Analysis of Tobacco for the *Alternaria* Toxins, Alternariol and Alternariol Monomethyl Ether

Forty samples of harvested and stored flue-cured tobacco obtained from four tobacco producing states during 1966 through 1970 were analyzed by gas–liquid chromatography for the presence of alternariol and alternariol monomethyl ether (AME). None of the collected samples revealed the presence of either mycotoxin. However, inoculated tobacco did contain small amounts of both toxins. Apparently these two toxins play little or no significant role as components of marketed flue-cured tobacco.

Brown spot, the most important disease of tobacco in North Carolina, is caused by *Alternaria alternata* (Fries) Keissler (previously *A. tenuis*) (Lucas, 1971). The fungus attacks plants before harvest, causing extensive necrosis and chlorosis of leaf tissues. Various *Alternaria* spp. are capable of damaging cured tobacco leaves after commercial curing and during the storage period.

A. alternata is known to produce in culture at least three bioactive dibenzo-α-pyrones, namely, alternariol, alternariol monomethyl ether (AME), and altenuene (Raistrick *et al.*, 1953; Pero *et al.*, 1971b; Pero and Owens, 1971). Mammalian toxicity of alternariol (Spalding *et al.*, 1970) and AME (Spalding, 1971) in lymphoma L 5178 Y and Hela cells in tissue culture systems have been demonstrated. ID_{50} values of 6 μg/ml and 8 μg/ml, respectively, have been established for alternariol and AME. Detailed *in vivo* histological and toxicological studies of the compounds following injections into mice also indicate their toxicity. These data will be reported elsewhere by the authors.

The apparent ubiquity of *Alternaria alternata* on tobacco prompted our investigation into the possible presence of the toxins alternariol and AME in harvested and stored tobacco.

EXPERIMENTAL PROCEDURE

Tobacco Samples. Flue-cured tobacco was obtained from markets in four states. This and additional tobacco harvested from 1966 through 1970 was classified for brown spot infection as very slight to very severe. Leaves of each of 40 samples were dried at room temperature to approximately 12% moisture and ground in a Wiley mill equipped with a 40 mesh screen; 40–100 g samples were used in analysis. Two 1-l. flasks each containing 50 g of shredded tobacco as used in commercial cigarettes were inoculated with *A. alternata* and incubated for 2 weeks as described by Pero and Main (1970). The contents of these flasks were used for analyses.

Analysis. Each sample was extracted with boiling acetone (100 g/400 ml), filtered, and the filtrate concentrated to dryness. The extract was dissolved in 5 ml of tetrahydrofuran, and 20 λ chromatographically separated on precoated, 250 μ thin-layer plates (silica gel G, Analtech, Inc.) in tetrahydrofuran:benzene (20:80 v/v). Analytical standards of purified alternariol and AME (0.2 μg and 1 μg) were used for cochromatography. The R_f values for alternariol and AME were 0.52 and 0.65. Scopoletin, a tobacco phenolic which was difficult to separate from the toxins in other solvent systems, had a $R_f = 0.24$.

Samples indicating the presence of alternariol or AME by tlc and cochromatography procedure were further purified by column chromatography (silica gel G) using the increasing tetrahydrofuran:benzene elution series reported by Pero *et al.* (1971a). Fractions suspected of containing alternariol (15% tetrahydrofuran:benzene) and AME (5% tetrahydrofuran:benzene) were concentrated to dryness, silyl derivatives prepared with *N,O*-bis-(trimethylsilyl)acetamide [BSA:trimethylchlorosilane:tetrahydrofuran, 6:2:9 (v/v/v)], and analyzed by gas–liquid chromatography (Varian Aerograph, model 2100) using 3% OV-17 (w/w) on Gas Chrom Q with temperature programing from 200–250° C at 2° C/min. Other gas–liquid chromatographic parameters have already been described (Pero *et al.*, 1971a). Results are reported as ppm or μg/g tobacco.

RESULTS AND DISCUSSION

None of the 40 tobacco samples (Table I) collected from the marketplace or from stored tobacco revealed the presence of alternariol and AME when analyzed by the tlc, column, and glc methods. Only 20 μl out of 5 ml of sample was spotted and analyzed by tlc, which establishes detection limits in tobacco for alternariol and AME at 25 μg/sample. Quantitative limit of glc detection using these methods was 0.1 μg for each toxin as reported by Pero *et al.* (1971a). Since the tobacco samples varied in weight from 40–100 g then actual detection limits varied from 0.625–0.25 μg/g of tobacco tissue. The sensitivity of detection could have been increased by preliminary column cleanup of the extracts prior to tlc separation. However, alternariol and AME concentrations below the detection limits selected were considered insignificant as a serious health hazard.

Shredded tobacco inoculated with *A. alternata* produced low yields of alternariol (2.1 and 2.3 μg/g tobacco) and AME (1.7 and 1.8 μg/g tobacco). Under environmental conditions favorable for fungus growth and with a high inoculum potential, tobacco evidently is a poor natural substrate for toxin production. These results substantiate the work of Pero

Table I. Tobacco Samples Analyzed for Alternariol and Alternariol Monomethyl Ether (AME)

Source	Year	No. of samples	Brown spot severity	Sample weight g
Marketplace	1970	1	Very light	50
Marketplace	1970	20	Slight	40–100
Marketplace	1970	1	Moderate	50
Marketplace	1970	1	Severe	50
Marketplace	1970	1	Very severe	50
Air cured	1970	2	Very slight	40
Flue cured	1970	2	Very slight	40
Air cured	1970	1	Slight	40
Flue cured	1970	1	Slight	40
Air cured	1970	1	Severe	40
Flue cured	1970	1	Severe	40
Stored	1969	1	Slight	50
Stored	1968	1	Slight	50
Stored	1968	2	Moderate	50
Stored	1967	1	Slight	50
Stored	1966	1	Slight	50
Stored	1966	2	Very severe	50
Inoculated tobacco	Unknown	1	Unknown	50
Inoculated tobacco	Unknown	1	Unknown	50

and Main (1970), who detected AME in shredded tobacco leaf culture but could not detect the purified toxin in live tobacco leaves 72 hr after hypodermic injection into the leaf lamina. Welty *et al.* (1968) demonstrated that the population of viable propagules of *Alternaria* spp. of field harvested leaves decreased with flue curing. However, a low number of propagules did survive the high temperature of commercial flue curing. They also showed that *Alternaria* are poor competitors, as evidenced by low population counts in stored tobacco (Welty and Lucas, 1969). Main and Pero (1970) failed to detect either toxin within brown spot lesions collected in the field. Therefore, contamination of processed tobacco with alternariol or AME during the growing or storage phase of tobacco production is counterindicated.

In conclusions, it seems that the mycotoxins, alternariol and AME, play little or no significant role as components of marketed flue-cured tobacco.

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LITERATURE CITED

Lucas, G. B., *Tobacco Sci.* **15,** 37 (1971).
Main, C. E., Pero, R. W., unpublished data (1970).
Pero, R. W., Main, C. E., *Phytopathology* **60,** 1570 (1970).
Pero, R. W., Owens, R. G., *Appl. Microbiol.* **21,** 546 (1971).
Pero, R. W., Owens, R. G., Dale, S. W., Harvan, D., *Biochem. Biophys. Acta* **230,** 170 (1971b).
Pero, R. W., Owens, R. G., Harvan, D., *Anal. Biochem.* in press (1971a).
Raistrick, H., Stickings, C. E., Thomas, R., *Biochem. J.* **55,** 421 (1953).
Spalding, J. W., NIEHS, P.O. Box 12233, Research Triangle Park, N. C., personal communication (1971).
Spalding, J. W., Pero, R. W., Owens, R. G., *J. Cell Biol.* **47,** 199a (1970).
Welty, R. E., Lucas, G. B., *Appl. Microbiol.* **17,** 360 (1969).
Welty, R. E., Lucas, G. B., Fletcher, J. T., Yang, H., *Appl. Microbiol.* **16,** 1309 (1968).

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