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# Ozone Injury in Soybeans

## ISOFLAVONOID ACCUMULATION IS RELATED TO NECROSIS[1]

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### ABSTRACT

**Fumigation of soybean leaves (*Glycine max* [L.] Merr. with ozone caused stippling and silvering at the same time that large accumulations of the isoflavonoid compounds daidzein, coumestrol, and sojagol occurred. Nitrogen dioxide and sulfur dioxide caused lesser accumulation of the isoflavonoids, and peroxyacetyl nitrate did not result in significant accumulation. Visible toxicity and chemical changes in ozone-fumigated leaves were similar to the hypersensitive disease defense reaction of soybean leaves to the pathogen *Pseudomonas glycinea*, except that the phytoalexin hydroxyphaseollin was not produced in the ozone-treated leaves.**

The hypersensitive resistant response is a necrotic reaction of plant tissue to invasion by pathogens and is regarded as an important disease defense mechanism in many plants. The chemical basis of hypersensitive resistance in soybeans (*Glycine max* [L.] Merr.) to the fungus pathogen *Phytophthora megasperma* Drechs. var. *sojae* A. A. Hildb. (3, 5) and to the bacterium *Pseudomonas glycinea* Coerper (4) appears to be the derepressed postinfectional production of several isoflavonoid compounds, the most notable of which is hydroxyphaseollin (Fig. 1), an antifungal and antibacterial phytoalexin. The related isoflavonoids daidzein, coumestrol, and sojagol (Fig. 1) also accumulate to high levels in the defense reactions to these pathogens (4, 5), but their antibiotic properties are less pronounced. The fact that production of the entire complex of isoflavonoids is observed in the resistant reactions suggested that, biochemically, resistance might result from a multivalent derepression of isoflavonoid biosynthesis (5). Based on previous research (5, 7, 10), the hypersensitive necrosis of host tissue that occurs in the defense reactions is believed to be directly caused by phytotoxicity of the high isoflavonoid levels. Because the hypersensitive necrosis of soybean leaves caused by *P. glycinea* (4) is superficially similar to injury caused by several air pollutants, especially ozone (12), we became interested in seeing if air pollutant injury might also be chemically related to the disease defense reaction. This paper tests that hypothesis.

Soybean plants (Harosoy 63) were grown from seed in growth chambers as described (3). At various ages, the plants were fumigated with air pollutants in chambers in the greenhouse (1). Isoflavonoids were extracted from the primary leaves of fumigated plants with ethanol and purified and quantitated as described elsewhere (4, 5). In this paper concentrations are expressed as $\mu$g/g fresh weight of leaves. The identity of the isoflavonoid compounds detected in ozone-fumigated leaves was established by UV and mass spectral data, and found to be identical to published values for authentic compounds (5). Primary soybean leaves were inoculated with the incompatible race 1 of *P. glycinea* by the methods in reference 4.

Ozone supplied at 0.25 to 1 $\mu$l/l for 1 to 4 hr gave varying degrees of foliar damage on soybeans which generally agreed with published symptoms (12). The initial symptom, especially with high dosages, was a rapid desiccation, bleaching, and necrosis of leaves within 1 to 3 hr after fumigation. This result was presumed to be due to an effect on permeability, causing excessive water loss. After 12 to 24 hr, stippling (red-brown pigmentation) and silvering of leaf undersurfaces was observed, especially with intermediate doses of ozone. This symptom developed into more pronounced stippling of lower and upper leaf surfaces after 24 to 28 hr, with considerable necrosis. Pronounced accumulation of coumestrol was observed in all ozone-treated leaves (Table I), and the concentrations attained were related to the severity of foliar symptoms. Unexpectedly, hydroxyphaseollin did not accumulate in any ozone treatment. Less accumulation of the isoflavonoids was observed when other air pollutants were used at damaging doses. Experiments with the photochemical pollutant peroxyacetyl nitrate (PAN) did not result in significant accumulation of coumestrol or other isoflavonoids (Table I), although considerable damage occurred. $NO_2$ and $SO_2$ both produced foliar injury and led to low rates of accumulation of coumestrol (Table I). None of the air pollutants elicited hydroxyphaseollin production. As previously observed, inoculation of soybean leaves with race 1 of *P. glycinea* caused accumulation of both coumestrol and hydroxyphaseollin (Table I).

In addition to coumestrol, the isoflavone daidzein and the coumestane sojagol accumulated in ozone-treated soybean leaves (Table II), similar to leaves inoculated with *P. glycinea*.

Doses of ozone from 0.40 to 0.80 $\mu$l/l for 2 hr all resulted in coumestrol accumulation; however, the highest ozone level caused lesser accumulation than did the two lower levels (Fig. 2). This was probably due to the greater extent of initial necrosis and desiccation produced on leaves fumigated with the highest level. The lower doses caused less early necrosis, but produced considerable silvering and stippling of leaves after 20 to 50 hr. Accumulation of coumestrol to high levels occurred at the same time as this "later necrosis" (Fig. 2). A

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similar pattern was observed when plants of various ages were fumigated with ozone. At 48 hr after exposure to 0.6 $\mu$l/l ozone for 2 hr, primary leaves of 9-day-old plants were relatively tolerant to $O_3$ injury and contained only 100 $\mu$g/g coumestrol; 13-day-old plants showed greater toxicity and contained 650 $\mu$g/g coumestrol, whereas 16-day-old plants were injured less and contained 450 $\mu$g/g.

The fact that ozone and to a lesser degree $NO_2$ and $SO_2$ induced the production of considerable coumestrol and daidzein in soybean leaves at the time when stippling and silvering were observed (Table II and Fig. 2) may indicate a cause-effect relationship. Experiments with plants of various ages showed that the degree of stippling and silvering of leaves was positively correlated with levels of the isoflavonoids. Although we did not further test this possibility, it

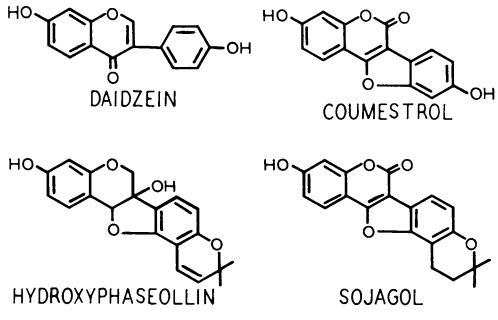


FIG. 1. Structures of soybean isoflavonoids.

Table I. *Hydroxyphaseollin and Coumestrol Levels in Soybean Leaves Exposed to Various Air Pollutants or Innoculated with an Incompatible Race of Pseudomonas glycinea*

Ozone was supplied for 2 hr at 0.60 $\mu$l/l; peroxyacetyl nitrate at 0.4 $\mu$l/l for 2 hr; $NO_2$ at 18 $\mu$l/l for 2 hr; $SO_2$ at 2.4 $\mu$l/l for 2 hr. *P. glycinea* race 1 cells were infiltrated into the leaves in water at $10^7$ cells/ml. All leaves were harvested at 48 hr after treatment.

| Treatment | Hydroxyphaseollin | Coumestrol |
|---|---|---|
| | $\mu$g/g | |
| Untreated control | <5 | 5 |
| Ozone | <5 | 690 |
| Peroxyacetyl nitrate | <5 | 11 |
| Nitrogen dioxide | <5 | 50 |
| Sulfur dioxide | <5 | 25 |
| *P. glycinea* | 1200 | 400 |

Table II. *Levels of Coumestrol, Daidzein, Sojagol, and Hydroxyphaseollin in Soybean Leaves Fumigated with Ozone or Inoculated with an Incompatible Race of Pseudomonas glycinea*

Twelve-day-old Harosoy 63 plants were used, and isoflavonoid extractions were made after 40 hr.

| Treatment | Coumestrol | Daidzein | Hydroxyphaseollin | Sojagol |
|---|---|---|---|---|
| | $\mu$g/g | | | |
| Untreated control | 5 | 50 | <5 | <1 |
| Ozone-fumigated[1] | 500 | 800 | <5 | 35 |
| Inoculated with *P. glycinea*[2] | 350 | 2000 | 810 | 40 |

[1] 0.7 $\mu$l/l ozone for 3 hr.

[2] Race 1 of *P. glycinea* infiltrated at $10^7$ cells/ml.

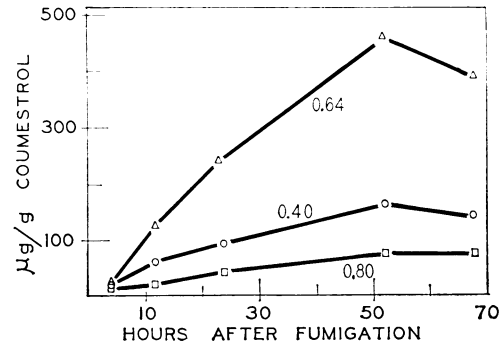


FIG. 2. Concentrations of coumestrol in primary leaves of 16-day-old soybean plants fumigated with three concentrations of ozone ($\mu$l/l) for 2 hr. Control plants that were not fumigated contained 10 $\mu$g/g fresh wt or less coumestrol.

is of interest that accumulation of the isoflavonoids by soybeans and other plants in response to pathogens, insects, or physiologic injury has been related to the occurrence of necrosis (3, 4, 7, 10). It is well accepted that the primary toxic effect of ozone in many plants is probably on membrane permeability (9, 11). We suggest that this effect directly accounted for the early desiccation and necrosis of ozone-treated soybean leaves, observed especially when high doses were used. It is not clear whether permeability alteration was directly responsible for the silvering and stippling of soybean leaves that occurred at times considerably after fumigation, but the permeability effect could conceivably cause these symptoms by triggering the accumulation of isoflavonoids.

In part, our data support the hypothesis that ozone-induced foliar injury in soybeans operates through invocation of the hypersensitive disease-resistance response. The visible appearance of ozone-treated leaves and the accumulation of coumestrol, daidzein, and sojagol are very similar in both ozone-injured and bacterial-inoculated leaves (Table II). A major point of departure, however, is the fact that ozone did not elicit production of the pterocarpanoid compound hydroxyphaseollin, considered a key element in the resistance of soybeans to fungi and bacterial pathogens. Production of coumestrol and daidzein, but not hydroxyphaseollin, in ozone-treated leaves therefore represents the first case in which coordinate production of the isoflavonoid compounds has not been observed in appropriately stressed soybean plants.

We are not aware of previous reports of isoflavonoid accumulation following exposure of plants to ozone. However, Nouchi and Odaira (8) noted that ozone led to accumulation of the anthocyanin cyanidin in morning glory plants, and Koukol and Dugger (6) observed anthocyanin accumulation caused by ozone in *Rumex crispus*. Howell (2) reported the accumulation of several fluorescent compounds in green beans after exposure to ozone, one of which was identified as caffeic acid. These papers and our findings therefore suggest that much of the toxicity associated with stippling and necrosis in ozone-damaged plants may be due to the post-treatment production of flavonoids and other phenolic compounds by the plant.

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