Pathogenesis and Halo Formation of the Tobacco Brown Spot Lesion

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ABSTRACT

Leaf lesions caused by *Alternaria tenuis* on susceptible tobacco cultivars are comprised of a fungal-invaded necrotic center, surrounded by a noninvaded chlorotic halo and biochemically detectable "prehalo" zone, beyond which is located healthy tissue. Alterations in noninvaded tissues are believed to result from the response of host tissue to diffusible fungal metabolite(s), and are associated in a cause and effect relationship gradient to the tissue zones within the lesion. Plastid pigments, starch, and reducing sugars decreased, whereas individual mono- and disaccharides increased progressively from healthy to halo tissue. Changes in total nitrogen, soluble protein, and amino acids indicate a disruption of protein metabolism. Free phenols decreased toward the lesion center, indicating polymerization and brown pigment formation. Cultivar tolerance in tobacco is related to the extent of chlorotic halo formation. The lesion is presented as a working model for the study of pathogenesis and the nature of tolerance to the brown spot disease. Phytopathology 61: 1437-1443.

Brown spot of flue-cured tobacco (*Nicotiana tabacum* L.) is a foliar disease caused by *Alternaria tenuis* Nees (11). Typical lesions on sensitive cultivars are comprised of a fungal-invaded necrotic center surrounded by an extensive, noninvaded chlorotic halo (Fig. 1-A). Cultivar tolerance has been related to extent of chlorotic halo and number of lesions per unit leaf area (14, 15, 30).

Alternaria tenuis isolated from tobacco produced pectic and cellulolytic enzymes in vitro (31). However, Lucas & Sherwood (12) failed to detect pectic enzyme activity in vivo, and concluded it was not involved in disease initiation or halo formation. Main & Pero (17, 19) isolated and identified alternariol monomethyl ether, alternariol, and altenuene from *A. tenuis* cultures. Infiltration bioassay in tobacco leaf tissue demonstrated that the three phytotoxins induced chlorosis resembling the halo surrounding natural lesions.

Riley (23) found that N-P-K nutrient ratio and fertility level in Rhodesian soils influenced the size of brown spot lesions. Root knot nematode damage caused by *Meloidogyne incognita* (Kofoid & White) Chitwood predisposed tobacco to infection and increased disease development (21). Ontogeny of the host and weather conditions (11) have considerable influence on the extent of chlorotic halo formation.

Characteristics of the lesion suggest that a gradient(s) of diffusible metabolite(s), probably of fungal origin, produces the halo. The halo thus provides a site for studying these effects and for comparing natural lesions versus those induced artifically by injection of specific fungal metabolites into healthy leaves.

Preliminary study had shown that incipient pathologic effects extended beyond the halo into adjacent green leaf tissues. This so-called "prehalo" zone (Fig. 1-B) was visually indistinguishable from surrounding healthy tissue, but could be detected biochemically. As the lesion expanded with time, the prehalo zone became chlorotic. During the flue-curing process, the lesions, including the halo and prehalo zones, became dark brown, and there was an increase in severely damaged leaf tissue and a corresponding reduction in quality and market value (15, 16).

Initially, certain biochemical and physiological alterations within the lesion were characterized without regard to specific causes. This knowledge was used to delineate important alterations for further assessment of their sequence and magnitude in an attempt to elucidate cause and effect relationships. The objectives of this study, therefore, were (i) to analyze the gradients of biochemical change occurring in each zone of the lesion; (ii) to relate them to the development of the disease syndrome; and (iii) to employ this pathological system to study the aggressiveness of certain isolates of the pathogen in various host cultivars under different environmental regimes and to determine, if possible, the nature of host resistance. A preliminary report on some of these studies has been published (13).

MATERIALS AND METHODS.—Lesions were collected from leaves of naturally infected, brown spot-sensitive cultivar Coker 298 tobacco plants grown at Oxford, N.C., in 1967-68. To standardize the status of lesion development, only lesions measuring ca. 30 mm in diam, including the halo, were mechanically separated into necrotic, halo, and prehalo tissues (Fig. 1-B). The prehalo zone was arbitrarily designated as a 5-mm zone adjacent to the outer edge of the halo. Healthy tissue was collected adjacent to, and outside, the prehalo zone. Tissues were freeze-dried or processed into acetone powders and stored over calcium chloride at 4 C in the dark. Chemical data were calculated on a dry weight basis and statistically analyzed.

Per cent dry weight.—Fresh tissue was weighed immediately after collection, dried for 48 hr in a forced air oven at 38 C, then reweighed. The per cent

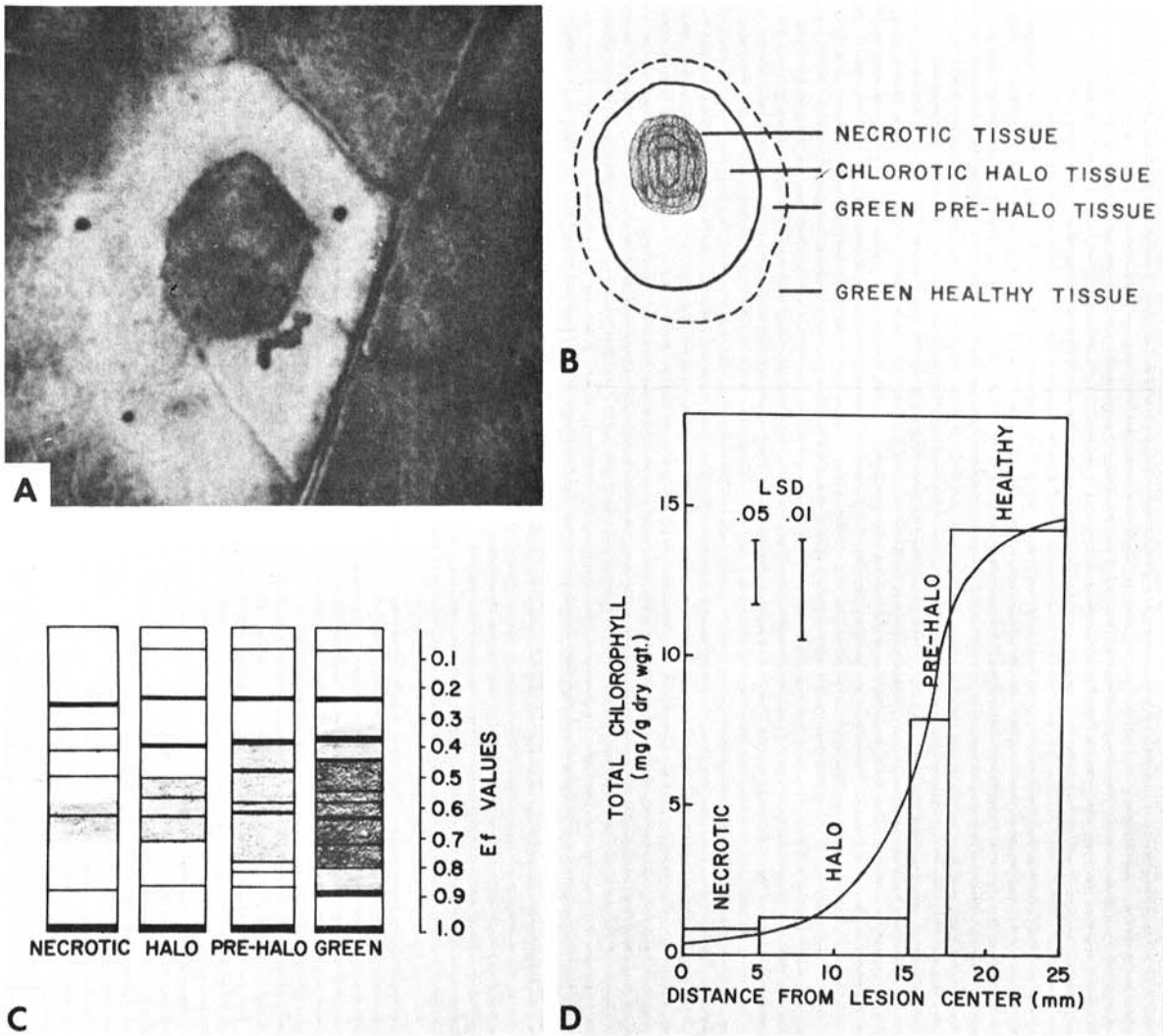

Fig. 1. Typical brown spot lesion collected from field-infected tobacco, and idealized model lesion showing biochemical alterations due to *Alternaria tenuis* infection. **A)** Brown spot lesion on sensitive cultivar Coker 298, showing necrotic center and chlorotic halo; **B)** diagrammatic representation of lesion showing necrotic, halo, and prehalo zones; **C)** electrophoretic protein profile of lesion and healthy tissue; and **D)** total chlorophyll content of lesion and healthy tissue.

dry weight of each type of lesion tissue was computed.

Pigment analysis.—Total chlorophyll was determined on freeze-dried samples. The tissue was extracted twice with cold 80% acetone and filtered, and the combined extract adjusted to 25-ml volume. Absorbance was measured at 663 and 645 nm on a Beckman DB spectrophotometer (Beckman Instruments, Palo Alto, Calif.). Data were calculated and expressed as mg total chlorophyll per gram dry weight using Arnon's simultaneous equation and specific absorption coefficients for chlorophyll A and B (2).

A more detailed pigment analysis was conducted using the method of Weybrew (32). Freeze-dried samples were extracted with 95% ethanol plus 500 mg calcium carbonate to neutralize the plant acids. The extract was filtered, and the residue extracted with acetone. One hundred twenty-five ml anhydrous, peroxide-free ether were added to the combined filtrates. The pigments were forced into the ether phase with water, and the aqueous phase was extracted a second time with ether. The total pigment solution was dried with anhydrous sodium sulfate and brought to 200 ml volume with ether. Absorbancies were recorded at 665, 649, 642.5, 485, 474, and 470 nm on a Beckman DU spectrophotometer, and pigment content was calculated, using Weybrew's estimating equation.

Starch.—Starch content was determined on freeze-dried samples by the method of Gaines & Meudt (6). The tissue was homogenized in water for 20 sec. The sample was quantitatively transferred to a 250-ml beaker, boiled for 20 min, and filtered through glass wool. The extracted tobacco was washed with boiling water, and the washings were combined with the

filtrate. The filtrate was heated again to boiling, and 95% ethyl alcohol added to precipitate the starch. The precipitate was recovered by centrifugation at 37,000 *g* for 30 min, dissolved in 20 ml of distilled water, and mixed with 0.5 ml of iodine reagent (2.0 g iodine and 20 g potassium iodide/liter of water) per 10-ml test solution. Color intensity of the reaction was determined at 600 nm on a Bausch & Lomb spectronic 20 colorimeter, and concentration estimated from a standard starch curve. The data are expressed as mg starch per gram freeze-dried tissue.

Sugars.—Water-soluble reducing sugars were extracted with distilled water, clarified with lead acetate, and oxidized with an excess of potassium ferricyanide. The resulting ferrocyanide ions were titrated with ferric sulfate to a Setopaline end point (8).

Sugars were determined chromatographically according to Tso et al. (28). Freeze-dried tissues were extracted 3 times with 25 ml hot 70% (v/v) ethanol, concentrated to 5 ml on a rotary evaporator, spotted on Whatman No. 1 paper, and chromatographed for 68 hr in *n*-butanol:ethanol water (4:1:5 v/v). The spots were visualized by spraying with *p*-anisidin reagent and heating at 100 C for 3 min. Semiquantitative estimation of sugar concentration was made by comparison with sugar standards.

Polyphenols.—A portion of the extract used for chromatographic sugar determination above was tested for phenol content. Phenol chromatograms were developed in either *T*-amyl alcohol:water (84:16 v/v) for 20 hr, or *n*-butanol:acetic acid:water (4:1:2.2 v/v) for 7 hr. The spots were detected, using Hoepfner's test (HNO_2 + alkali) for the major phenols and periodate nitroprusside piperazind for quinic and shikimic acid (3). Semiquantitative estimation was made, using phenol standards. Scopolin concentration was rated on a scale of 0-10 based upon relative spot intensity.

Total alkaloids.—Total alkaloids were determined by the Griffith procedure (7). Freeze-dried samples were extracted with 20% (w/v) aqueous NaOH saturated with NaCl and steam-distilled in a Griffith apparatus (Consolidated Glass Co., Kingsport, Tenn.). The distillate was trapped in 10 ml of 3 *N* H_2SO_4/240 ml distillate, and absorbancies for nicotine and nicotine-related alkaloids were measured at 236, 282, and 259 nm, using a Beckman DU-2 spectrophotometer. Results were calculated and expressed as per cent total alkaloids per gram dry weight.

Total nitrogen, protein, and amino acids.—Total nitrogen content was determined by the micro-Kjeldahl procedure (1), using freeze-dried tissue. Protein analysis and electrophoretic separation of proteins were conducted on acetone powders. Samples were extracted in 10 ml of pH 6.0, 0.05 *M* sodium phosphate buffer at 4 C, passed through cheesecloth, and centrifuged at 37,000 *g* for 30 min in a refrigerated Sorvall RC-2 centrifuge. Half the extract was dialyzed against 16 liters of extracting buffer for 24 hr at 4 C. Soluble protein content was determined on dialyzed and nondialyzed extracts by the Lowry procedure (10), and results were expressed as mg protein per gram acetone powder, based upon a bovine serum albumin standard curve. Sucrose was added to the extracts to achieve a concentration of 0.4 *M*. Polyacrylamide gel disc electrophoresis (5, 18) was used to qualitatively separate the protein in each extract. Current was regulated at 3 milliamperes per gel during stacking in the coarse gel, and adjusted to 5 milliamperes per gel during separation through the small gel. The gels were fixed with 7% (v/v) acetic acid and stained with 1% (w/v) amido Schwarz reagent (Buffalo Black NBR). After destaining, densitometric traces were obtained using a Canalco Model D electrophoresis microdensitometer (Canal Industrial Corp., Rockville, Md.). Motility values (E_f) were calculated as ratio of distance each band moved from the origin compared to the dye marker distance.

Amino acids were extracted from freeze-dried samples with 1% HCl (w/v) in the proportion of 1:20 (w/v), as described by Weybrew & Matzinger (34). The extract was stored at 4 C overnight and centrifuged at 1,340 *g* for 15 min. The supernatant was filtered and deproteinized with hot 1% picric acid and centrifuged for 8 min at 885 *g*. Excess picric acid was removed by absorption on Dowex 2-X8 resin. Separation and analysis of individual amino acids was accomplished on a Spinco amino acid analyzer, Model 120B (Spinco Division, Beckman Instruments, Palo Alto, Calif.) operated at 55 C with flowrates of 68 and 34 ml/hr of buffer and ninhydrin, respectively. Quantification of peak areas was obtained from replicate analyses of mixtures containing 0.5 μmole quantities of pure compounds. Data were expressed as micrograms amino acid per gram dry weight.

RESULTS.—Per cent dry weight increased significantly in necrotic tissue compared to healthy tissue (Table 1). Although halo and prehalo dry weight decreased slightly, differences were not significant.

Total chlorophyll (Fig. 1-D) decreased progressively from the healthy tissue toward the necrotic center of the lesion. Healthy and prehalo tissues differed significantly from each other and from the halo and necrotic tissue. Halo tissue was not different from necrotic tissue. The ratio of chlorophyll A to B decreased from healthy to necrotic tissue (Table 2). Decreases in xanthophyll and carotene occurred, but the differences were not significant. When xanthophyll was high, carotene was low, and vice versa. When combined xanthophyll and carotene content was considered, all lesion tissues differed significantly from the healthy tissue and from one another. Xanthophyll to carotene ratio increased progressively toward the necrotic center, indicating that carotene was affected to a greater extent.

Starch decreased progressively from healthy to necrotic tissue (Table 1). Lesion zones differed significantly in starch content from each other and from surrounding healthy tissue. Reducing sugars decreased significantly from healthy tissue in each type of lesion tissue (Table 1). Glucose increased in all lesion tissues. Fructose increased in the halo and necrotic tissue, but not in the prehalo tissue. Sucrose

TABLE 1. Biochemical alterations within the brown spot lesion

Analysis	Lesion zone				LSD	
	Necrotic	Halo	Prehalo	Adjacent healthy tissue	.05	.01
Dry wt (%)	45.9	14.1	14.7	15.5	2.3	3.2
Reducing sugars (5)	1.6	6.1	11.3	11.9	0.2	0.3
Starch (mg/g dry wt)	17.4	63.6	97.2	122.6	16.4	24.8
Glucose (mg/g dry wt)	11.3	28.8	8.3	2.8	4.7	8.7
Fructose (mg/g dry wt)	28.8	42.5	11.3	5.0	7.2	13.3
Sucrose (mg/g dry wt)	2.5	18.8	14.3	12.5	2.3	4.2
Total alkaloids (%)	2.3	3.5	3.1	2.9	0.3	0.5
Total N (%)	3.1	2.7	3.1	3.2	0.1	0.2
Soluble protein[a] (mg/g dry wt)						
Nondialyzed	279.0	214.0	192.0	104.0	15.1	22.8
Dialyzed	65.0	45.0	41.0	33.0	16.4	
Phenols (mg/g dry wt)[b]						
Chlorogenic acid	1.0	17.0	14.5	16.0	2.1	3.9
Rutin	t[c]	13.5	14.0	16.5	0.6	1.1
Scopoletin	0.2	0.1	t	t	NS[d]	
Scopolin	t	9+	9+	7+		
Free quinic acid	2.0	5.0	4.0	2.0	NS	

[a] Expressed as bovine serum albumin equivalents.

[b] Semiquantitative chromatographic estimation based on standards except scopolin which is represented as relative intensity of spots. Caffeic, sinapic, *p*-coumaric, ferulic, shikimic acid, and quercetin were not detected.

[c] Trace.

[d] Not significant at 95% level of confidence.

increased in the halo tissue, but the prehalo content was not statistically different from healthy tissue. All three sugars, particularly sucrose, decreased in necrotic tissue compared to halo tissue.

Although the chromatographic method for phenol analysis was semiquantitative (Table 1), it provided an indication of relative phenol alteration within the brown spot lesion. The greatest change occurred in necrotic tissue where chlorogenic acid, rutin, and scopolin were decreased drastically. Scopoletin increased slightly in the halo and necrotic tissues. Free quinic acid increased in halo and prehalo tissues, but remained unchanged in the necrotic tissue.

Total alkaloids, of which ca. 98% is nicotine in flue-cured tobacco, were increased in halo tissue and decreased the necrotic tissue (Table 1).

Total nitrogen decreased in all lesion tissues, but only the halo tissue was significantly different from the other zones and from the healthy tissue (Table 1). Soluble protein content increased progressively toward the center of the lesion in both dialyzed and nondialyzed extracts (Table 1). Only necrotic tissue differed significantly from healthy tissue in dialyzed extracts, while protein differed significantly between all tissues for nondialyzed extracts.

Electrophorograms indicate both qualitative and quantitative protein alterations within the lesion when compared to healthy tissue (Fig. 1-C). The number of bands decreased progressively toward the necrotic center. Two bands were unique to necrotic tissue, and may represent protein contributed by the fungus which is confined to the necrotic tissue.

TABLE 2. Plastid pigment alteration within the brown spot lesion[a]

Pigment	Lesion zone				LSD	
	Necrotic	Halo	Prehalo	Adjacent healthy tissue	.05	.01
Chlorophyll A	6.6	59.5	578.0	1,912.0	124.3	188.4
Chlorophyll B	11.6	49.3	214.5	714.8	83.6	126.6
Xanthophyll	112.8	177.5	185.0	296.8	NS[b]	
Carotene	23.1	60.1	87.0	167.1	NS	
Carotene + xanthophyll	136.0	237.5	272.0	464.0	79.4	120.3
Ratio chlorophyll A:B	0.6	1.2	2.7	2.7		
Ratio xanthophyll:carotene	4.9	2.9	2.1	1.8		

[a] Data expressed as μg/g dry wt.

[b] Not significant at 95% level of confidence.

Background-staining of healthy and prehalo profiles indicated the presence of phenolic material which reacted with the amido-Schwarz reagent after dialysis.

Six amino acids increased progressively from healthy to necrotic tissue, whereas aspartic acid, threonine, glutamic acid, proline, and phenylalanine decreased (Table 3). Seven amino acids either did not change, or changed in a nonprogressive manner in either direction. Ammonia increased from healthy to halo tissue, but was unchanged in the necrotic tissue. ϵ-amino acid-N decreased progressively from healthy to necrotic tissue. Cystine, glucosamine, homocystine, and β-alanine were not detected in any of the tissues. Arginine appeared only in necrotic tissue, and may be a constituent of the fungus.

DISCUSSION.—The brown spot lesion consists of three biochemically distinguishable zones. The pathogen is confined to the necrotic, central zone (26). Biochemical alterations in halo and prehalo zones show an intensity gradient outward from the center not related precisely to zonal boundaries. Although specific biochemical mechanisms responsible for these changes are not known, they are thought to be caused by diffusible fungal metabolites.

A decrease in chlorophyll content was the most striking alteration of halo tissues. Corresponding decreases in carotene and xanthophyll suggest that the chlorophyll loss was due to metabolic disruption rather than to direct destruction. The chlorotic halo may be regarded as a kind of pathologically induced senescence similar to that described by Weybrew (33).

Progressive decrease in starch and coincident increase in sucrose and monosaccharides toward the necrotic center probably result from altered photosynthetic and respiratory rates. *Alternaria tenuis* is known to produce amylases (29) and to utilize sucrose, fructose, and glucose as energy sources (30, 35). The latter could explain low levels of sugars found in the necrotic tissue. Pectolytic and cellulolytic enzyme activity has been demonstrated in cultures of *A. tenuis* (12, 31), but has not been found in the halo zone.

Alternaria tenuis (20) and related species (9) produce proteases; if produced in vivo they could cause some of the observed alterations in total nitrogen, proteins, and amino acids. Total amino acid and soluble protein contents decreased toward the center of the lesion. Fungal mycelium probably contributed to the protein profile in the necrotic zone.

Decrease in phenolic constituents toward the

TABLE 3. Amino acid alterations within the brown spot lesion[a]

Amino acid	Lesion zone			
	Necrotic	Halo	Prehalo	Adjacent healthy tissue
Lysine	76.0	46.8	43.8	32.2
Histidine	37.2	34.2	12.4	9.4
Arginine	73.2	t[b]	t	t
Cysteic acid	189.2	270.4	229.8	175.8
Taurine	t	t	t	t
Aspartic acid	263.6	362.0	571.6	713.4
Threonine	t	t	95.2	92.8
Serine	266.0	176.6	157.6	197.6
Glutamic acid	338.4	335.4	664.8	1,038.6
Proline	25.4	69.0	99.0	145.0
Glycine	33.0	19.6	19.6	21.0
Alanine	94.4	78.4	83.8	80.2
Cystine				
Valine	28.2	t	16.4	16.4
Methionine	23.8	47.8	38.8	38.8
Isoleucine	49.8	84.0	78.8	60.4
Leucine	18.4	13.2	10.4	7.8
Norleucine	7.8	18.4	13.2	15.8
Tyrosine	3.6	21.8	18.2	21.8
Phenylalanine	10.0	26.4	46.2	46.2
Glucosamine				
Homocystine				
Galactosamine	t	t	t	t
β-alanine				
γ-aminobutyric acid	90.8	497.0	614.4	519.6
Total amino acids	1,628.8	2,101.0	2,814.0	3,232.8
ϵ-amino acid-N	343.4	438.2	473.0	503.2
Ammonia	188.8	231.2	199.6	186.6

[a] μg/g dry wt.
[b] Trace.

lesion center could have resulted from polymerization to polyphenols, melanins, and brown pigment (27). This binding might account for some of the changes in protein mobility patterns obtained by electrophoretic separation. Phenol oxidase activity of each type of lesion tissue differed when assayed using tyrosine, phenylalanine, or dihydroxyphenylalanine as the substrate (24). Protein content followed the total nitrogen pattern from healthy to necrotic tissues when first separated by Sephadex G-25 gel filtration (25).

Changes in plastid pigments, starch, protein, and the level of certain amino acids that were detected in the prehalo zone indicate the kinds of initial degenerative changes that extend beyond the visible lesion. Histological studies by Stavely & Slana (26) showed degeneration of chloroplasts in the prehalo area. Since this zone does not yet show the secondary and more drastic changes found in the visible zones of the lesion, it should provide suitable material for studying early stages of pathogenesis.

The role of phytotoxins in pathogenesis and halo formation has been investigated. Three biphenyl derivatives and a large molecular-weight material were isolated and characterized from *A. tenuis* isolated from brown spot lesions (17, 19). All four toxins induced chlorosis when bioassayed by hypodermic injection-infiltration into tobacco leaf lamina. However, these compounds have not been isolated from natural lesion tissue. Alternariol monomethyl ether was apparently metabolized following infiltration, as it was not recovered from the leaf tissue more than 1 hour after injection. The phytotoxins may be involved as precursors in the formation of polyphenols.

Total alkaloids and reducing sugar content are two important criteria established as minimum standards for commercial acceptance of flue-cured tobacco cultivars and breeding lines (22). Alterations in these constituents, and others, could influence release of new cultivars if brown spot incidence should be severe. The pathogen can alter chemical constitution sufficiently (16) to render as unacceptable a candidate breeding line normally acceptable under disease-free conditions.

The economic loss caused by brown spot depends largely upon the extent of halo formation. This is known to be influenced by various factors, among them the host cultivar (4, 14, 30), soil fertility (23), nematode damage (21), and weather conditions.

The brown spot lesion, as herein described, provides a coordinated system for studying the effects of certain characteristics of the pathogen, the host, and the environment, either singly or in various combinations, upon specific pathological processes.

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