# 2nd Annual Progress Report

on

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by

Eva J. Pell, Associate Professor
of Plant Pathology
THE PENNSYLVANIA STATE UNIVERSITY
226 Fenske Laboratory
University Park, Pennsylvania 16802

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## Introduction

During the last two years we have been conducting studies to determine the effect of acute, toxic exposures of ozone to alfalfa, potato and soybean plants. The objective has been to correlate the foliar response with alterations in quality of the edible portion of the plant viz. the leaf, tuber and seed of alfalfa, potato and soybean, respectively.

In 1977 we (1) modified our fumigation facilities, (2) developed protocol for studies with alfalfa and potato and (3) conducted studies on flavonoid status of alfalfa and a series of parameters of potato tubers. In 1978 we (1) conducted more indepth studies with alfalfa, (2) repeated the potato study, (3) began to develop protocol for measuring additional parameters of alfalfa and potato quality and (4) developed protocol for cultivating and exposing soybean plants.

## Progress

Alfalfa        In 1977 we demonstrated that ozone did not induce the estrogenic isoflavonoid, coumestrol, in the foliage of Buffalo cultivar of alfalfa. Another flavonoid, 4',7-dihydroxyflavone, was induced in the foliage of alfalfa by ozone. The intensity of the symptom was directly correlated to the degree of visible ozone injury to the foliage.

The observations above, which have been confirmed for 'Buffalo', have important implications for ozone phytotoxicology. Coumestrol can have deterious effects on pasture crops. It has been reported that ozone induces coumestrol production in soybean foliage. The inability of ozone to induce coumestrol formation in alfalfa foliage can be viewed positively. The induction of 4',7-dihydroxyflavone by ozone is undoubtedly related to symptom development. This compound is a flavonoid. Other flavonoids are also induced by ozone and if we could understand the role which ozone plays in stimulating intermediary metabolism we might be able to explain the pathway of ozone toxicity.

The implications of the observations cited above made it imperative that we ascertain that these changes were characteristic of alfalfa and not unique to one cultivar viz. 'Buffalo'. A series of experiments were conducted in which the impact of ozone on concentration of coumestrol and 4',7-dihydroxyflavone in four cultivars of alfalfa viz. Medicago sativa L. 'Moapa', 'Sonora', and 'Ladak' and Medicago sativa L. x Medicago falcata L. 'Vernal' were studied. The protocol for exposure and analysis of tissue were enumerated in the 1st Annual Progress Report, January 1978 and in two manuscripts which have been accepted for publication (see Hurwitz et al. and Skärby and Pell cited at the end of this report). When cultivars were exposed to 0.40 ppm ozone for 3 hours foliar necrosis was induced. Forty-eight hours after exposure, all foliage exhibiting injury was harvested for analysis of flavonoid status. Injured tissue was selected for study since we had previously shown optimal response to visibly affected tissue. Coumestrol was not induced in foliage of any of the cultivars. 4',7-dihydroxyflavone was induced in all four cultivars (Table 1). Concentration of the flavonoid was not statistically different in any of the cultivars. While severity of foliar injury prevented statistical separation of susceptibility of the four cultivars, it appeared that 'Moapa' and 'Sonora' sustained higher levels of ozone injury than did 'Ladak' or 'Vernal' cultivars.

Table 1. Concentration of 4',7-dihydroxyflavone (ppm dry weight) in alfalfa foliage from four cultivars, 48 hr after exposure to 773 $\mu g/m^3$ (0.40 ppm) ozone for 3 hr.[a]

| Cultivar | Trial[b] I | Trial[b] II | Trial[b] III |
|---|---|---|---|
| 'Ladak' | 60 ± 16[c] | 50 ± 19 | 42 ± 17 |
| 'Sonora' | 41 ± 16 | 64 ± 28 | 35 ± 11 |
| 'Vernal' | 43 ± 18 | 47 ± 3 | 46 ± 19 |
| 'Moapa' | 44 ± 20 | 53 ± 15 | 47 ± 20 |

[a]Nonozonized control tissue was analyzed in conjunction with ozonized tissue. 4',7-DHF was never detected in control tissue.

[b]Each value is the mean of four separate analyses. Means for each cultivar and trial are similar based on an analysis of variance (P = 0.05).

[c]Standard deviation.

Early in our efforts to detect coumestrol in ozonized alfalfa foliage, we observed induction of many compounds which had some characteristics of flavonoids. Since some flavonoids in addition to coumestrol are estrogenic, we thought important to determine whether any of them might be present in higher concentrations in alfalfa foliage exposed to ozone. 'Moapa' was selected for further studies. This cultivar was quite susceptible to ozone and grew well under our regimen. Through a series of thin layer and paper chromatography trials we have been testing the hypothesis that ozone induces daidzein, genistein and formononetin in injured leaves of 'Moapa'. The former two compounds are apparently not induced. One compound appears similar to formononetin. Fluorescent color and $\underline{R}_F$ values, with a number of solvent systems, are consistent for this unknown compound, extracted from ozonized alfalfa foliage, and authentic formononetin. We are presently purifying the unknown compound in order to compare its ultraviolet spectrum with that of authentic formononetin. If the compound should be formononetin it will signify the ability of ozone to induce increased concentrations of an estrogenic isoflavonoid in an important pasture crop.

Potato In 1977 the protocol for studying ozone impact on tuber quality was developed and has been enumerated in the 1st Annual Progress. Some data from the first experiment appeared in that Report. In 1978 the experiment was repeated and additional data is enumerated in Tables II and III. As in 1977 tuber weight and number, and total solids were reduced in both cultivars in response to ozone exposure. Sugars were separated, identified and quantified as described in the first Progress Report. Results have been reported on a wet and dry weight basis (Table III). Ozone apparently stimulated elevated levels of each of the reducing sugars

Table II. Quantitative and qualitative parameters of tubers harvested from 'Norland' and 'Kennebec' potato plants exposed to 0.20 ppm ozone for 3 hr once every 2 weeks for 110 and 130 days, respectively[a,b,c].

| | Norland | | | Kennebec | | |
|---|---|---|---|---|---|---|
| | $+O_3$ | | $-O_3$ | $+O_3$ | | $-O_3$ |
| wet wt (g) | 8,664 | * | 12,075 | 4,748 | * | 6,803 |
| # of tubers | 70 | * | 90 | 44 | * | 65 |
| total solids (% dry matter) | 14.59 | * | 15.96 | 14.88 | * | 17.16 |
| total glycoalkaloid (mg/100 g wet wt) | 2.14 | * | 3.45 | 2.57 | | 2.81 |
| Total glycoalkaloids mg/g dry wt | 0.14 | | 0.17 | 0.17 | | 0.16 |

[a]Experiment conducted between March and August, 1978.

[b]Each number is a mean of ten separate determinations

[c]an * denotes significance at the $P = 0.05$ level based on an unpaired "t" test

Table III. Concentration of major sugars in tubers of 'Norland' and 'Kennebec' plants exposed to 0.20 ppm ozone for 3 hours once every second week for 110 and 130 days respectively [a,b,c].

| | 'Norland' | | | | | | | | 'Kennebec' | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1977 | | | 1978 | | | | | 1977 | | | 1978 | | | | |
| | $+O_3$ | | $-O_3$ | $+O_3$ | | $-O_3$ | | | $+O_3$ | | $-O_3$ | $+O_3$ | | $-O_3$ | | |
| **mg sugar/g fresh weight** | | | | | | | | | | | | | | | | |
| Fructose | 0.90 | | 0.98 | 0.21 | * | 0.10 | | | 0.86 | | 0.72 | 0.13 | * | 0.08 | | |
| α-glucose | 0.47 | | 0.44 | 0.21 | * | 0.13 | | | 0.30 | | 0.22 | 0.12 | | 0.11 | | |
| β-glucose | 0.69 | * | 0.58 | 0.30 | * | 0.21 | | | 0.66 | | 0.47 | 0.23 | | 0.19 | | |
| Sucrose | 0.79 | | 0.66 | 1.24 | | 1.27 | | | 0.28 | | 0.30 | 0.97 | | 1.08 | | |
| **mg sugar/g dry weight** | | | | | | | | | | | | | | | | |
| Fructose | 8.54 | | 7.87 | 1.42 | * | 0.61 | | | 7.79 | | 5.93 | 0.88 | * | 0.49 | | |
| α-glucose | 4.50 | * | 3.48 | 1.43 | * | 0.86 | | | 2.75 | | 1.63 | 0.84 | | 0.64 | | |
| β-glucose | 6.61 | * | 4.65 | 2.05 | * | 1.35 | | | 6.00 | | 3.91 | 1.52 | * | 1.12 | | |
| Sucrose | 7.32 | * | 5.19 | 8.54 | | 7.94 | | | 2.48 | | 2.50 | 6.80 | | 6.85 | | |

[a]Experiments were conducted between May and October, 1977 and March and August, 1978.

[b]Values are means of 33 and 38 analyses of Norland samples for 1977 and 1978, respectively and of 9 and 36 analyses of Kennebec samples for 1977 and 1978, respectively.

[c]*denotes significance at the $P = 0.05$ level based on unpaired "t" test.

viz. α-glucose, β-glucose and fructose, in tubers of both cultivars in 1977 and 1978; this increased concentration appeared whether results were reported on a wet or dry weight basis. Because of variation, statistical significance was not always apparent. Significant increases were more apparent for the 'Norland' cultivar. Since the foliage of this cultivar was more ozone susceptible than that of 'Kennebec', the greater magnitude of the increase in reducing sugars was predictable. Since increases in total reducing sugars lower tuber quality, we contrasted the levels in ozone exposed versus nonexposed plants. When expressed on a dry weight basis, the total reducing sugar content of tubers of ozone exposed plants was significantly ($P = 0.05$) greater than of nonexposed plants in both cultivars in both years. In 1978 but not 1977, total reducing sugars were significantly greater in both cultivars when concentrations were expressed on a wet weight basis. Sucrose levels were unaffected in all but one case. Sucrose content, expressed on a dry weight basis, increased significantly in 'Norland' tubers exposed to ozone in 1977.

Glycoalkaloid levels were measured in the tubers and results were reported on a wet and dry weight basis (Table II). On a wet weight basis, glycoalkaloids decreased significantly ($P = 0.05$) in tubers of 'Norland' plants exposed to ozone. No other effects were observed.

Soybean Seeds of 'Chippewa' were cultivated in 10" diameter pots on April 20, 1978 in a 2:1 sand:mushroom composte soil. All seeds were innoculated with Rhizobium japonicum and root nodulation was later verified. Plants were fertilized weekly with a 9:45:15 (NPK) mixture. A systemic insecticide-disyston was applied to the soil initially. We verified that disyston did not influence sensitivity of soybean plants to ozone.

Twenty plants were exposed to 0.25 ppm ozone for 3 hours every second week beginning May 9, 1978. Twenty additional plants were maintained in the greenhouse as controls. In mid July ozone exposures ceased and in mid August pods were harvested and yield quantified.

Ozone exposure caused a characteristic fleck of susceptible foliage at each fumigation. The symptoms were uniform for all exposed plants. Ozone significantly reduced the weight and number of seeds per plant and number of pods per plant (Table IV). Only number of seeds per pod were not affected. Our results are consistent with published efforts. The seeds have been stored for analysis of lipids, proteins and carbohydrates.

Table IV. Impact of ozone on yield of soybean plants[a,b,c].

| | $+O_3$ | | $-O_3$ |
|---|---|---|---|
| total weight of seeds (g)/plant | 18.39 | * | 28.78 |
| # seeds/plant | 124.10 | * | 164.30 |
| # pods/plant | 54.70 | * | 72.45 |
| # seeds/pod | 2.28 | | 2.15 |

[a]Plants were exposed to 0.25 ppm ozone for 3 hr once every two weeks from May - June, 1978.

[b]Each number is a mean from 20 plants.

[c]* denotes significance at the P = 0.05 level based on an unpaired "t" test.

2 & 3. Deferred and Additional Efforts

Alfalfa Research Confirmation of the nature of the compound which is hypothesized to be formononetin will be completed.

We have begun to establish the protocol for conducting electrophoretic studies to determine qualitative changes in major proteins of alfalfa. While there are reports that total protein levels of alfalfa are reduced by ozone, no one has considered the impact which the gas may have on the kinds of protein produced.

Potato Since reducing sugars increased it is reasonable to speculate that starches decreased. This speculation is supported by the reduction in total solids which was measured on the same tubers. Samples from tubers analyzed in the 1978 experiment have been lyophilized and will be subject to starch analysis. The protocol for this analysis has been receiving major attention for the last few months. When we are satisfied that the method is quantitative we will analyze the samples. Another constituent of total solids is protein; while not as important as starch, protein can be a valuable factor in tubers. Hence, we intend to quantify total protein in these samples by the Coumassie blue technique.

Soybeans A second set of plants will be grown and exposed to ozone this summer. Photoperiod and light quality apparently influence soybean susceptibility to ozone to a great extent. Hence, we felt it would be preferrable to conduct a second experiment at the same time of year as the 1978 experiment cited earlier in this report. The seeds will be analyzed for lipids, proteins and carbohydrates. The development of the protocol for these analyses will constitute a major portion of the 1979-1980 research effort.

Manuscripts

Hurwitz, B. 1978. Influence of ozone on concentration of coumestrol and 4'7-dihydroxyflavone in alfalfa foliage. M.S. Thesis, The Pennsylvania State University, University Park. 36 p.

Hurwitz, B., E. J. Pell and R. T. Sherwood. 1977. Does ozone induce coumestrol formation in alfalfa. Proc. Amer. Phytopathol. Soc. 4:88.

Skärby, L. and E. J. Pell. Concentrations of coumestrol and 4',7-dihydroxyflavone in four alfalfa cultivars after ozone exposure. J. Environ. Quality (Tentative acceptance for publication pending revision).

Hurwitz, B., E. J. Pell and R. T. Sherwood. Status of coumestrol and 4',7-dihydroxyflavone in alfalfa foliage exposed to ozone. Phytopathology (accepted pending revision).

Pell, E. J., W. C. Weissberger and J. Speroni. Altered tuber quality of potato plants exposed to ozone. (Abstract). To be presented at the National American Phytopathological Society meeting in Washington, D. C. - August, 1979. Subsequent publication of the abstract will appear in Phytopathology.