Status of Coumestrol and 4′,7-Dihydroxyflavone in Alfalfa Foliage Exposed to Ozone

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ABSTRACT

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Alfalfa plants, 7 wk old, were exposed to 387 $\mu g/m^3$ (0.20 ppm) ozone for 2.5 hr, and the most severely injured leaves were harvested 48 hr after exposure. In a second experiment, 8.5-wk-old plants were exposed to 580 $\mu g/m^3$ (0.30 ppm) ozone for 2 hr and middle-aged leaves were harvested 0 and 24 hr after exposure. Coumestrol was never detected in ozonized leaves or nonozonized controls from either experiment. At least seven fluorescent compounds accumulated in alfalfa foliage in response to ozone exposure. One of the compounds was identified as 4′,7-dihydroxyflavone. As symptom severity increased, the concentration of 4′,7-dihydroxyflavone in the foliage increased. Foliage exhibited more ozone-induced injury and accumulated higher levels of 4′,7-dihydroxyflavone in trials conducted during December, January, and February than in trials conducted in August.

Coumestrol, an estrogenic flavonoid compound, accumulates in soybean foliage injured by ozone (11). This observation raises concern that ozone injury to leguminous species used for hay or pasture could adversely affect the quality of the feed. Alfalfa (*Medicago sativa* L.), the principal forage legume in the United States, is sensitive to ozone (4,9,12) and accumulates coumestrol and other flavonoids, including 4′,7-dihydroxyflavone (4′,7-DHF), when the foliage is infected by fungi (3,7,14–16).

In this paper, we report on the effect of ozone exposure on the concentration of coumestrol and 4′,7-DHF in alfalfa foliage. Also considered are the relation of flavonoid content to symptom severity and length of time from leaf injury to leaf harvest.

MATERIALS AND METHODS

M. sativa L. 'Buffalo' seed was surface-sterilized with 0.1% mercuric chloride and planted in Jiffy Mix (E. C. Geiger Co., Inc., Harleysville, PA 19438), a peat/vermiculite mixture. Four seeds were planted per 7.6-cm diameter plastic pot and seedlings were thinned to one per pot. Plants were fertilized with 3.8 ml of Peters Peat-Lite Special (Robert B. Peters Co., Inc., Allentown, PA 18101) per 2 L of water 2 wk after seeding and subsequently once per week. Insects were controlled with a weekly spray of Malathion 5E-55 (Ortho-Chevron, San Francisco, CA 94120) or Resmethrin (SBP-1382 EC) (E. C. Geiger Co., Inc.). Plants were grown in a greenhouse with supplemental incandescent and fluorescent lighting of 4 klux and a 15-hr photoperiod during the winter. In experiments between October and February, minimum greenhouse temperatures ranged from 2 to 20 C and maximum from 17 to 34 C; between May and August, minimum and maximum temperature ranges were 11–23 C and 22–39 C, respectively.

Plants were placed in the ozone or control exposure chamber (22) the afternoon before treatment and returned to the greenhouse at 0 or 4–5 hr after ozonization. The chamber was maintained at 21 C and 70% relative humidity, with a 15-hr photoperiod of 25 klux, beginning at 0600 hr. Ozone was produced and monitored as previously described (6). Control plants were subject to identical chamber conditions, with the exclusion of ozone production.

Symptom severity study. This experiment was conducted four times: trials I and II were completed in December and January, respectively, and trials III and IV were completed in August. In trials I, II, and III, 7-wk-old plants were exposed to 387 $\mu g/m^3$ (0.20 ppm) ozone for 2.5 hr. In trial IV plants were exposed to 677 $\mu g/m^3$ (0.35 ppm) ozone for 3 hr. In the four trials, 117, 98, 100, and 100 plants were ozonized, respectively. There were 30 nonozonized control plants in trials I, II, and III, and 35 in trial IV. The foliar symptoms were rated 48 hr after exposure as follows: 1 = no visible injury, 2 = slight stipple on adaxial leaf surface, 3 = chlorosis and/or necrosis, and 4 = extensive necrosis. Only the most severely injured leaves of each plant were harvested and all leaves of a similar rating were pooled for tissue analysis. In trials I and II, samples were taken from the injury severity classes 1 through 4. In trial III samples from classes 1 through 3 were used, whereas only samples from class 4 were used in trial IV.

Time study. Trials I, II, and III were completed in January, February, and July, respectively. When plants were 8.5-wk-old, 30, 72, and 40 plants, in trials I, II, and III, respectively, were exposed to 580 $\mu g/m^3$ (0.30 ppm) ozone for 2 hr. For each trial, 30 plants were maintained as controls. Immediately, and 24 hr after ozone exposure, all middle-aged leaf lamina were harvested from the center one-third of each shoot and were pooled.

Tissue extraction. Replicated 1-g (fresh weight) samples of pooled foliar tissue were cut into small pieces and placed in vials with 15 ml of 80% methanol. After 3 or more days, the samples were filtered and washed. The combined filtrate was reduced in vacuo at 40 C to near dryness, then brought to 2 ml in 80% methanol as previously described (16). This solution is referred to as the crude extract. Additional 1-g samples from both studies were used to determine percent dry weight.

Detection of coumestrol. Ten-microliter portions of crude extracts were spotted on silica gel G plates, 0.75 mm thick, along with samples of authentic coumestrol (0.04 $\mu g/\mu l$). The plates were developed in hexanes/ethyl acetate/methanol (60:40:1 v/v) and viewed under long-wave UV light.

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Identification of 4′,7-DHF. To identify 4′,7-DHF, silica gel G plates, 1.0 mm thick, were streaked with 0.5–2.0 ml of crude extract and developed in toluene/ethyl formate/formic acid (5:4:1). The bright whitish blue fluorescent band was scraped from the plates, eluted with methanol, dried, and redissolved in 8 or 18 ml of methanol. A second purification was performed by descending chromatography on Whatman 3MM paper in 30% acetic acid. After drying for about 18 hr, the fluorescent band was eluted for 10 min in spectroscopic grade methanol. The solution was filtered and centrifuged to remove paper particles, and analyzed immediately with a Gilford 2400-S spectophotometer (Gilford Instrument, Inc., Oberlin, OH 44074). Spectra in methanol, supplemented with five reagents, were obtained as described by Mabry et al (13). Spectra of authentic 4′,7-DHF (provided by A. L. Livingston and B. E. Knuckles, USDA, Western Regional Research Laboratory, Albany, CA 94706) also were obtained. The purified methanol solution of the alfalfa compound and authentic 4′,7-DHF were compared by silica gel thin-layer chromatography (TLC) and by two-dimensional paper chromatography (2-D PC). Solvents for TLC included: hexanes/ethyl acetate/methanol (60:40:1), benzene/ethyl acetate (3:1), toluene/ethyl formate/formic acid (5:4:1), and chloroform/methanol (3:1). Solvents for 2-D PC were tertiary butanol/acetic acid/water (3:1:1) and 15% acetic acid. After development of the paper chromatograms, the color of fluorescence of the compound was noted before and after exposure to ammonia vapors.

Quantification of 4′,7-DHF. Relative concentrations of 4′,7-DHF were estimated by fluorometry. Ten-microliter aliquots of crude extracts were spotted on silica gel plates, 0.75 mm thick. The plates were developed in hexanes/ethyl acetate/methanol (60:40:1). Relative fluorescence of the 4′,7-DHF spots was measured with a photofluorometer as described by Bailey (1), except that we used a Photovolt Model 520-M Photometer (Photovolt Inc., New York, NY 10010) with a 2-mm aperture. From two to 13 samples (usually four to seven samples) were tested for each treatment by trial combination. Data were analyzed by one-way and two-way analyses of variance (17). Square root transformations of the data were necessary because data were nonlinear.

During the latter part of the study when identification of 4′,7-DHF was completed and authentic material was available, TLC plates were spotted with known amounts of authentic material as well as with crude extract samples, to estimate actual amounts of 4′,7-DHF in the samples. The minimum level of detection was 2 μg/g dry wt. A galvanometer reading of 0.5 indicated 11.6 μg of 4′,7-DHF per gram of tissue (dry wt), and a reading of 53.5 indicated about 1,370 μg/g. The relationship of galvanometer deflection to actual amounts of compound was not linear.

RESULTS

Symptoms. Injured leaves exhibited chlorosis or slight necrosis 24 hr after exposure and later more extended necrosis developed. The pattern of injury varied, sometimes occurring on the leaf margins and interveinal areas of small, isolated patches and other times only on tissue adjacent to the veins. Slight injury appeared as a faint, chlorotic stipple on the adaxial surface. Dark, watersoaked spots, seen immediately after ozone exposure, often occurred on foliage on which more severe injury developed later.

In the symptom severity study, percentage of leaves in each injury class varied among plants harvested 48 hr after ozone exposure, but the distributions for trials I, II, and IV were generally similar (Table 1). The foliage from plants in first two trials, completed in December and January, exhibited 87% visible injury (injury classes 2–4). In trial III, completed in August, only 44% of the foliage was visibly injured. Plants in trial IV, also conducted in August but with a higher dosage of ozone, had 79% visibly injured leaves.

In trials I and II of the time study, conducted in January and February, 67% and 81% of the leaves, respectively, were visibly injured (Table 1). Only 46% of the leaves in trial III, conducted in July, showed visible symptoms.

Identification of compounds. Coumestrol was not detected in any sample (control or experimental) from either experiment. The minimum level of detection of coumestrol was 2 μg/g dry wt.

Fluorescence on 2-D paper chromatograms of ozone-injured leaf samples appeared in seven different areas but was not present in crude extract samples from healthy foliage (10). The most conspicuous whitish blue fluorescent area was identified as 4′,7-DHF. When the compound was exposed to ammonia vapors, there was a color change to yellow-green fluorescence under UV light, as reported previously (2,13). The compound extracted from alfalfa gave UV spectra in six reagents that were identical to published spectra (13) and to spectra obtained from an authentic sample (10). The unknown compound and authentic 4′,7-DHF had identical R_f values after silica gel TLC with all solvent systems used: hexanes/ethyl acetate/methanol, R_f = 0.08; benzene/ethyl acetate, R_f = 0.07; toluene/ethyl formate/formic acid, R_f = 0.52; and chloroform-methanol, R_f = 0.85. With 2-D PC, R_f values were 0.83 and 0.09 in tertiary butanol acetic acid/water and 15% acetic acid, respectively. After PC in 30% acetic acid, R_f was 0.42. This compared favorably with other reports of 0.40 and 0.37 (14,21).

Concentration of 4′,7-DHF. Both symptom severity and date of the trial affected the concentration of 4′,7-DHF (Table 2). Two-way analysis of variance of the square roots of individual observations showed that both factors were significant (5% level). Nonozonized controls and ozonized leaves showing no visible injury had either no 4′,7-DHF or relatively low levels. Leaves ozonized in December or January, and showing chlorosis or necrosis, had relatively high levels. Concentrations were highest in severely injured leaves; one sample had approximately 1,370 μg of the flavone per gram of tissue. The leaves treated in August had only low levels of the flavone, however, regardless of symptom severity. In a second

TABLE 1. Percent of alfalfa leaves showing different symptoms of ozone injury in symptom severity and time study trials

Symptoms	Symptom severity study trials[a]				Time study trials[b]		
	I Dec. (%)	II Jan. (%)	III Aug. (%)	IV Aug. (%)	I Jan. (%)	II Feb. (%)	III Aug. (%)
No visible injury	13	13	56	21	33	19	54
Slight stipple	39	18	30	32	29	28	32
Chlorosis and/or necrosis	30	28	12	22	28	28	11
Extensive necrosis	18	41	2	25	10	25	3

[a]Plants were exposed to 387 μg/m^3 (0.20 ppm) ozone for 2.5 hr, but plants in trial IV were exposed to 677 μg/m^3 (0.35 ppm) ozone for 3 hr. Samples were harvested for tissue analysis and symptoms were rated 48 hr after exposure.

[b]Plants were exposed to 580 μg/m^3 (0.30 ppm) ozone for 2 hr. Samples were harvested at 0 and 24 hr for tissue analysis; symptoms were rated 24 hr after exposure.

TABLE 2. Relative fluorescence of 4′,7-dihydroxyflavone extracted from alfalfa leaves showing different symptom severity 48 hr after 2.5 hr exposure to 387 μg/m^3 (0.20 ppm) ozone

Symptoms	Trials[a]		
	II Dec.	II Jan.	III Aug.
Control (no ozone)	1.7[a] (5)[b]	5.1 (5)	0.0 (7)
No visible injury	0.0 (3)	6.5 (3)	1.0 (7)
Slight stipple	5.8 (4)	12.5 (2)	1.7 (6)
Chlorosis and/or necrosis	27.6 (4)	32.5 (5)	1.5 (4)
Extensive necrosis	46.5 (2)	37.0 (3)	...[c]
Standard deviation of means[d]	5.6	2.4	0.6

[a]Galvanometer deflections produced by fluorescent areas on thin layer chromatography plates and quantified with a photometer. Deflection of zero indicates concentration below 2 μg/g (dry wt) or nil. Deflection of 12.5 indicates approximately 100 μg/g of flavone.

[b]Number of samples per value is indicated in parentheses.

[c]Insufficient material for assay.

[d]Pooled standard deviations within the trial.

trial in August, in which plants were exposed to 0.35 ppm ozone for 3 hr to induce extensive necrosis (Table 1), 4′,7-DHF was relatively low in severely injured leaves.

In the time study, nonozonized controls contained no detectable 4′,7-DHF in January and August and low levels of the flavone in February (Table 3). Ozonized plants also had little or no 4′,7-DHF immediately after exposure. In August, a relatively low level of 4′,7-DHF was detected 24 hr after exposure, but in January and February there was a significant increase 24 hr after exposure, compared with content immediately after exposure.

DISCUSSION

The foliar symptom response of individual alfalfa plants varied within a single ozone exposure. Because alfalfa is an open-, insect-pollinated crop, plants are not genetically identical within one cultivar (5), and variable response to ozone would be expected. In general, the symptoms were similar to those reported by others (4,9,12).

The plants in trials I and II of both experiments were more sensitive to ozone than plants in trial III. The variation might be attributable to differences in the greenhouse environment before or after the period of ozone treatment. The plants for trials I and II were grown in the winter when total light energy and day and night temperatures were lower than during the summer when plants for trials III and IV were grown. Growth, temperature, and light quantity and quality influence ozone sensitivity of various plants (8,20).

Although Keen and Taylor (11) detected coumestrol in ozone-damaged soybean leaves, we did not find the compound in alfalfa. The concentrations of coumestrol they detected (11) were within the limits of detection of our methods. Furthermore, the technique for extracting coumestrol was effective because we detected the isoflavonoid in samples of fungal-infected alfalfa foliage (Hurwitz, *unpublished*). Coumestrol has been detected in healthy alfalfa foliage (7,18,19), but absence of the isoflavonoid in control tissue, as we observed, is consistent with other reports (14,16). An earlier report (16) indicated no accumulation of coumestrol in alfalfa leaves exposed to another oxidant, formaldehyde gas.

The observed accumulation of 4′,7-DHF in foliage injured by ozone is consistent with reports that abiotic stresses (heavy metal toxicants) induce accumulation of 4′,7-DHF in alfalfa (15,16). Unlike the reports in which no 4′,7-DHF aglycone was detected in untreated control leaves, we found 4′,7-DHF in some extracts of our control plants. We do not know whether the accumulation of 4′,7-DHF in injured leaves occurred by de novo synthesis or by release of the aglycone from constitutive glycosides of this compound (14). Similar results were obtained with another method in which tissue was frozen in liquid nitrogen and subsequently extracted in boiling methanol (14). We feel confident, therefore, that 4′,7-DHF in control tissue was not an artifact resulting from release of the aglycone during extraction.

In trials I and II of the symptom severity study, there was an apparent positive association between severity of foliar symptoms and concentration of 4′,7-DHF. The association was not seen either in trial III or in severely damaged necrotic class 4 leaves of trial IV. Apparently the environment before and after ozone exposure influence both foliar injury and flavonoid accumulation.

The flavonoid accumulation in response to ozone-induced injury was not solely a function of observable injury. It was possible to induce severe injury by ozone without necessarily inducing large accumulations of 4′,7-DHF, but we never found the latter without injury. The results of the first two trials of the time study suggest that ozone exposure of alfalfa plants for 2 hr induced accumulation of 4′,7-DHF between 1 and 24 hr thereafter. Flavone levels in injured foliage 24 hr after treatment were probably higher than reported. The uninjured middle-aged leaves in the sample diluted the flavone concentration. The lower amounts of 4′,7-DHF in trial III than in trials I and II may be explained by the lower percentage of injured leaves in the pooled samples of trial III than in the first two trials.

In ozonized alfalfa foliage accumulation of six other compounds in addition to 4′,7-DHF has been reported (10). Once these ozone-induced compounds have been identified, we may more readily be able to determine specific biochemical pathways that this oxidant alters in alfalfa. Our results with cultivar Buffalo indicate there should be no concern that ozone will adversely affect the content of the estrogenic compound coumestrol in alfalfa.

TABLE 3. Relative fluorescence of 4′,7-dihydroxyflavone from alfalfa leaves harvested 0 and 24 hr after 2-hr exposure to 580 $\mu g/m^3$ (0.30 ppm) ozone

Treatment	Trials I Jan.	II Feb.	III Aug.
Control			
0 hr	0[a] (4)[b]	4.8 (5)	0 (7)
24 hr	...[c]	4.6 (9)	0 (7)
Ozone			
0 hr	1.2 (7)	5.6 (7)	0 (7)
24 hr	15.4 (13)	27.9 (9)	1 (7)
Standard deviation of means[d]	2.6	3.3	0.2

[a]Galvanometer deflections produced by fluorescent areas on thin layer chromatography plates and quantified with a photometer. Deflection of zero indicates concentration below 2 $\mu g/g$ (dry wt) or nil. Deflection of 15.4 indicates approximately 110 $\mu g/g$.
[b]Number of samples per value is indicated in parentheses.
[c]Insufficient material for assay.
[d]Pooled standard deviation within the trial.

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