Response of Soybean Foliage to Reciprocal Challenges by Ozone and a Hypersensitive-Response-Inducing Pseudomonad

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ABSTRACT

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Primary leaves of soybean inoculated with a concentration of bacteria which elicited a low level of hypersensitive response (HR) were subsequently challenged with an ozone dose which induced a severe water-soaking symptom in noninoculated foliage. Leaves inoculated with bacteria 24 hr prior to ozone exposure exhibited significant reduction in ozone symptoms. Leaves inoculated with bacteria 4 hr prior to ozone exposure displayed an enhanced response. The response to the interaction was unique and was characterized by some properties of each stress. Soybean plants were exposed to a dose of ozone which elicited a slight flecking response and inoculated 24, 48, 96, 144, and 168 hr after exposure with bacterial concentrations sufficient to cause severe HR. Ozone-treated plants did not display the bacteria-induced HR at any inoculation times. The absence of significant differences between ozone-treated and control plants at 168 hr probably was due to increased resistance of the host to the bacteria with increasing leaf age.

The host response resulting from the interaction between biotic stresses and ozone has been investigated by a number of researchers. Infection by *Pseudomonas phaseolicola* has been reported to prevent ozone injury in the chlorotic halos around the necrotic tissue of Red Kidney beans (9). Infections by *Puccinia graminis* f. sp. *tritici* on wheat, *Microsphaera alni* on lilac, and *Uromyces phaseoli* on bean protected host tissue from subsequent ozone exposure (5, 6, 26). There have been numerous reports of protection from ozone injury induced by viruses on a wide spectrum of plant species (1, 3, 18).

Research to determine the effect of ozone on subsequent microorganism infection of vegetation has been more limited. Ozone injury enhanced *Botrytis cinerea* infection of potato (17), inhibited *Botrytis gladiolorum* infection of gladiolus flowers (15), and had no influence on *Botrytis cinerea* infection of poinsettia bracts (16).

Certain abiotic stresses other than ozone have prevented bacteria induced hypersensitive response (HR). Exposure to high temperature (10), long periods of darkness (11), and calcium salt (2) could prevent HR. The response of soybean foliage to ozone injury resembles the HR to incompatible bacteria (8) and both ozone and incompatible bacteria stimulate production of coumestrol, daidzein, and sojagol in soybean leaves (8). Because of the similarity of these responses we hypothesized that a treatment with either ozone or HR-inducing bacteria might induce protection against subsequent injury by the alternate stress.

This study was undertaken to determine whether (i) an inoculation of soybean foliage with HR-inducing bacteria would ameliorate subsequent ozone challenge and (ii) an exposure of soybean foliage to ozone would alter subsequent challenge with HR-inducing bacteria. Both forms of protection were observed in preliminary studies (13, 19).

MATERIALS AND METHODS

Glycine max (L.) Merr. 'Chippewa' was grown in a mixture composed of sand and Hagerstown silty clay loam soil (2:1, v/v), two plants per 10.2 cm diameter pot, in a greenhouse protected with charcoal filters. The soil mix was amended with a complete fertilizer once a week. When the first trifoliolate leaf was one-third expanded (approximately 2 wk after planting) the leaves were inoculated with an incompatible bacterium or treated with ozone and subsequently subjected to the alternate stress.

Inoculation with bacteria.—The bacterium selected for this study was a *Pseudomonas* sp. [originally isolated from alfalfa roots (12)] which causes the rapid collapse of leaves associated with the HR (10). In each experiment, one primary leaf of one plant per pot was infiltrated with bacteria by submerging the leaf in the test suspension and creating a partial vacuum in a large desiccator. The entire plant was in the desiccator under vacuum. The leaf was kept submerged with a metal or plastic screen. The

periods of infiltration were varied as needed, but approximately 3 min was usually long enough to water-soak the entire leaf. The opposite leaf of the inoculated plant and both primary leaves of the companion plant served as controls. To confirm that the alterations observed were not due to infiltration with water per se, one primary leaf of each plant in additional pots was infiltrated with distilled water, and the response was compared with that of the noninfiltrated opposite leaves. Concentrations of bacteria were estimated photometrically and verified by standard plate-count procedures, and adjusted by dilution to either 3×10^7 cells/ml H_2O or 5×10^6 cells/ml H_2O. Following infiltration, plants were placed in plastic bags which were removed 1 hr prior to ozone exposure. Leaves were evaluated for intensity of the HR on a scale of 1 to 7 with 1 = 0%, 2 = 1-5%, 3 = 5-25%, 4 = 25-50%, 5 = 50-75%, 6 = 75-95%, 7 = 95-100% of the surface area affected.

Ozone exposure.—Soybean plants were placed in a growth chamber where they were exposed to either ozonized or charcoal-filtered air. The chamber and method of exposure have been described elsewhere (20). Plants were exposed to 499, 598, 694, or 793 $\mu g/m^3$ (0.25, 0.30, 0.35, or 0.40 ppm) ozone for 2, 3, or 4 hr at 20 C and 70% relative humidity (RH). Because plants were grown in the greenhouse and experiments were conducted throughout the year, it was necessary to vary the ozone dosage from 694 to 793 $\mu g/m^3$ (0.35 to 0.40 ppm) for 2-4 hr in order to induce water-soaking or from 499 to 598 $\mu g/m^3$ (0.25 to 0.30 ppm) for 3-4 hr to induce a low intensity "flecking" response. Forty-eight hr after exposure the leaves were evaluated for degree of injury. An injury index was calculated by multiplying a symptom intensity factor: viz., 1 = slight, 2 = moderate, 3 = severe, by a factor representing the total leaf surface area affected; viz., 0-10 when 0 = absence of symptom, 1 = 1-10% of leaf surface, 2 = 11-20% etc.

Experimental sequences.—Three experiments were conducted. In the first experiment, primary leaves were infiltrated with 5×10^6 bacterial cells/ml, 24 hr prior to exposure to 694 or 793 $\mu g/m^3$ ozone (0.35 or 0.40 ppm) for 2 to 4 hr. The experiment was conducted five times with five plants per treatment. In the second experiment, plants were treated similarly, but were exposed to ozone 4 hr after bacterial inoculation. This experiment was repeated four times, three times with five plants per treatment and once with 12 plants per treatment. In the third experiment, plants were exposed to 499 or 598 $\mu g/m^3$ ozone (0.25 or 0.30 ppm) for 3-4 hr and subsequently inoculated with 3×10^7 cells/ml 24, 48, 96, 144, and 168 hr after exposure. The number of trials and number of plants observed per time period are shown in Table 3.

Comparisons between HR responses of ozone-treated and nontreated primary leaf tissue were conducted by using an unpaired *t*-test (22). The ozone response of inoculated and noninoculated leaves, and of those infiltrated with water were compared by an analysis of

TABLE 1. Response of primary leaves of 2-wk-old soybean plants to an ozone treatment 24 hr after inoculation with 5×10^6 cells/ml of an incompatible strain of *Pseudomonas* sp.

	Bacterial response[b]		Ozone response[c,d]				
Number of trials[a]	$+O_3$	$-O_3$	+Bact.	−Bact.	Comp.	$+H_2O$	$-H_2O$
5	2.4	2.1	6.2	18.8 x	18.2 x	16.6 x	18.4 x

[a]Five plants per trial.

[b]Bacterial response was evaluated on a scale of 1 to 7 with 1 = 0%, and 7 = 95-100% of the leaf surface area necrotic. Means in rows followed by the same letter are not significantly different according to an unpaired *t*-test ($P = 0.01$).

[c]Ozone response was calculated by multiplying a symptom intensity factor by a factor representing the leaf surface affected. Means followed by the same letters are not significantly different according to a Waller and Duncan modified (Bayesian) least significant difference test value (K = 500) approximating $P = 0.01$.

[d]Ozone response of primary leaf inoculated with bacteria (+ Bact.), of the opposite primary leaf not inoculated with bacteria (− Bact.), of the average of two primary leaves of noninoculated companion plants (Comp.), of primary leaf infiltrated with water ($+H_2O$) and of its opposite leaf not infiltrated with water ($-H_2O$).

TABLE 2. Response of primary leaves of 2-wk-old soybean plants to an ozone treatment 4 hr after inoculation with 5×10^6 cells/ml of an incompatible strain of *Pseudomonas* sp.

	Bacterial response[c,d]		Ozone response[e]				
Number of trials	$+O_3$	$-O_3$	+Bact.	−Bact.	Comp.	$+H_2O$	$-H_2O$
3[a]	5.0	2.4 x	−[c]	10.5	10.5	10.1	11.2
1[b]	3.4	1.8 x	−[c]	11	...	...	...

[a]Five plants/trial.

[b]Twelve plants per trial.

[c]The symptom on leaves infiltrated with bacteria was only evaluated as bacterial response since it represented an interaction between the bacterial and ozone responses rather than either type of symptom separately.

[d]Bacterial response was evaluated on a scale of 1 to 7 with 1 = 0%, and 7 = 95-100% surface area affected. Means in rows followed by the same letter are not significantly different at the $P = 0.01$ level according to an unpaired *t*-test.

[e]Ozone response was calculated by multiplying a symptom intensity by a factor representing the leaf surface affected. Ozone response of primary leaf inoculated with bacteria (+ Bact.), of the opposite primary leaf not inoculated with bacteria (− Bact.), of the average of two primary leaves of uninoculated companion plants (Comp.), of primary leaf infiltrated with water ($+H_2O$) and of its opposite leaf not infiltrated with water ($-H_2O$).

variance, and significant differences between treatment means were determined by the Waller and Duncan (24) modified test for least significant difference ($P = 0.01$).

RESULTS

Pretreatment (24-hour) with bacteria.—When leaves were treated with bacteria 24 hr before exposure to ozone, inoculated leaves showed a significant reduction in ozone injury (Table 1). Inoculated leaves had less ozone injury than any of the noninoculated leaves (viz. opposite primary leaf), primary leaves of companion plants, or primary leaves infiltrated with water. All noninoculated leaves exposed to ozone were watersoaked at the termination of the exposure. There were no differences in the bacterial HR symptoms expressed by control groups or those exposed to ozone.

Pretreatment (4-hour) with bacteria.—Primary leaves treated with ozone 4 hr after bacterial inoculation, exhibited a significant increase in symptom severity (Table 2). Although we expressed symptom intensity with the rating for HR, the symptom was unique, and comprised some characteristics of both the ozone and HR symptoms. The distribution of the symptoms was interveinal and regular, similar to that of severe tissue collapse induced by ozone (23). The brown coloration of the symptom was reminiscent of bacteria-induced HR. All leaves treated with ozone but not inoculated with bacteria exhibited typical ozone injury (23). Primary leaves infiltrated with water and exposed to ozone had symptoms similar in appearance and intensity to others that had not been infiltrated. Water infiltration alone did not cause any symptoms.

The HR in plants pretreated with ozone.—In this experiment, plants exhibited low intensities of ozone injury characterized by a red-brown stipple. There were no significant differences between the intensities of ozone injury on any of the primary leaves (Table 3). Plants were infiltrated with 3×10^7 cells/ml 24, 48, 96, 144, and 168 hr after ozone exposure. At all times except the 168-hr period, plants pretreated with ozone exhibited significantly lower intensity of HR than nontreated plants (Table 3). The HR of plants not exposed to ozone decreased over time from 24 to 168 hr.

To determine whether leaf age influenced the HR, two experiments were conducted in which primary leaves of plants not exposed to ozone were inoculated either when the plants were 2 wk old; i.e., the same age as 24-hr inoculated plants or when the plants were 3 wk old; i.e. the same age as 168 hr inoculated plants. The average HR rating for primary leaves of 2-wk-old plants was 5.9 whereas the response of analogous leaves of 3-wk-old plants averaged 2.3. These differences were based on 22 observations per time period and were significantly different ($P = 0.01$).

DISCUSSION

Protection against ozone injury was elicited by bacteria-induced HR. The ozone tolerance of plants infected with HR-inducing bacteria probably resulted from physiological and biochemical changes to the plant cells rather than cell death per se since less than 10% of the foliage exhibited HR (Table 1).

Plants exposed to ozone 4 hr after bacterial infiltration, exhibited enhancement of symptom expression. The symptoms were more intense than the HR of plants not exposed to ozone and more extensive than the ozone symptoms of noninfiltrated plants. Coloration of the symptom associated with the HR-ozone interaction resembled the bacteria-induced HR, but its distribution was similar and more extensive than that of the ozone symptom alone. This enhancement may be due to the similar mode of action of the bacteria and ozone. Both stresses have been reported to affect cell membranes (2, 4, 21). If the bacteria had begun to alter cell membranes at the time of ozone exposure, it seems possible that the membranes were more vulnerable to injury by ozone. It also was possible that the watersoaking initiated by ozone may have increased the presence of substrate for bacterial multiplication and that an enhanced bacterial response resulted. One or both of these explanations may have accounted for the dramatic increase in symptom expression.

From data in Table 3 it was apparent that low levels of ozone injury in the leaf tissue prevented HR induction by the bacteria. Although the protection persisted for 144 hr after exposure, the HR diminished in intensity over time and by 168 hr it was at such a low level that protection was not demonstrated (Table 3). In another experiment we demonstrated that the decrease in ozone-induced protection against the HR over time occurred because the intensity of the HR was greatly reduced in older leaves.

TABLE 3. Response of primary leaves of soybean foliage to inoculation with 3×10^7 cells/ml of an incompatible strain of *Pseudomonas* sp. at 24, 48, 96, 144, and 168 hr following exposure to ozone

No. of trials	Time	Bacterial response[a]		Ozone response[b]		
Total no. of plants	(hr)	$+O_3$	$-O_3$	+Bact.	−Bact.	Comp.
4/23	24	2.0	4.4 x	9.6	9.8	8.3
4/23	48	1.7	5.1 x	8.6	9.4	9.3
3/18	96	1.6	2.9 x	7.8	6.9	8.3
2/12	144	1.8	3.8 x	6.3	7.2	6.5
1/6	168	2.7	2.7	4.0	4.0	3.2
1/12	168	2.6	2.6	10.0	10.0	...

[a]Bacterial response was evaluated on a scale of 1 to 7 with 1 = 0%, and 7 = 95-100% surface area affected. Means in rows followed by the same letter are not statistically different according to an unpaired *t*-test ($P = 0.01$).

[b]Ozone response was calculated by multiplying a symptom intensity factor by a factor representing the leaf surface affected. Ozone response of primary leaf inoculated with bacteria (+ Bact.), of opposite leaf not inoculated with bacteria (− Bact.) and of the average of two primary leaves of noninoculated companion plants (Comp.).

The reduction in HR level owing to increase in plant age was not expected, as it has been reported that senescent, etiolated leaves of White Burley tobacco still displayed HR when inoculated with *Pseudomonas syringae* (10). However, the results of another study also show that older tissue would be less likely to develop HR induced by fungal inoculation (25). As ozone induces premature senescence (21) and since older leaves, in general, are not responsive to the HR-inducing bacteria, the mechanism of ozone-induced protection of soybean foliage from the HR may be the same as that which occurs when plant tissue ages.

Keen and Taylor (8) reported that exposure to ozone induced an increase in concentration of a number of isoflavonoid compounds in soybean. These compounds also increased in concentration in leaves inoculated with an incompatible strain of *Pseudomonas glycinea* (7). Isoflavonoid compounds such as coumestrol have been shown to inhibit growth of *P. mors-prunorum*, *P. phaseolicola* (14), and *P. glycinea* (7). Although the increase in isoflavonoid concentration could explain ozone-induced protection against HR, the isoflavonoid hydroxyphaseollin, considered to possess phytoalexinlike properties, did not increase in concentration in response to ozone (8). The mechanism by which low levels of bacteria-induced HR protect plants from subsequent ozone injury and by which low levels of ozone injury may protect foliage from subsequent HR, may be similar or the same. The two symptoms are similar in appearance and the ozone response has been referred to as a hypersensitive response (8). It is possible that a compound induced by the bacteria or ozone is responsible for inhibition of either stress in the reciprocal challenges.

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