Symptom Expression and Free Sterol and Fatty Acid Composition of Flue-cured Tobacco Plants Exposed to Ozone

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ABSTRACT

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Two flue-cured tobacco cultivars, North Carolina 88 and Coker 86, were fumigated with 0 to 0.30 ppm ozone (O_3) for 6 hr. Both cultivars were highly sensitive to O_3. Plants fumigated with concentrations of O_3 greater than 0.15 ppm displayed typical weather fleck symptoms. Fumigation for 6 hr with 0.25 or 0.30 ppm O_3 resulted in a significant increase in the lipid concentration but a decrease in the concentration of free sterols and triglyceride fatty acids in all leaf tissues. These changes were not correlated with injury ratings. It is postulated that triglyceride fatty acids and other cellular components were being utilized for membrane repair.

Genetic variability of ozone (O_3) sensitivity exists among and within plant species and that of *Nicotiana tabacum* cultivars has been studied and reported perhaps more than of any other species (7,12).

Several reports have been published concerning the effects of O_3 on the lipid components of plants; in pinto bean exposed to O_3, there was a decrease in total free sterols (17,23) and a simultaneous increase in sterol glycosides and acylated sterol glycosides (23). Analysis of individual sterols revealed that only a change in the cholesterol content was significant (17). Application of cholesterol or benzimidazole to the rooting medium prior to exposure significantly decreased the sensitivity of bean to O_3, but application of a steroid inhibitor significantly increased sensitivity. Neither treatment, however, significantly affected foliar sterol levels. Spotts et al (17) concluded that cellular changes were the result of altered membrane permeability.

Tomlinson and Rich (23) reported an increase in linolenic acid concentration in ozonized bean leaves. They (21) also reported a 50% loss of saturated fatty acids from tobacco leaves exposed to high concentrations of O_3. Tomlinson and Rich (23) proposed that synthesis of bound sterols occurred at the expense of essential diglycerides. Mudd et al (14) found that, in an in vitro system, synthesis of digalactosyl and trigalactosyl diglycerides was inhibited both by sulfhydryl reagents and O_3.

This study was conducted to determine the O_3 sensitivity of two flue-cured tobacco cultivars and to characterize changes in certain major lipid classes following O_3 fumigation.

MATERIALS AND METHODS

Determination of injury threshold and characterization of response under controlled fumigation. Two flue-cured tobacco cultivars, North Carolina 88 (NC 88) and Coker 86, were used. Plants were grown as previously described (11) and maintained, with daily watering, in a charcoal-filtered greenhouse at a mean temperature of 25 C. When the fifth or seventh leaf had reached a length of 3 cm, plants were exposed once to a specific concentration of O_3 for 6 hr. Fumigations were initiated at 1000 hr in the fumigation chambers described previously (6).

Ozone was generated by ultraviolet light (Ultra-Violet Products Inc., San Gabriel, CA 91778) and measured with a Bendix Chemiluminescence Ozone Monitor Model 8002 (Bendix Corp., Ronceverte, WV 24970). The O_3 monitor was calibrated with 1% neutral-buffered KI (1). Fumigation chambers were illuminated with high-pressure sodium lamps (Harvey Hubbell Inc., Lighting Division, Christiansburg, VA 24073) which provided 28–30,000 lux at the plant surface. The temperature and relative humidity in each chamber were recorded hourly during fumigation. The mean temperature in all chambers was 25 C and the mean relative humidity was 66%. Ozone concentrations were administered in increments of 0.05 ppm with 0.05 and 0.10 ppm, 0.15 and 0.20 ppm, and 0.25 and 0.30 ppm in individual fumigations. The 0.05, 0.10, 0.15, 0.20, 0.25, and 0.30 ppm O_3 were equivalent to 98, 196, 294, 392, 490, and 588 $\mu g/m^3$ ozone, respectively, at 25 C, 760 mm Hg. On the third day following fumigation, leaves were numbered consecutively from the base of the plant and those exhibiting fleck were rated for injury. The injured area of each leaf was expressed as a percentage of the total leaf area (0–100% in 5% increments). Data from all fumigation experiments represented combined effects of six individual exposures of three plants each of both cultivars.

Chromatographic determination of free sterols and fatty acids. To determine the effects of O_3 injury on the lipid content of the two cultivars, plants were fumigated with 0.25 or 0.30 ppm O_3 for 6 hr. Three days after fumigation, when maximum symptom expression was observed, the leaves of each cultivar were removed and separated into top (T), middle (M), and bottom (B) groups. All leaf tissue was quick-frozen, freeze-dried for 72 hr, then ground in a Wiley mill to pass a 0.97-mm (20-mesh) screen. Samples were stored in a desiccator at −20 C until analyzed.

Milled samples (approximately 200 mg) were placed in tared Whatman No. 1 filter paper and inserted into prewashed cellulose extraction thimbles. Samples were extracted with 90 ml of 2:1 chloroform-methanol ($CHCl_3$-MeOH) in Soxhlet extractors for 8 hr.

After cooling, extracts were concentrated in vacuo at 35 C, dissolved in $CHCl_3$, and filtered through Whatman No. 1 filter paper. The filtered extracts were transferred to screw-cap test tubes and then evaporated to dryness under nitrogen (N_2) at 35 C. Approximately 100 μl of hexane was added to dried extracts and the solubilized extracts were applied to 0.5 mm thick silica gel G thin layer plates (Applied Science Laboratories Inc., State College, PA 16801). Chromatography of extracts and standards was performed with hexane/diethyl ether/acetic acid (80:20:1, v/v). After development, the plates were sprayed with 2′,7′ dichlorofluorescein and viewed under long wave UV light. Fluorescing lipid bands were located by parallel chromatography with known lipid standards, scraped from the plate, extracted with diethyl ether, and filtered through a sintered glass funnel. These

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extracts were stored in the dark at 4 C.

Major lipid fractions separated by thin layer chromatography, were analyzed with a Bendix Model 2600 (The Bendix Corporation, Lewisburg, WV 24901) gas chromatograph and peak areas were determined with a Spectra Physics (Spectra Physics, Santa Clara, CA 95051) minigrator.

Chloroform was added to dry sterol samples which were transferred to 1-ml reaction vials and evaporated under N_2. Trimethylsilyl (TMS) derivatives of free sterols were prepared by adding 60 μl of BSA[N, O-*bis* (trimethylsilyl) acetamide] (Supelco Inc., Bellefonte, PA 16823) and heating the vials at 55 C for 1 hr. Sterols were separated on a 90 × 2 cm (OD) glass column packed with 3% SE-30 on chromosorb Q (Applied Science Laboratories Inc.). Samples were run isothermally at 245 C. Sterol concentrations were expressed as equivalents of an external standard containing TMS derivatives of the four major free sterols of tobacco tissue: cholesterol, campesterol, stigmasterol, and sitosterol.

Volatile derivatives of free fatty acids were prepared by adding 3 ml of boron trichloride-methanol (BCl_3-MeOH) (Applied Science Laboratories Inc.) to dry samples in screw-cap test tubes. Tubes were heated at 55 C for 5 min, and after cooling, an equal amount of hexane was added and the mixture was shaken briefly. A double layer formed; the top hexane layer was drawn off. This procedure was repeated three times. The hexane extracts were combined, evaporated to dryness in N_2, and redissolved in 60 μl of hexane. Separation of fatty acid derivatives was accomplished with a glass column as described above and packed with 10% EGS on 80/100 chromosorb W AW (Supelco Inc.). Samples were run isothermally at 170 C and concentrations were expressed as methylstearate equivalents.

For the methanolysis of triglycerides a modified method described by Morrison and Smith (13) was employed. Three milliliters of BCl_3-MeOH was added to a dry sample of triglycerides, heated for 45 min at 100 C, and after cooling, 7 ml of hexane and 3 ml of distilled water were added. The mixture was shaken briefly, then centrifuged for 15 min at 6,000 *g* until both layers were clear. The top layer of hexane containing methylesters was removed, placed in a screw-cap test tube, and treated as outlined above.

RESULTS

Response under controlled fumigation and determination of injury threshold. Both NC 88 and Coker 86 plants were sensitive to O_3. Under conditions of this study fleck symptoms were first observed when the plants were fumigated for 6 hr with 0.15 ppm O_3. Coker 86 was, in general, more sensitive to ozone than NC 88.

Fumigation with 0.05 or 0.10 ppm O_3 resulted in no visible injury. Although plants were sensitive at 0.15 ppm, injury was minimal. Fumigations at 0.20 ppm O_3 resulted in increased numbers of injured leaves and a higher percent leaf surface damaged than at lower concentrations. However, only two or three leaves were injured and 10–15% of the leaf surface was damaged. When the plants were exposed to 0.25 ppm O_3, typical weather fleck symptoms were observed. The oldest leaves at this concentration exhibited fleck symptoms at their base. The most recently maturing leaves had fleck symptoms uniformly distributed over the leaf surface. Young, expanding leaves were sensitive only in the area of the leaf apex at the margins. Fumigations at 0.30 ppm resulted in irregular distribution of symptoms over the surface of nearly expanded leaves. The oldest and the recently matured leaves, as well as the young nearly expanded leaves, had symptoms that were characterized by the presence of bifacially necrotic lesions.

Chromatographic determination of free sterols and fatty acids. Both nonfumigated and fumigated Coker 86 plants had significantly higher concentrations of total extractable lipid as percent dry weight than comparable foliage of NC 88 plants (Table 1). Sterol as percent dry weight did not differ significantly between the two cultivars. Within each treatment there were no differences in lipid as percent dry weight with respect to stalk position. Top leaves of both nonfumigated and fumigated plants contained higher sterol concentrations expressed as percent lipid than M or B leaves. Fumigation with either 0.25 or 0.30 ppm O_3 increased the lipid content of tobacco leaves, but decreased the sterol content expressed either as percent dry weight or as percent lipid. The most drastic changes occurred in M and B leaves, where a 48–57% reduction in sterols as percent dry weight or as percent lipid was recorded.

Cholesterol, campesterol, stigmasterol, and sitosterol were the major sterols identified (Table 2). Nonfumigated NC 88 contained a significantly higher level of sterol, expressed as μg sterol/mg lipid, than nonfumigated Coker 86. In nonfumigated tissue, T leaves contained the highest concentration of total free sterols, followed by M and then B leaves. Fumigation with 0.30 ppm O_3 resulted in a 40–57% reduction in total free sterol concentration in leaves of both cultivars. The largest reduction occurred in the stigmasterol concentration (44–65%), but a reduction (19–52%) of the other three sterols also occurred.

The relative proportions of individual free fatty acids were generally consistent for all samples. Palmitic, stearic, oleic, linoleic, and linolenic acids comprised 70% of the total free fatty acids extracted from tobacco leaf tissue. Concentrations of the free fatty acid were, however, highly variable between repetitions of a particular treatment and among replications within treatments. No conclusions could be made with respect to the effects of O_3 on free fatty acid concentration.

Five major fatty acids were detected in the triglyceride fraction significant differences were found with respect to cultivar, fumigation, and leaf position (Table 3). Nonfumigated NC 88 had a significantly higher level of fatty acids than nonfumigated Coker

TABLE 1. Total lipid and free sterol content of North Carolina 88 (NC 88) and Coker 86 tobacco leaves after 6 hr of exposure to ozone

		NC 88 at ozone conc:			Coker 86 at ozone conc:		
	Leaf position	0 Control	0.25 ppm	0.30 ppm	0 Control	0.25 ppm	0.30 ppm
Lipid % dry wt.	T	6.3 B[a]	6.1 B	8.1 A	7.5 C	12.8 A	9.6 B
	M	7.3 B	8.8 A	7.0 B	7.2 B	11.0 A	8.1 B
	B	6.7 B	8.1 A	8.3 A	6.6 B	10.1 A	8.8 B
	Average	6.8	7.7	7.8	7.1	11.3	8.8
Sterol % dry wt. ($\times 10^{-2}$)	T	20.9 A	12.6 B	12.1 B	21.2 A	20.3 A	15.9 B
	M	22.7 A	10.8 B	9.8 B	19.9 A	14.2 B	11.7 C
	B	18.9 A	11.5 B	10.4 B	15.0 A	15.5 A	11.1 B
	Average	20.8	11.6	10.7	18.7	16.6	12.9
Sterol % lipid ($\times 10^{-1}$)	T	34.0 A	22.1 B	14.6 C	28.7 A	15.5 C	17.2 B
	M	31.6 A	13.6 B	13.6 B	27.6 A	12.8 C	14.4 B
	B	29.0 A	12.3 B	12.7 B	24.5 A	12.2 B	12.8 B
	Average	31.5	16.0	13.6	26.9	13.5	14.8

[a] Within a row, means for each cultivar followed by the same letter were not significantly different at $P = 0.05$ according to Duncan's multiple range test.

86. Bottom leaves of nonfumigated plants contained appreciably higher levels of fatty acids than M or T leaves. At 0.25 and 0.30 ppm O_3 the concentrations of all five fatty acids were reduced. There was an overall disproportionate decrease in fatty acid concentration of B leaves compared to that of M or T leaves of plants fumigated with 30 ppm O_3.

DISCUSSION

This study confirmed that both NC 88 and Coker 86 are highly sensitive to O_3. Field observations in Virginia have shown that these cultivars are among the most sensitive of all flue-cured cultivars (12,19, and J. J. Reilly and L. D. Moore, *unpublished*). Both NC 88 and Coker 86 are recent releases which have moderate to high levels of resistance to the major fungal and bacterial pathogens (8) Coker 86, furthermore, is resistant to tobacco mosaic virus. There is a need to incorporate air pollution tolerance into these commercial cultivars. The need is further demonstrated by the fact that growth rate, value, and yield of flue-cured tobacco are inversely correlated with severity of weather fleck damage (12). Increases in total lipid concentrations of plants fumigated with O_3 have not been reported previously, although several researchers have reported changes in the sterol and fatty acid content of ozonized plants (10,14,16–18,21, 23,24). The increase in lipid expressed as percent dry weight occurred in both cultivars at both O_3 dosages, although differences between fumigation with 0.25 and 0.30 ppm O_3 were not always significant. The increase in lipids could not be associated with increased concentrations of free sterol or triglyceride fatty acids because the concentrations of these two groups of compounds

TABLE 2. Effect of 6 hr exposure to ozone on individual free sterol levels of North Carolina 88 (NC 88) and Coker 86 tobacco plants

		Free sterols (μg sterol/mg lipid [$\times 10^{-1}$]) in tobacco cultivars:					
		NC 88 at ozone conc:			Coker 86 at ozone conc:		
Sterol	Leaf position	0 Control	0.25 ppm	0.30 ppm	0 Control	0.25 ppm	0.30 ppm
Cholesterol	T	1.6 A[a]	1.7 A	1.2 B	1.1 A	0.7 B	0.7 B
	M	1.5 A	0.9 B	1.0 B	1.2 A	0.6 B	0.8 B
	B	1.7 A	0.9 B	0.8 B	1.0 A	0.7 B	0.6 B
Campesterol	T	2.1 A	2.1 A	1.6 B	1.5 A	0.9 B	1.1 B
	M	2.4 A	1.4 B	1.4 B	1.4 A	0.8 B	1.2 B
	B	2.4 A	1.3 B	1.3 B	1.2 A	1.0 B	0.9 B
Stigmasterol	T	22.3 A	14.5 B	7.9 C	21.6 A	11.1 B	12.1 B
	M	21.9 A	10.2 B	8.3 C	21.1 A	9.5 B	9.7 B
	B	19.7 A	9.1 B	8.0 C	19.3 A	12.2 B	9.2 C
Sitosterol	T	7.9 A	5.9 B	4.0 C	4.5 A	2.8 C	3.2 B
	M	5.9 A	2.6 B	3.0 B	3.9 A	2.2 B	2.7 B
	B	5.2 A	2.3 B	2.5 B	3.0 A	2.2 B	2.1 B
Totals	T	34.0	24.2	14.6	28.6	15.5	17.2
	M	31.6	15.1	13.7	27.6	13.1	14.4
	B	29.0	13.6	12.7	24.5	16.2	12.8

[a]Within a row, means for each cultivar followed by the same letter were not significantly different at $P = 0.05$ according to Duncan's multiple range test.

TABLE 3. Effect of 6 hr exposure to ozone on individual fatty acids hydrolyzed from triglycerides of North Carolina 88 (NC 88) and Coker 86 tobacco plants

		Fatty acids (μg fatty acid/mg lipid [$\times 10^{-1}$]) in tobacco cultivars:					
		NC 88 at ozone conc:			Coker 86 at ozone conc:		
Acid	Leaf position	0 Control	0.25 ppm	0.30 ppm	0 Control	0.25 ppm	0.30 ppm
Palmitic	T	19.7 A[a]	12.6 B	7.4 C	13.2 A	8.9 B	5.8 C
	M	15.6 A	5.9 C	8.3 B	20.2 A	5.5 B	6.3 B
	B	27.0 A	7.8 C	10.4 B	27.4 A	10.4 B	3.9 C
Stearic	T	6.7 A	3.7 B	2.6 B	6.3 A	2.7 B	2.5 B
	M	9.1 A	2.1 B	2.3 B	9.0 A	2.5 B	1.3 C
	B	10.8 A	2.6 B	2.5 B	9.8 A	1.8 B	2.1 B
Oleic	T	9.4 A	5.3 B	2.5 C	5.8 A	3.3 B	1.0 C
	M	10.0 A	1.8 B	2.3 B	4.8 A	1.7 B	1.2 B
	B	18.9 A	1.9 B	2.6 B	11.6 A	2.5 B	1.3 C
Linoleic	T	12.5 A	9.4 B	3.6 C	11.4 A	3.4 B	0.7 C
	M	11.0 A	3.5 B	3.1 B	12.2 B	2.3 B	2.5 B
	B	17.6 A	3.5 B	2.6 B	12.7 A	2.4 C	4.3 B
Linolenic	T	14.2 A	14.8 A	5.6 B	11.5 A	6.7 B	3.6 C
	M	15.0 A	4.9 B	6.1 B	12.9 A	3.6 C	6.0 B
	B	31.2 A	11.4 B	5.3 C	19.9 A	9.7 B	2.8 C
Totals	T	62.6	45.9	21.7	48.3	25.1	13.6
	M	60.7	18.2	22.2	59.2	15.6	17.4
	B	105.5	27.2	23.4	81.6	26.8	14.5

[a]Within a row, means for each cultivar followed by the same letter were not significantly different, $P = 0.05$, according to Duncan's multiple range test.

decreased in fumigated plant tissues. The increase in lipids in ozonized tissue may have been an injury response; the plants may have responded, in part, by increasing lipid synthesis. Lösel (9) reported that rust infection (*Puccinia poarum* Niels.) caused an accumulation of lipid in Kentucky bluegrass leaves.

The decrease in free sterols represented what appeared to be a general response by all leaf tissue regardless of stalk position or degree of injury. Although leaves in the T group exhibited no symptoms of O_3 damage, the reduction in sterol concentration was equal to that in the B leaf tissue. At both levels of fumigation the M group of leaves not only had the highest injury rating but also the largest loss of sterol expressed as percent dry weight. Decreases in total free sterols in ozonized tobacco have been reported previously (23,24). Menser et al (10) proposed that oxidants produced the same sterol changes in tobacco as those observed in bean.

Spotts et al (17) reported a significant reduction only in the cholesterol concentration in ozonized bean plants. In our study a significant reduction in all four major free sterols occurred. In fact, there was a greater reduction in stigmasterol concentration than in the concentration of the other three sterols. Reduction in concentration of cholesterol, the sterol most often associated with membrane stability (3,4) was greater in the M and B leaf tissue in which the largest amount of O_3 injury occurred. The loss of free sterols, particularly cholesterol, is most likely related to the loss of membrane permeability that occurs in ozonized plant tissue (2,15,16,18,20,25).

Ozonization of tobacco caused an appreciable reduction in the concentration of fatty acids hydrolyzed from triglycerides. Both saturated and unsaturated fatty acid concentrations were reduced appreciably. Swanson et al (18) and Tomlinson and Rich (22), on the other hand, recorded little alteration in the total fatty acid concentration of tobacco and pinto bean fumigated with 0.25 or 0.30 ppm O_3 for 2 or 3 hr. In a previous paper, however, Tomlinson and Rich (21) reported a 50% loss in total saturated fatty acids and a 10% loss in unsaturated fatty acids in tobacco fumigated with 1.0 ppm O_3 for 0.5 to 1 hr. Because bottom leaf tissue not only had the greatest reduction in fatty acids, but the highest injury rating, it is possible that triglyceride fatty acids are being employed by the plant for membrane repair. The interconversion of triglycerides and polar lipid is well documented (5).

This investigation demonstrated that significant changes in tissue composition occurred even when the tissue exhibited no obvious symptoms of O_3 damage. More detailed studies are needed, however, to determine the effect of air pollution on various lipid components.

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