Incidence of Tobacco Leaf Microflora in Relation to Brown Spot Disease and Fungicidal Treatment

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ABSTRACT

The brown spot pathogen, *Alternaria alternata* (Fr.) Keissl., and 18 or more other microorganisms (fungi, yeast, and bacteria) may colonize tobacco leaves without producing visible effects. The influence of this large and variable population of leaf microflora on yield and quality is not yet understood. The principal fungal species which occurred within the leaf were *Alternaria* and *Cladosporium*, being found in 67 and 25% of the leaves examined, respectively. *Alternaria* was found more often in the interior of lower and middle leaves, and *Cladosporium* more often in the interior of middle leaves. Yeasts and bacteria were most numerous in dilution plate cultures of leaf homogenates, in which organisms from both the interior and exterior of the leaves were recovered. Colonies of *Cladosporium* were the most numerous of the species observed. *Cis*-N-[(1,1,2,2-Tetrachloroethyl)thio]-4-cyclohexene-1,2-dicarboximide (Difolatan) was most effective for the control of brown spot and leaf microflora, and effectively inhibited the mycelial growth of several tobacco fungi in vitro. Benomyl reduced the internal incidence of *Cladosporium*. Other fungicides controlled brown spot but not the leaf microflora. The efficacy of several chemicals for the control of brown spot and leaf microflora are tabulated and provide a basis for future chemical control studies.

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Alternaria alternata (Fr.) Keissl., the cause of brown spot, is the most destructive foliar pathogen of tobacco in North Carolina. Many other microorganisms colonize tobacco leaves but produce no visible symptoms or signs, and their effects on yield and quality are not well understood (8, 9, 10). Brown spot itself is a perplexing disease, and reports on infection, effect of age on susceptibility, and seasonal development of the disease under field conditions are conflicting (3, 4, 5, 6). No effective control measures are currently available.

In this study, we sought to determine the incidence of the brown spot organism and associated microflora of tobacco leaves as affected by leaf position during the season. Trials were also conducted to determine whether fungicidal applications would give practical control of the brown spot fungus and other organisms occurring on the leaf surface and within the leaf.

MATERIALS AND METHODS.—*Field design and*

chemical application.—A planting of flue-cured tobacco (*Nicotiana tabacum* L.) at Oxford, N.C., was divided into three blocks. Each block included one replicate (20 plants) of 30 treatments. Fungicidal spray treatments (100 gal/acre) were applied 4 times during the season, beginning when the plants were about 61 cm high. Plants were sprayed thoroughly to cover both leaf surfaces. Normal production practices were followed except that the plants were topped and suckered by hand. Carbaryl was applied twice, and endosulfan once, for insect control. The following fungicides were tested: Alar (succinic acid, 2,2-dimethyl hydrazide); benomyl; Bordeaux mixture (2 parts cupric sulfate:1 part calcium hydroxide); Botran (2,6-dichloro-4-nitroaniline); Difolatan [(*cis*-N-(1,1,2,2-tetrachloroethyl)thio]-4-cyclohexene-1,2-dicarboximide); maneb; Du-Ter (triphenyl tin hydroxide); Dyrene [2,4-dichloro-6(*o*-chloroanilino)-*s*-triazine]; ferbam; Fore (a coordination product, manganese, 16%; zinc, 2%; ethylene bisdithiocarbamate ion, 62%); Fungi Sperse Cu-S (4% basic copper, 41.6% sulfur); Fungi Sperse Z-S (9.2% zineb, 45.0% sulfur); Isobac (monosodium salt of 2,2′ methylenebis 3,4,6-trichlorophenol); Kocide 101 (copper hydroxide); Ortho lawn disease control (25% captan, 50% pentachloronitrobenzene, 2% cadmium carbonate); Nabac [2,2′methylenebis (3,4,6-trichlorophenol)]; Polyoxin B (antibiotic substance, The Japan Monopoly Corporation, Tokyo); Polyram (a mixture of 5.2 parts by weight of ammoniates of [ethylenebis (dithiocarbamato)] zinc with 1 part by weight ethylenebis [dithiocarbamic acid], bimolecular and trimolecular cyclic anhydrosulfides and disulfides); thiram; Turf [0.75% cycloheximide (beta-[2-(3,5-dimethyl-2-oxocyclohexyl)-2-hydroxyethyl]-glutarimide), 75% thiram]; carboxin.

Evaluation of brown spot.—Incidence of brown spot was evaluated on middle (20 August) and upper leaves (28 August). Twenty leaves, one/plant, were selected from each replicate, and the identifiable brown spot lesions were counted. The average number of lesions per leaf was computed from three replicates.

Detection of microflora.—Two isolation methods were used to determine the number and kinds of microflora associated with tobacco leaves. The leaf disc method permitted estimates of organisms within the leaf tissue, whereas the dilution plate method indicated the total number of propagules on the exterior and interior of the leaf.

Leaf-disc method.—Leaves were folded along the midrib and folded again. Fifty 9-mm discs were cut with a cork borer from leaves from one treatment. The discs were immersed in 1.0% sodium hypochlorite for 30 sec, rinsed in sterile distilled water, and plated 10/dish on each of two media: Rose bengal streptomycin sulfate agar (RBSS) and Czapek solution agar (Difco) plus 6% NaCl (Cz + 6). These media provided a range of conditions suitable for different fungi (10). Dishes were incubated at 23 to 38 C for 10 to 14 days. Microflora growing from the discs were identified in the original culture whenever possible. Those not identified in situ were subcultured for later study. Each species growing from a leaf disc was counted as one, since there was no way to ascertain that other colonies of the same species were different isolates. Per cent frequency was computed by dividing the number of leaf discs from which a species was isolated by the number of discs cultured and multiplying the quotient by 100.

Dilution plate method.—Fifty grams of tobacco leaf tissue were chopped in 500 ml of a sterile solution of 0.15% agar in a blender for 30 sec. Dilutions of this suspension were made, and 1 ml of each dilution was placed in each of two petri dishes. Melted and cooled (52 C) agar, RBSS, and Cz + 6 were added to separate dishes. Dishes were swirled to distribute the suspension, and the agar was allowed to solidify. Colonies that grew in each medium were counted, multiplied by the dilution factor, and expressed as the number of colonies per gram of tobacco. Microflora were identified and counted.

Variations in the morphology of certain fungal isolates were observed, especially within the genus *Alternaria*. Because we could not identify species, the data were tabulated as *Alternaria* spp.

RESULTS.—*Brown spot control.*—Incidence of brown spot disease in the field plots was uniformly heavy. The average number of brown spot lesions per leaf on the middle stalk position (9 days after the last spray application) was reduced significantly by all treatments. Seventeen days after the last spray application, fungicidal control was still evident on the leaves in the upper stalk position with 16 treatments maintaining significant disease reduction. Thus, spray applications of fungicidal chemicals can control tobacco brown spot (Table 1).

Several of the treatments injured leaves. Turf produced necrotic lesions even after the spray concentration was halved. Carboxin, alone and in combination with Difolatan or Botran, produced a characteristic marginal chlorosis on the leaves. The highest rate of Kocide 101 produced small necrotic flecks.

Microflora recovered from within leaf tissue.—Six fungicidal treatments (Table 1) and the untreated control were selected for an evaluation of microflora control. Leaves from the selected treatments were harvested at 14-day intervals, and portions of the tissues were cultured as described above to determine the microflora. Leaves from the lower, middle, and upper stalk positions were harvested on 1 July, 5 August, and 19 August, respectively.

Of the six fungicides selected for the evaluation, only benomyl was effective. It reduced the isolation frequency of *Cladosporium* from 23% in the control to 3% on RBSS medium, and from 28 to 4% on Cz + 6 medium. No change was observed in the incidence of other microflora. Since the selected fungicides were ineffective against other microorganisms, the data were combined with the untreated control data (Table 2).

Alternaria was isolated frequently from the lower and middle leaves, whereas *Cladosporium* was isolated most frequently from middle leaves (Table 2). The

TABLE 1. Control of brown spot in middle and upper tobacco leaves from spray applications of fungicidal chemicals

Fungicide, formulation[a]	Rate (lb. per acre)		Lesions per leaf[c]	
	Formulation	Active ingredient	Middle	Upper
Alar, 85W	1.00	0.85	22	29
Benomyl, 50W	0.50	0.25[b]	26	34
Bordeaux Mixture		0.42[b]	28	42
Bordeaux Mixture		0.84	25	24
Bordeaux Mixture		1.68	25	34
Botran, 75W	2.00	1.50	31	41
Chlorothalonil, 75W	1.00	0.75	15	13
Difolatan, 80W	2.00	1.60	4	8
Maneb, 80W	2.00	1.60	9	12
Du-Ter, 47.5W	0.25	0.12	11	23
Dyrene, 50W	2.00	1.00	9	23
Ferbam, 76W	2.00	1.52[b]	16	31
Fore, 80W	2.00	1.60	11	18
Fungi Sperse Cu-S, 45.6L	0.50	0.22	26	26
Fungi Sperse Z-S, 54.2L	0.50	0.27	22	36
Isobac, 20EC	0.25	0.05	27	17
Kocide 101, 86W	0.50	0.43[b]	20	19
Kocide 101, 86W	1.00	0.86[b]	20	34
Kocide 101, 86W	1.50	1.29	22	23[d]
Lawn Disease Control, 78W	1.00	0.78	25	32
Nabac, 25EC	0.25	0.06	35	46
Polyoxin B, 10W	0.50	0.05	25	23
Polyoxin B, 10W	1.00	0.10	15	18
Polyram, 80W	2.00	1.60	15	24
Thiram, 65W	2.00	1.30	18	18
Turf, 75.75W	1.00	0.76	23	27[d]
Carboxin, 75W	1.00	0.75[b]	31	37[d]
Difolatan + Botran, 80W + 75W	1.0 + 1.0	0.80 + 0.75	14	15[d]
Difolatan + Carboxin, 80W + 75W	1.0 + 1.0	0.80 + 0.75	13	22[d]
Carboxin + Botran, 75W + 75W	1.0 + 1.0	0.75 + 0.75	28	34[d]
Untreated		0[b]	52	48

[a] W = wettable powder; T = technical; L = liquid; EC = emulsifiable concentrate. See text for details.
[b] Treatments sampled for microflora detection.
[c] LSD (.05) middle leaves = 14; LSD (.05) upper leaves = 23; middle leaves evaluated 9 days after last spray application; upper leaves evaluated 17 days after last spray application.
[d] Treatments causing injury.

"other fungi" category shown in Table 2 includes genera and species isolated too infrequently for separate statistical analysis. *Epicoccum* sp., *Trichoderma* sp., and *Cytosporella* sp. were isolated on RBSS but not on Cz + 6, the latter medium favoring the isolation of *Aspergillus* and *Penicillium* species. Otherwise the isolation frequency patterns were similar for both media.

Numbers of microflora recovered from within and on leaf tissue.—Although an abundance of microorganisms grew from blended leaf tissue, quantitative differences found among the six fungicidal treatments evaluated were not significant. Results were similar with both media, except for more "yeasts and bacteria" on the Cz + 6 medium than on the RBSS medium. Thus, only the results on RBSS medium are presented in Table 2. The predominant microorganisms were yeasts and bacteria. Among the fungi, *Cladosporium* was most abundant. Stalk position had no significant effect upon the frequency of *Alternaria* or "unidentified fungi" isolated from the leaves; but significantly more *Cladosporium* were isolated from upper than from either the middle or lower leaves. More "yeasts and bacteria" were isolated from middle and upper than from the lower leaves.

Because Difolatan-sprayed tobacco had the fewest brown spot lesions per leaf, plants from this treatment were sampled (28 August) and found to yield fewer fungi on either media than did the untreated controls (Table 3, Fig. 1).

Fungicidal activities of several chemicals were compared by an agar incorporation test with fungi isolated from tobacco leaves. Chemicals were incorporated into melted potato-dextrose agar in petri dishes to obtain concentrations of 1, 10, 100, and 1,000 ppm. Plugs of mycelium (3-mm diam) were planted on the media in the center of the dishes. After incubating the dishes for 1 week at 26 C, linear fungal growth was measured. This is considered a severe test, since mycelial growth is more difficult to control than other stages of fungal growth. Difolatan was one of the most active fungicides in concurrence with field tests (Table 4).

TABLE 2. The microflora isolated from within field-grown tobacco leaves and the numbers of microflora on and in tobacco leaves

Microorganism	Stalk position: Lower	Middle	Upper	LSD .05
Microflora within leaves–leaf disc method (% frequency[a])				
	Rose bengal streptomycin sulfate medium			
Alternaria sp.	85.3	79.1	40.1	8.7
Cladosporium sp.	0.7	56.3	12.8	8.7
Other fungi[b]	18.6	18.1	3.8	5.1
Unidentified fungi	16.3	14.3	15.5	NS
	Czapek + 6 NaCl medium			
Alternaria sp.	96.0	75.9	26.4	11.8
Cladosporium sp.	6.8	62.8	15.5	6.2
Other fungi[b]	4.8	13.2	2.3	2.6
Unidentified fungi	13.9	30.4	10.2	6.1
Microflora on and in leaves–dilution plate method (thousands of colonies[c])				
	Rose bengal streptomycin sulfate media			
Alternaria sp.	15	2.0	15	NS
Cladosporium sp.	1	0.3	39	26
Unidentified fungi	16	3.0	6	NS
Yeasts and bacteria	332	760.0	801	274

[a] Percentage values were computed by dividing the number of leaf discs from which a fungus was isolated by the number of discs cultured and multiplying the quotient by 100.

[b] Including *Aspergillus flavus, A. niger, Cytosporella* sp., *Epicoccum nigrum, Penicillium* sp., and *Trichoderma* sp.

[c] Each value represents the number in thousands of colonies isolated per gram wet weight of leaf tissue.

TABLE 3. Fungi isolated by two methods from tobacco leaves sprayed with Difolatan

Fungus	Culture media: RBSS Control	RBSS Difolatan	Cz + 6 Control	Cz + 6 Difolatan
Percentage frequency of fungi cultured from surface-sterilized leaf discs[a]				
Alternaria sp.	25.3	3.3	34.0	6.7
Cladosporium sp.	4.7	0.7	35.3	3.3
Penicillium sp.	7.3	1.3	0.7	0.0
Nigrospora sp.	3.3	0.0	1.3	0.0
Nodulosporium sp.	3.3	0.0	0.0	0.0
Cytosporella sp.	10.6	0.0	0.0	0.0
Aspergillus niger	4.0	0.7	0.0	0.0
Unknown fungi	27.3	24.7	14.7	5.3
Thousands of fungi per gram of tissue cultured by dilution plate technique[a]				
Alternaria sp.	1.6	1.6	4.6	1.3
Cladosporium sp.	4.0	8.0	2.3	0.6
Unknown fungi	18.6	4.6	0.3	1.6
Total fungi	24.3	15.6	7.3	3.6

[a] See Table 2 for a detailed explanation of the computation procedures.

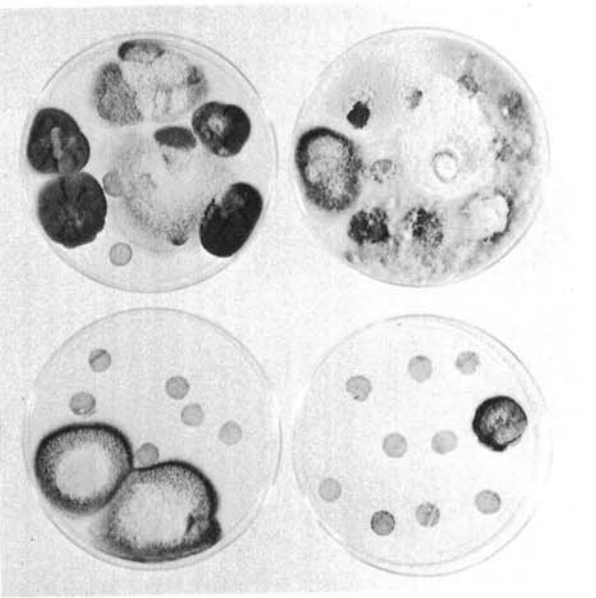

Fig. 1. Tobacco leaf discs from controls (top row) and from tobacco sprayed with Difolatan (bottom row). Note the reduction in fungal colonies emerging from Difolatan-treated tobacco.

DISCUSSION.–The incidence of brown spot increased 0.2% from 1968 to 1969 for an estimated loss of $5.6 million to North Carolina tobacco growers (7). This was the largest seasonal loss from a specific disease. Current control suggestions are to rapidly eliminate crop residues from the field and to plant the most tolerant cultivar. Fungicidal sprays might provide an economical means of control, as indicated by the efficacy of several treatments. All of the fungicidal treatments reduced the amount of brown spot. The increase in brown spot from the first evaluation on the middle leaves to the second evaluation on the upper leaves could relate to (i) decreasing protection with time following the last spray application; and (ii) increasing pressure from the formation of additional spores at established infection sites.

Impressive numbers and kinds of microorganisms are both within and on tobacco leaves during the growing season. These populations are dynamic, changing during the season. *Cladosporium* isolated from within leaf tissue increased from the lower to the middle leaves (0.7 to 56.3%) and decreased in upper leaves to a 12.8%. *Alternaria* spp. were isolated most frequently (average of 68%) during the season. A significant decrease in *Alternaria* spp. was noted in the upper leaves at a time when brown spot was developing. These *Alternaria* colonies in the leaf may be directly related to the amount of brown spot, or should they represent species other than the brown spot pathogen, they may predispose the plant to infection by the latter. However, the control of brown spot by the six fungicidal treatments without a corresponding reduction in the isolation of *Alternaria*

TABLE 4. Fungicidal dosages (ppm) which inhibited by 50% the mycelial growth on potato-dextrose agar of certain fungi originally isolated from tobacco leaves

Chemical	*Alternaria*	*Epicoccum*	*Trichoderma*	*Penicillium*	*Aspergillus*
Alar	>1,000	>1,000	>1,000	1,000	>1,000
Benomyl	1,000	10	10	1	10
Bordeaux	>1,000	>1,000	>1,000	100	>1,000
Botran	10	10	>1,000	100	
Chlorothalonil	1,000	>1,000	>1,000	1	>1,000
Difolatan	1	10	100	1	10
Maneb	10	100	1,000	1,000	100
Du-Ter	10	1,000	10	10	1
Dyrene	10	100	100	>1,000	100
Ferbam	10	10	1,000	100	1,000
Fore	10	10	1,000	1	100
Isobac	1	1	10	>1,000	10
Kocide 101	100	1,000	1,000	1,000	>1,000
Nabac	1	1	10	1,000	10
Polyoxin B	100	1,000	1,000	>1,000	>1,000
Polyram	100	1,000	1,000	100	>1,000
Thiram	10	10	100	10	10
Turf	10	100	100	10	100
Carboxin	100	100	100	100	100

indicates that different forms of the fungus are present in and on the leaves. Only six treatments were examined for microflora because time and materials were limiting factors. Carboxin and benomyl treatments were selected because these chemicals are systemic; the others because they are broad in their spectrum of fungicidal activity. Unfortunately, the choices were poor and only *Cladosporium* was controlled by benomyl. Difolatan treatments were evaluated late in the season because of their good performance in controlling brown spot. This fungicide reduced the microflora in and on tobacco leaves and inhibited the mycelial growth of several tobacco fungi in the agar incorporation test. The control of fungi in tobacco leaves by Difolatan seems to result from both its fungicidal activity and its noninjurious penetration of leaves. This activity offers promise as a tool for investigating the relationship of these leaf microorganisms to leaf quality and disease. Residual fungicide on the cultured tissue may have prevented some fungi from growing out of the tissue; however, the 8-day interval after the last spray application probably eliminated most of the active fungicide.

Using the dilution plate technique, every viable organismal unit is expected to produce a colony. The results substantiate this expectation, as seen by the high numbers of yeasts and bacteria. A dramatic increase in *Cladosporium* as observed in the upper leaves indicates that the fungus has sporulated. This evaluation gives information about the kinds of organisms both on and in the plant, and their relative numbers during the season.

In summary, a large and variable population of microorganisms or "invisible invaders" is present in tobacco leaves during the growing season which may influence leaf quality and disease incidence. Some ideas concerning these influences in plant disease have been reviewed (2). Also, the presence of these microflora could affect the health of tobacco users. A few tobacco fungi were tested and found to be nontoxic to mice (1). Additional toxicological studies may be made in the future. In the event that it becomes desirable to control brown spot and leaf microflora, the use of chemicals constitutes a practical approach.

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