Chlorosis of Tobacco Induced by Alternariol Monomethyl Ether Produced by Alternaria tenuis

R. W. Pero and C. E. Main

Research Associate and Assistant Professor, respectively, Department of Plant Pathology, North Carolina State University, Raleigh 27607. Present address of senior author: National Institute of Environmental Health Sciences, Cell Biology Section, Research Triangle Park, North Carolina 27709.

ABSTRACT

Alternariol monomethyl ether (AME) was isolated from cultures of *Alternaria tenuis* grown on autoclaved rice grains supplemented with yeast extract and Czapek's broth. A simplified method for extraction and isolation of AME was developed. AME was also isolated from flue-cured tobacco cultures inoculated with *A. tenuis*, but was not detected in naturally infected tobacco leaves. AME induced a chlorosis when injected into the leaves of intact tobacco plants. The chlorosis was similar to the characteristic chlorotic halo surrounding natural brown spot lesions. Evidence presented suggests that AME was metabolized by live tobacco leaves. Alternariol monomethyl ether was identified by comparison with authentic AME as to melting point, IR spectral absorption, and chromatographic behavior in four solvent systems. Mass spectrum analysis of isolated AME conformed to the molecular wt and structure reported in the literature. The possible significance of AME in pathogenesis and its relation to tolerance in the brownspot disease of tobacco is discussed. Phytopathology 60:1570-1573.

Additional key words: phytotoxin, brown spot disease, *Nicotiana tabacum*, pathogenicity.

Tobacco varieties sensitive to brown spot, caused by *Alternaria tenuis* Nees, develop larger chlorotic halos around lesions than tolerant varieties (3, 5). These yellow halos coalesce on the sensitive cultivar Coker 298 and affect large portions of the leaf, which dies prematurely. The breeding line NC 80383-5-9 is tolerant to brown spot as indicated by the lack of chlorotic halo around the lesions. Main (4) and Spurr & Main (7) have described metabolic and biochemical alterations to this disease related to lesion development and varietal tolerance.

An antibacterial toxin (alternariol) is produced by *A. tenuis* (6). Alternariol monomethyl ether (AME), another metabolite of *A. tenuis*, causes necrosis in leaves of Japanese pear (11). Such a broad spectrum of biological activity for these related fungal metabolites stimulated interest in their potential to induce chlorosis of tobacco similar to that observed for natural infection. The purpose of this investigation was to develop a simple method for production, extraction, and isolation of AME, and to consider the potential of this compound as a factor in pathogenesis by *A. tenuis* on tobacco.

MATERIALS AND METHODS.—Hirokata Torikata (Nagoya University, Nagoya, Japan) kindly supplied an authentic sample of AME isolated from *Alternaria kikuchiana* (Tanaka) which was used as the reference standard in this study. The identity of the AME isolated from cultures of *A. tenuis* was determined by comparison with authentic AME relative to melting point, chromatographic behavior, IR spectra, and mass spectrum analysis. The IR spectra for the isolated and reference AME were obtained in KBr pellets with a Beckman Model IR-8 infrared spectrophotometer.

Thin-layer chromatography was carried out on precoated 0.25-mm Silica Gel F-254 aluminum-backed TLC sheets (Brinkman Instruments, Inc., Westbury, N. Y.). The solvent systems used for separating AME from scopoletin and other tobacco constituents were absolute methanol:water (60:40, v/v), chloroform: absolute methanol (95:5, v/v), benzene:absolute ethanol (95:5, v/v), and ethyl acetate:benzene:methyl ethyl ketone:formic acid (50:30:10:5, v/v). AME was detected on the chromatograms by use of long- and short-wave ultraviolet lamps and by reaction with diazotized sulphanilic acid (1). The mass spectrum of the isolated AME was obtained at 70 electron volts of energy and 100 microamperes of current in a Hitachi-Perkin Elmer mass spectrometer.

The isolate of *A. tenuis* (M1) used in this study was obtained from naturally infected tobacco in 1965. Slant cultures of the isolate were maintained on potato-dextrose agar (PDA). A high yield of AME was obtained by growing *A. tenuis* in 1-liter flasks on a sterilized medium containing 50 g of polished whole rice moistened with 75 ml of a nutrient solution. The nutrient solution consisted of 2% (w/v) yeast extract in commercial Czapek's Dox broth (Fisher Scientific Co.). The flasks were inoculated with 2 mycelial discs cut with a No. 4 cork-borer from the edge of 5-day-old PDA plate cultures of *A. tenuis*. The inoculated flasks were incubated in stationary culture for 28 days at 25 C in the dark.

Alternariol monomethyl ether was extracted by homogenizing the entire culture in a Waring Blendor for 1 min with acetone:water (70:30, v/v) and allowing the slurry to stand for 15-20 min. The slurry was filtered under suction through glass wool and Whatman No. 42

filter paper. The filtrate was concd to 25% of its original volume with an air stream and placed in the cold at −18 C for 24 hr or until a precipitate formed. The precipitate was collected by filtration, redissolved in hot dioxane, filtered again, and placed at room temp until crystals formed. The crystals were dried over P_2O_5 and sublimed in a high vacuum at 200 C.

A 150-g sample of shredded, flue-cured tobacco leaves was sterilized in 1-liter flasks and inoculated with a 75-ml mycelial spore suspension (50 spores/ microscopic field at ×450 magnification) of *A. tenuis* to determine if AME was produced by *A. tenuis* with tobacco as the substrate. After incubation in the dark at 25 C for 2 weeks, the cultures were extracted with acetone, concentrated to 10 ml, and 20 µliters of the conc spotted on thin-layer plates. Naturally infected tobacco leaves (*Nicotiana tabacum* 'Coker 298') were also tested for the presence of AME. The diseased tobacco leaves were dried in an oven at 55 C for 24 hr. A 150-g sample was extracted with acetone, concd to 10 ml, and 20 µliters of the conc were spotted on thin-layer plates. Two concn of isolated AME, 0.7, and 5.0 µg were cochromatographed with the inoculated and diseased tobacco samples.

Alternariol monomethyl ether was tested for its ability to induce chlorosis in the leaves of 6- to 8-week-old, cultivar Coker 298 tobacco plants. The plants were grown in the greenhouse of the phytotron at North Carolina State University and transferred to a growth chamber programmed for 9 hr of daylight at 34 C, 15 hr of darkness at 30 C, and 99% relative humidity. The light intensity was 4,400 ft-c at plant level. The plants were allowed to acclimate to the growth chamber environment for 3 days before AME was injected into the lateral veins of the three lower leaves in each plant, using a 26-gauge hypodermic needle. Because of low solubility in water, 10 mg of crystalline AME were ground to a fine powder in a test tube homogenizer and 10 ml distilled water added to give a suspension containing 1,000 µg/ml. Appropriate serial dilutions were prepared to provide suspensions containing 100, 50, 25, and 12.5 µg AME/ml. Approximately 0.5 ml of each suspension were injected through each of four lateral veins on two replicate plants until a water-soaked zone approx 15-20 mm in diam formed adjacent to the injection site. The water-soaked zone disappeared within 1 hr after injection. Symptom development was recorded 72 hr after injection and rated on a 0-4 scale depending on the intensity of chlorosis. Distilled water was injected as a control.

Another experiment was designed to determine if attached leaves of Coker 298 tobacco plants could metabolize AME. A suspension of 10 µg/ml AME was injected into eight tobacco leaves as already described except that the water-soaked areas were outlined with ink so that the injection sites could be accurately distinguished. After 1 hr, the injected tissue of four tobacco leaves was cut out, extracted with acetone, concd to 1 ml, and 50 µliters of the conc spotted on a thin-layer plate. After 72 hr, the injected tissue on the four

remaining leaves was cut out and analyzed in the same manner. The detection of AME in the two samples was carried out by co-chromatography with 0.4 µg and 0.8 µg of reference AME.

RESULTS.—Alternariol monomethyl ether was identified from cultures of *A. tenuis* by comparison of the isolated crystalline material with a reference standard of AME and by its mass spectral pattern. The isolated and reference AME had identical melting points of 267 C (cor). When the isolated and reference AME preparations were cochromatographed in four separate systems, their R_F values were identical, as were their fluorescence patterns and color development with diazotized sulphanilic acid. The R_F value of the AME preparations in benzene:ethanol was 0.37; in chloroform:methanol, 0.78; in ethyl acetate:benzene: methyl ethyl ketone:formic acid, 0.70; and in methanol:water, 0.60. Good separation from other phenolic constituents in the tobacco extracts was achieved with all four solvent systems. In methanol:water, however, poor separation was achieved when 10-µg quantities or larger were used. Alternariol monomethyl ether fluoresced blue-white in long-wave ultraviolet light, sky-blue in short-wave ultraviolet light, and yielded a wine-colored product when sprayed with diazotized sulphanilic acid. The long-wave ultraviolet fluorescence pattern was intensified and changed to a brighter white when the plates were exposed to ammonia vapors. The lower limit of detection for AME was 0.05-0.1 µg with ultraviolet radiation and 0.5-1.0 µg with diazotized sulphanilic acid.

The IR spectra for isolated AME and reference AME were essentially the same with only minor peak variations, presumably due to small amounts of impurities in one or both AME samples (Fig. 1). The fragmentation patterns and parent molecular ion observed on the mass spectrum of the isolated AME conformed to the reported mol wt of 272, and was consistent with the molecular structure for AME (10). The aforementioned chemical evidence conclusively identifies the crystalline material isolated from rice cultures of *A. tenuis* as AME.

Chlorosis developed when AME was injected into the veins of tobacco leaves, and the intensity of the reaction was related to the concn of AME injected (Fig. 2). Chlorosis was observed at all concn tested except at 12.5 µg/ml. The chlorotic response was confined to the water-soaked zone around the injection site. Chlorosis was first detected after 24 hr at the higher concn, but required 36-48 hr for the lower concn. No necrotic response was observed in any of the treatments. A wide range of environmental conditions was found satisfactory for the development of chlorosis, but it was most rapid and intense under the previously described conditions.

Alternariol monomethyl ether was detected chromatographically in extracts from cultures containing shredded tobacco, but not in extracts from naturally diseased tobacco leaves (Fig. 3). The lack of AME in naturally diseased tobacco might be due to its degrada-
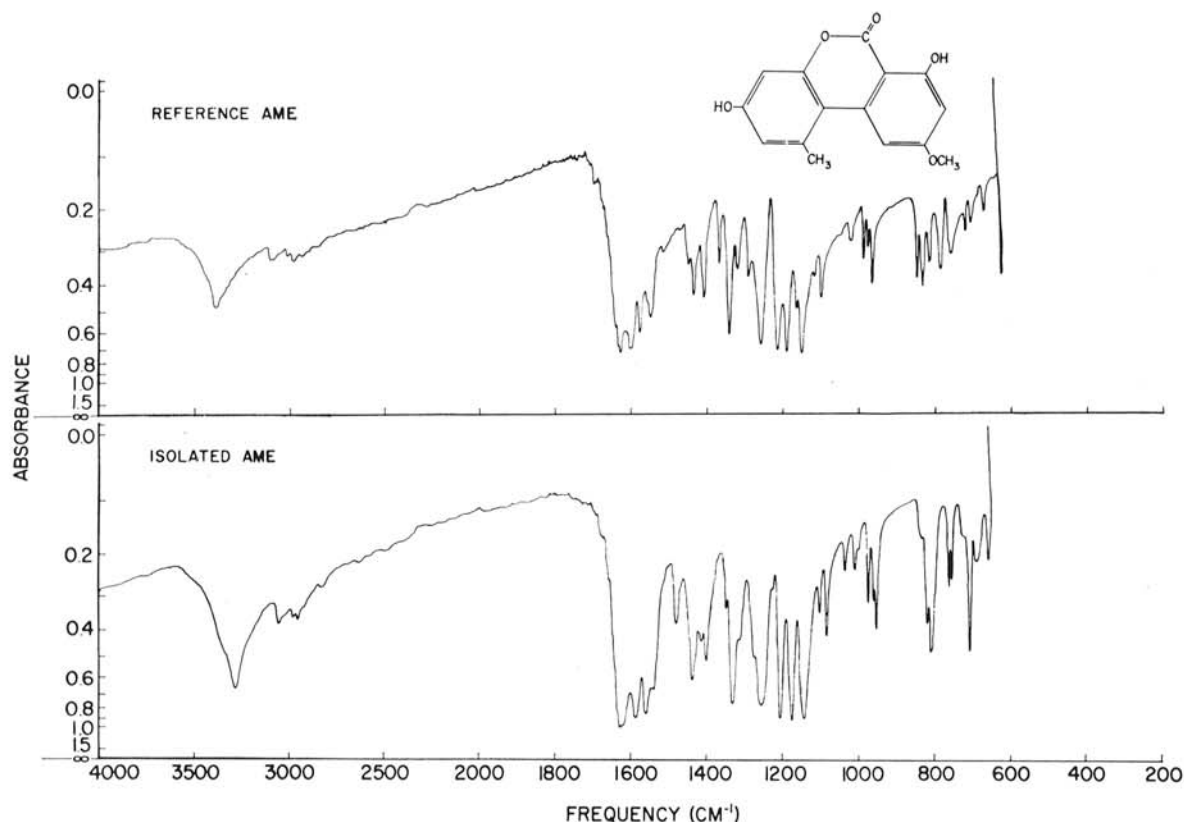
Fig. 1. Infrared spectra for reference alternariol monomethyl ether (AME) obtained from Dr. Torikata and AME isolated from rice cultures of *Alternaria tenuis*.

tion in the living tissue. This possibility is supported by data in Fig. 4, which shows that injected AME disappears in leaves after 72-hr incubation, compared with the amount present after 1-hr incubation; however, no intermediary breakdown products were detected on the chromatograms.

DISCUSSION.—A new and simplified method for the production, isolation, and purification of AME has been described. The advantages of this method over those previously reported (6, 11) are (i) high production of AME on the enriched rice medium; (ii) that the use of a solid medium requires no volume reduction of liquid cultures prior to extraction with an organic solvent; (iii) that no additional purification step is required to separate AME from alternariol; and (iv) little loss of AME during the purification.

Many of the chemical constituents of live tobacco and shredded, cured tobacco leaves are the same. Since AME was produced by *A. tenuis* on shredded leaf culture, the prospect of live tobacco leaves serving as a satisfactory substrate for AME production is suggestive. The production of AME on live tobacco leaves and the AME-induction of chlorotic halos are prerequisites for the phytotoxin to be of importance in brown spot lesion development.

The chlorotic response to AME that developed in attached tobacco leaves was confined to the injected water-soaked zone. The size of the zone was kept between 15-20 mm in diam in an effort to control the relative amounts of AME injected into the leaf tissues; however, the actual amount of AME injected could not be quantitated by the method used. Therefore, the min concn of AME necessary to cause chlorosis in live tobacco leaves has not yet been critically determined. The concn of AME used in this study indicate the basic parameters for further, more detailed studies.

The disappearance of AME when injected into live tobacco leaves suggests some degradative or metabolic activity on the part of the leaf. The production of polyphenolic compounds in response to injury and microbial infection is well known. Main (4) showed that free phenols were decreased in the halo tissue of naturally occurring lesions. Spurr & Main (7) demonstrated that acetone powder extracts of halo tissue from these lesions formed black pigments and increased oxygen uptake as compared to healthy tissue extracts when tyrosine, dihydroxyphenylalanine, and chlorogenic acid were used as substrates. Alternariol monomethyl ether, a biphenyl derivative, may be involved as a precursor in the metabolic pool which gives rise to polyphenols.

Templeton et al. (9) reported a cyclic tetrapeptide (tentoxin) as a chlorosis-inducing agent present in cultural filtrates of *A. tenuis*. The chlorosis-inducing ac-
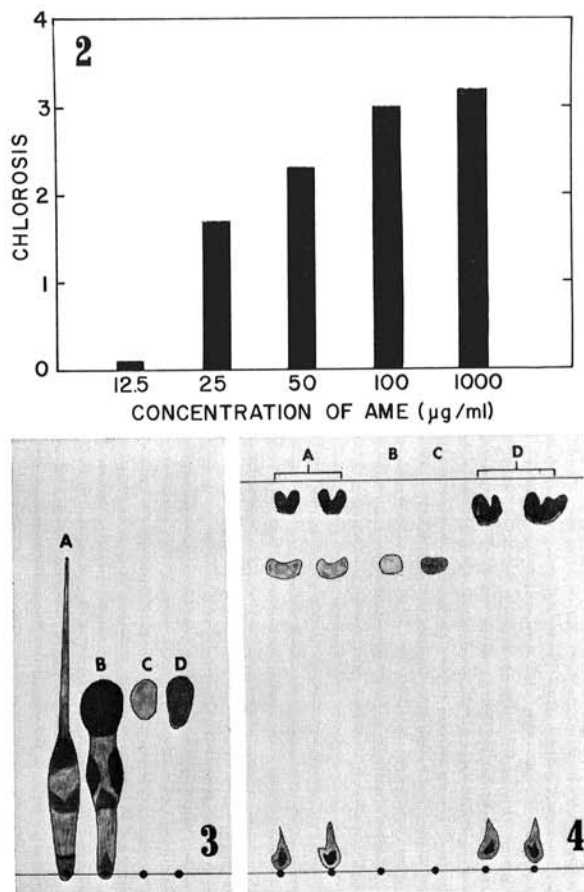
Fig. 2-4. 2) Chlorosis induced on Coker 298 tobacco plants injected into the leaf lamina with alternariol monomethyl ether (AME) isolated from *Alternaria tenuis*. **3, 4)** Diagrammatic representation for thin-layer chromatograms showing alternariol monomethyl ether (AME) isolated from *Alternaria tenuis* and diseased tissue. **3)** Acetone extracts of naturally infected tobacco leaves and artificially inoculated, shredded leaf culture. **A)** Naturally infected leaves; **B)** tobacco leaf culture seeded with *Alternaria tenuis* (M-1); **C)** AME standard at 0.7 µg; **D)** AME standard at 0.5 µg. **4)** Recovery of AME injected into leaf lamina. **A)** One hr following injection; **B)** AME standard at 0.4 µg; **C)** AME standard at 0.8 µg; **D)** 72 hr following injection.

tivity of tentoxin (8) in cucumber seedlings was comparable to that of AME in tobacco, being 10 and 25 µg/ml, respectively. Fulton et al. (2) demonstrated that other isolates of *A. tenuis* and other species of *Alternaria*, including an isolate of *A. longipes* from North Carolina, produced tentoxin. The chlorosis response was demonstrated in other host plants such as

bean, cotton, garden pea, okra, and watermelon. As yet no report concerning tentoxin has been made, however, which determines whether it can be detected in diseased plants or produced in culture when host tissue was used as the substrate. Tentoxin was shown to inhibit chlorophyll production (2). In the case of AME, further studies are underway to determine whether chlorophyll production is inhibited or the existing chlorophyll is destroyed.

Brown spot was the most important tobacco disease in North Carolina in 1969. The use of tolerant varieties to control this disease is important because no fungicidal control program is presently recommended. An understanding of the role of AME and other phytotoxins in pathogenesis and the tolerance mechanisms involved is necessary to our studies on disease control and alterations resulting from infection.

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