21 C, 80% relative humidity and 26 K lx illumination with a 12-hr photoperiod. All plants were watered uniformly and exposed to light for 1.5 hr prior to exposure to O_3. Plants then were transferred to a modified growth chamber (24) where they were exposed to O_3 from 0800 to 1200 hours, under the same environmental regime used previously. Ozone was produced with an Orec Model 03V5-0 O_3 generator (Ozone Research and Equipment Corp., Phoenix, AZ 85019) and measured continuously with a calibrated McMillan 1100 chemiluminescence monitor (McMillan Electronics Company, Houston, TX 77036). Concentrations of O_3 during exposure varied ±19 to 29 $\mu g/m^3$ (0.01 - 0.015 ppm) from the desired concentration. Immediately following exposure, the plants were returned to the initial chamber where they were maintained for 2 days, and then transferred to the greenhouse.

Ozone injury was evaluated 7 days after exposure by visual estimates of symptom intensity. Reference charts similar to those used by Kohut et al. (8) were employed to standardize estimates of percent leaf area affected and frequency of the symptom. The index ranged from 0 (none) to 100 (most severe).

Inoculation procedure.—Soybean plants were inoculated at six different times with *P. glycinea* by applying 0.5 ml/leaf or leaflet of standardized bacterial suspension with a DeVilbiss No. 15 atomizer. Inoculation times ranged from 2 days before O_3 exposure to 16 days after exposure. Noninoculated control plants were treated identically except that sterile, distilled water was substituted for bacterial suspension. The number of bacterial lesions per leaf was determined 14 days after inoculation.

Experimental design.—*Dose-response experiments.* A completely randomized design was utilized with five replications of three plants each per treatment. Plants were exposed to O_3 at each of five concentrations and four time periods. The entire experiment was conducted twice. Appropriate controls were maintained. Regression analysis was used to determine the relationship between pollutant concentration, length of exposure, and severity index.

Ozone-Pseudomonas glycinea interaction experiments.—Factorial experiments in randomized, complete block design involving two leaves per plant, three plants per pot, and four blocks were utilized. All but one of the experiments were repeated three times; the remaining experiment was repeated twice. To stabilize the variance, the data were (re-expressed) as the square root of the number of bacterial lesions per leaflet and were analyzed by factorial analysis of variance. Non-transformed O_3 symptom severities were analyzed in a similar fashion. Relationships of treatment means were examined using two sample *t*-tests and the Bonferroni inequality (4) which adjusts the test to reflect the number of treatment comparisons made.

RESULTS

Relationship of O_3 concentration and length of exposure to symptom severity.—The response of Chippewa 64 soybean was evaluated to determine the relationship between five pollutant concentrations, four periods of exposure, and symptom severity. Two types of symptoms were commonly observed: a purple stippling of the adaxial surface, obtained only at an O_3 concentration of 392 $\mu g/m^3$ (0.20 ppm) for 2 to 4 hr, and a necrotic fleck, also on the upper surface, which occurred at higher concentrations. Bi-facial necrosis was observed on some plants fumigated with 588 $\mu g/m^3$ (0.30 ppm) for 4 hr.

A three-dimensional plot revealed that a true arithmetic dose-response relationship was not present since equal concentration × length of exposure products (doses) did not result in similar responses (Fig. 1). It was evident that as either concentration or length of exposure increased, injury became more severe until, at 588 $\mu g/m^3$ (0.30 ppm) for 4 hr, 80% of the leaf area became necrotic.

Based on these results, combinations of 490 $\mu g/m^3$ (0.25 ppm) for 4 hr, which produced a uniform necrotic fleck on exposed leaves, and 157 $\mu g/m^3$ (0.08 ppm) for 4 hr, which did not produce visible injury, were chosen for use in the experiments described below.

Ozone - P. glycinea interactions.—Twenty-one days after planting, when primary leaves of plants were almost fully expanded, they were exposed to 490 $\mu g/m^3$ (0.25 ppm) O_3 for 4 hr. These leaves were inoculated with *P. glycinea* at six times: 2 days, 1 day, and 1 hr before and 1 hr, 1 day, and 2 days after exposure. Conditioning periods, exposure, and inoculation procedures were as described in Materials and Methods. The experiment was repeated three times.

Ozone symptom severity was recorded 1 wk after exposure. The number of bacterial lesions per leaf was recorded 2 wk after inoculation. Comparisons were made only between exposed and nonexposed plants at the same time of inoculation. The changes in susceptibility of the plant to the bacterium with age, and our inability to precisely quantify viable inoculum made meaningful comparisons between times of inoculation impossible. When compared to controls, fewer lesions per primary leaf were observed in exposed plants inoculated at 1 day and 1 hr before exposure and at 1 hr, 1 day, and 2 days after exposure. In many cases, these differences were significant as indicated by *p*-values in Fig. 2-A. It is apparent that the differences and trends were consistent across experiments although the presence of a significant repetition × time of inoculation interaction did not permit the repetitions to be combined for analysis.

The length of time over which the decrease in bacterial infection persists was determined by extending the time of inoculation. Primary leaves were inoculated at 1 hr, 1 day, 2 days, 4 days, 8 days, and 16 days after exposure. In addition, trifoliolate leaflets which were beginning to expand at the time of exposure were inoculated 8 and 16 days after exposure. The experiment was repeated three times.

A lower mean number of lesions per leaf again was observed. At 8 days after exposure, primary leaves of

both exposed and nonexposed plants were beginning to senesce and were not susceptible to infection. At 8 and 16 days after exposure, exposed trifoliolate leaflets also were found to have fewer lesions per leaflet (Fig. 2-B).

Based on results of these experiments, further investigations were made to determine the effect of O_3 on *P. glycinea* infection in trifoliolate leaves. The times of inoculation ranged from 2 days before to 2 days after exposure as previously described. Plants were fumigated 28 days after planting, when trifoliolate leaves were almost fully expanded. The experiment was repeated three times.

Differences were not found in mean number of lesions per leaflet between exposed and nonexposed plants when inoculated 2 days prior to exposure. Thereafter, in all but one case, exposed plants had fewer lesions per leaflet than did nonexposed plants (Fig. 2-C).

Experiments identical to those previously described were made with an exposure of 157 $\mu g/m^3$ (0.08ppm) for 4 hr. This exposure regime did not result in visible injury to plants and was used to investigate effects of asymptomatic O_3 stress on a plant-parasite interaction.

In the first of these experiments, inoculations were made from 2 days before to 2 days after exposure. Plants were exposed 21 days after planting, when primary leaves were almost fully expanded. The experiment was repeated three times.

The results of this experiment were similar to those at the higher concentration exposure (Fig. 3-A). Differences in mean number of lesions per leaf were observed at 1 day and 1 hr before exposure and 1 hr, 1 day, and 2 days after exposure (Fig. 3-A).

When the time of inoculation was extended to 4, 8, and 16 days after exposure, it was again noted that susceptibility of primary leaves to the bacterium dropped sharply 8 days after exposure in both exposed and nonexposed leaves. Differences were generally smaller than those previously observed and the almost total suppression of bacterial infection noted before was absent at the lower concentration exposure. There was, however, a reduction in mean number of lesions per leaflet on the exposed trifoliolate leaflets inoculated 8 and 16 days after

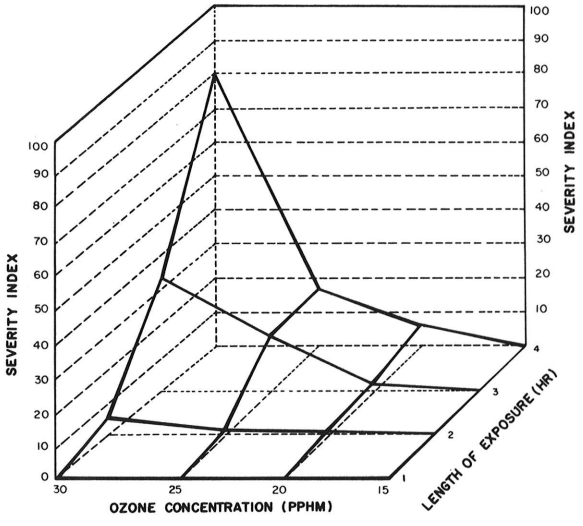

Fig. 1. Relationship of O_3 concentration and length of exposure to O_3 symptom severity on Chippewa 64 soybeans. 1 pphm = 19.6 $\mu g/m^3$.

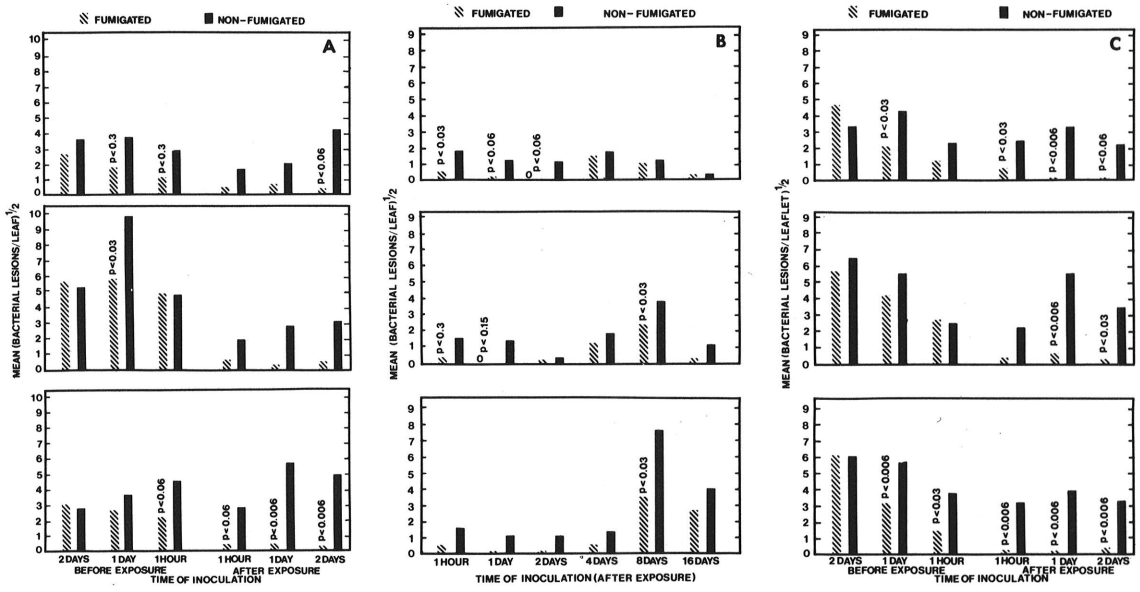

Fig. 2-(A to C). Effect of O_3 exposure (490 $\mu g/m^3$, 4 hr) on infection of soybean by *Pseudomas glycinea*. Absence of a *p*-value indicates $p>0.3$. Data are expressed as the mean of the square root of the number of bacterial lesions observed on leaves of exposed and non-exposed Chippewa 64 soybean plants. **A)** Primary leaves inoculated from 2 days before to 2 days after exposure. **B)** Leaves inoculated from 1 hr to 16 days after exposure. Inoculations at 8 and 16 days were of trifoliolate leaves, just beginning to emerge at the time of exposure. **C)** Trifoliolate leaves inoculated from 2 days before to 2 days after exposure.

exposure, thus showing a trend similar to that observed at the higher concentration (Fig. 3-B).

Trifoliolate leaves also were exposed to 157 $\mu g/m^3$ (0.08 ppm) for 4 hr and inoculated as before. The results were more variable than those in previous experiments. The differences in mean number of lesions per leaflet were generally small and the response observed showed no consistent trend in that, occasionally, exposed leaves had more lesions than did nonexposed leaves (Fig. 3-C).

DISCUSSION

The O_3-*P. glycinea* interaction experiments indicate that O_3 at the dosages used has a detrimental effect on bacterial infection of primary and trifoliolate leaves. It does not appear as though a lack of tissue available for infection was the cause of this reduction since the response occurred at levels of O_3 that caused light-to-moderate, or no visible injury. In addition, when leaves were inoculated 2 days prior to exposure, the same amount of O_3 injury was observed and there was no difference in number of bacterial lesions present on exposed versus nonexposed plants.

A possible explanation for the difference in bacterial infection is the production, by the plant, of a bacteriostatic or bactericidal compound, or compounds, in response to O_3 exposure. Keen and Taylor (6) have reported the production of the isoflavonoid compounds daidzein, coumestrol, and sojagol in soybean foliage following exposure to concentrations of O_3 which produced visible symptoms. The soybean phytoalexin, glyceollin, was not produced. They found that elevated concentrations of coumestrol began to occur about 10 hr after exposure. This observation would include the accumulation of this compound in the time period needed for infection to take place in a leaf inoculated 1 day prior to, but in most cases, not 2 days prior to exposure. Lyon and Wood (11) have found coumestrol to be bacteriostatic or bactericidal when tested against *Pseudomonas* sp.

The production of peroxidases occurs in soybean following O_3 fumigation (2, 3). These compounds also could be contributing to the difference in bacterial infection observed.

The reduced infection of trifoliolate leaves 8 and 16 days after exposure suggests either that (i) compounds inhibiting infection can be produced in young leaf tissue, or (ii) that materials produced in primary leaves are translocated to trifoliolates where their effect becomes evident.

In contrast to other reports (7, 12, 18), differences were not detected in severity of O_3 injury on infected versus noninfected leaves. It is probable that the inhibition of O_3 injury surrounding infection sites (7) occurs when an active, well-established infection is present. In comparison to studies where localized protection has been observed, the inoculation procedure used in our studies would probably result in a very small number of bacterial cells being introduced into the leaf. If an accumulation of materials produced by the plant or bacterium is necessary to inhibit O_3 injury, it is possible that the reduced number of infections would result in a delayed buildup of the necessary compounds and subsequently, there would be no effect on O_3 injury.

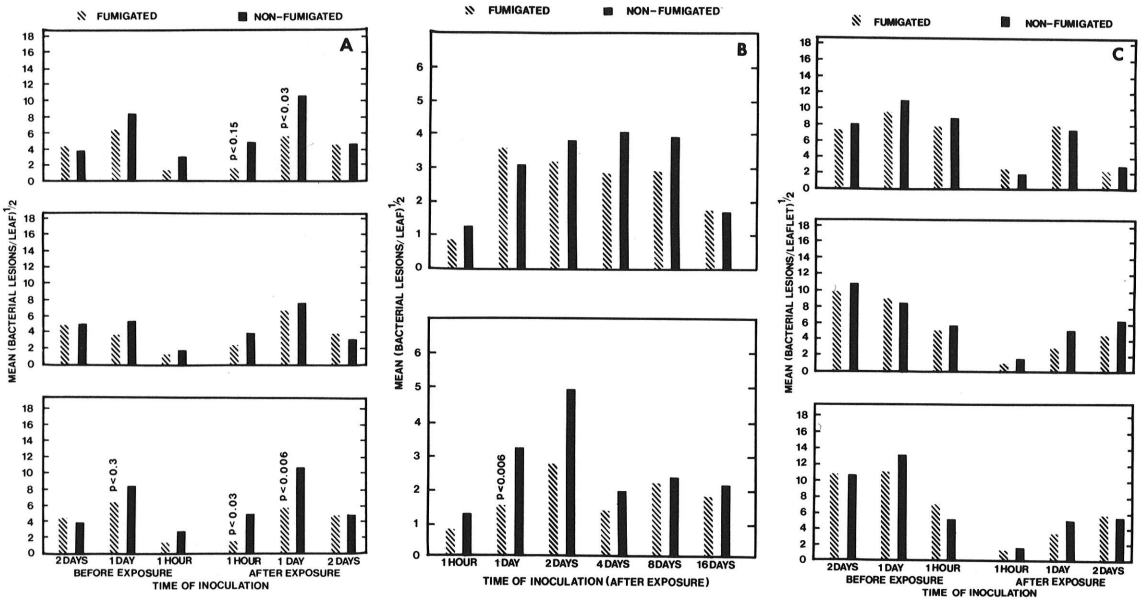

Fig. 3-(A to C). Effect of O_3 exposure (157 $\mu g/m^3$, 4 hr) on infection of soybean by *Pseudomonas glycinea*. Absence of a *p*-value indicates $p>0.3$. Data are expressed as the mean of the square root of the number of bacterial lesions observed on leaves of exposed and nonexposed Chippewa 64 soybean plants. **A)** Primary leaves inoculated from 2 days before to 2 days after exposure. **B)** Leaves inoculated from 1 hr to 16 days after exposure. Inoculations at 8 and 16 days were of trifoliolate leaves, just beginning to emerge at the time of exposure. **C)** Trifoliolate leaves inoculated from 2 days before to 2 days after exposure.

The response of Chippewa 64 soybean to O_3 exposure was similar to that reported by Tingey et al. (22) except that a lower level of injury was observed. The occurrence of stippling at lower concentrations was similar to that seen in field-grown soybeans in Minnesota following exposure to ambient O_3 at concentrations of 294 - 392 $\mu g/m^3$ (0.15 - 0.20 ppm) for 4 to 6 hr (S. Krupa, *personal communication*. It appears that the response of this cultivar of soybean to O_3 exposure is not linear, suggesting that if O_3 concentrations increase in rural areas, a substantial increase in injury might be expected.

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