Characterization of Endophytic Fungi in Healthy Leaves of Nicotiana spp.

Harvey W. Spurr, Jr. and Ronald E. Welty

Research Plant Pathologists, Southern Region, Agricultural Research Service, U.S. Department of Agriculture, Oxford Tobacco Research Laboratory, Oxford, N. C. 27565; and North Carolina State University, Raleigh 27607, respectively. Also, Professor and Associate Professor of Plant Pathology.

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ABSTRACT

Endophytic fungi were studied in healthy appearing tobacco leaves from field-grown plants. Surface sterilization with sodium hypochlorite was reaffirmed as adequate for removing or destroying epiphytes on leaves. Proximal and distal portions of the leaves had similar endophytic populations. Endophytic fungi were frequently observed in cross sections of tobacco leaf tissues, and in sections with the epidermis removed. They were varied in morphology, were found in all leaf tissues, and increased in number with leaf growth and development. Certain differences in endophytic populations appeared to be host-specific. *Alternaria* spp. were frequent endophytes in all of the *Nicotiana* spp. examined. *Penicillium, Aspergillus,* and *Cladosporium* spp., respectively, were next most frequent. Most endophytic *Alternaria* isolates from healthy appearing leaf tissues were unable to induce symptoms under conditions favoring the brown-spot pathogen, *A. alternata.* Endophytes were not found in young seedlings in the plant bed protected with ferbam fungicide. After these seedlings were transplanted and the fungicidal treatment discontinued, endophytic fungi became established in the leaves. The number of endophytes changed as the tobacco leaves grew and developed. *Alternaria* spp. steadily increased in number or frequency whereas *Cladosporium* spp. fluctuated. The frequency pattern of endophytic fungi was similar in leaves from four different stalk positions. The influence of endophytic fungi in disease development and senescence are discussed.

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Although internal cells and tissues of healthy plants are generally considered sterile, bacteria (7, 14, 19), flagellates (4), and fungi (including yeasts) (1, 3, 7, 13), have been isolated from them. More recently, endophytic fungi were isolated from healthy appearing leaves of field-grown tobacco *(Nicotiana tabacum* L.) (8, 17, 21, 22).

Epiphytic microorganisms, described by Leben (5) as residents or casuals, in the phyllosphere of plants have been the subject of increased research interest (9, 10). These epiphytes must be removed or killed before the microflora associated with the inner plant tissue, endophytes, can be studied. Surface disinfection with aqueous sodium hypochlorite, alcohol, or mercuric chloride is a technique commonly used to eliminate epiphytes (8, 18).

In previous studies, *Alternaria* and *Cladosporium* were identified frequently as endophytes of tobacco leaves (8, 17, 22). Our objectives for this study were (i) to reassess the efficacy of surface sterilization for eliminating epiphytes, (ii) to make histological observations and records of endophytic fungi, (iii) to analyze the leaves of several species of *Nicotiana* for endophytic fungi, (iv) to quantitate endophytic *Alternaria* and *Cladosporium* during the growth and development of tobacco leaves in the field, and (v) to determine the relationship of endophytic *Alternaria* to brown-spot disease caused by *Alternaria alternata* (Fries) Keissler.

MATERIALS AND METHODS.—*Efficacy of surface disinfection.*—Disinfection efficacy was reexamined on 9 mm diameter disks cut from leaves of

greenhouse-grown tobacco and from Whatman no. 1 filter paper. Both were sprayed with an aqueous suspension of *Alternaria* spores and dried just prior to cutting the disks. The disks were placed on Difco Czapek's solution agar with 6% NaCl (Cz6) (17):(i) without prior treatment, (ii) after washing disks in running tap water for 30 minutes, or (iii) after agitating the disks in 0.5% or 1.0% sodium hypochlorite (NaClO) for 30, 60, or 120 seconds. Disks were observed for fungal growth at 2-day intervals for 10 days. Ten disks were used for each treatment, and the experiment was done twice. Disks of field-grown tobacco were placed on Cz6 after agitation in 1% NaClO for 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 6, 7, 8, 9, 10, 15, 20, 25, and 30 minutes. Also, 50 disks were cut from each of 10 leaf areas between the midrib and margin, avoiding the large veins, to determine if *Alternaria* was associated with certain portions of the leaf.

Histological observations of endophytic fungi.—Leaf disks (9-mm diameter) were cut from tobacco plants (cultivar Coker 298) in the field as described below under quantification of endophytic fungi. Twenty-five disks were cut and placed immediately into a vial containing a killing and fixing solution: 5 ml 40% formaldehyde: 5 ml acetic acid: 90 ml alcohol (FAA) and held in the FAA until examined. The disks were sectioned for histological examination with a Lab-Line/Hooker Plant microtome (Lab-Line Instruments, Inc., Melrose Park, Ill.). A fresh carrot section was cut 35 mm long, 3 mm thick and 8 mm wide with the skin (epidermis) on one long (35 mm) side. The section was cut through the center of the 3 mm thickness to the skin - leaving the skin to act as a hinge. The carrot section was hinged open, a stack of five leaf disks inserted, and then hinged closed onto the leaf disks. The carrot section with the disks was placed in the microtome holder with the open side opposite the hinged side and toward the blade. Sections were cut with the speed of the microtome blade set at 4. Leaf tissue sections 24 microns thick and 8 mm long were removed from the water bath of the microtome and placed in a drop of lactophenol containing 0.025% cotton blue on a microscope slide, covered with a cover slip and examined with a microscope.

A count of endophytic fungi in tobacco leaf tissue was made using this histological technique. Fifty cross-sections were examined from each replicate in the experiment described below under "Quantification of endophytic fungi during tobacco leaf growth and development." Samples were obtained from various stalk positions during the growing season. All positively identified endophytic fungi were counted. A colony was not counted when symptoms of lesion formation or tissue damage were observed in association with the fungus. The number of endophytes was expressed as the mean per 50 cross-sections per sample.

Endophytic fungi of Nicotiana spp.—Four species of tobacco were grown during 1968 in the field at the Oxford Tobacco Research Station, Oxford, N. C. These included two cultivars of *Nicotiana tabacum:* brown-spot susceptible Coker 298 and resistant PD 121. Plants were topped at 20 leaves, and lateral bud growth was controlled with two spray-applications of maleic hydrazide. The other species were *N. glutinosa, N. rustica,* and *N. sylvestris*. These were neither topped nor sprayed. A field plot contained five consecutive plants of each species or cultivar, with the five plots randomized within a row. Five randomized rows served as replications. Leaves from the lower stalk position were sampled in mid-July: twenty disks were cut from each plot in each row, transported to the laboratory in an ice chest, immersed in 1% NaClO for 30-60 seconds, rinsed 3 times in sterile distilled or running tap water, and placed on Cz6 or rose bengal streptomycin (RB) agar in petri dishes, five or ten disks/dish (17). The disks were viewed every 2 or 3 days until the 10th day, when the final observations for filamentous fungi were made. Identifications were made in the original dish, but when this was not possible, fungi were subcultured and incubated for further study. In a nonreplicated field-sampling of six other *Nicotiana* spp., and a reciprocal (4N) hybrid, 20-60 leaf disks from each were examined on Cz6.

Quantification of endophytic fungi during tobacco leaf growth and development.—The endophytic fungi of cultivar C 298 were studied beginning with young seedlings in the plant bed to harvest time during 1970. The first leaf disks were cut 15 May from 15-to-20-cm seedling plants in a plant bed treated weekly with ferbam to control blue mold *(Peronospora tabacina* Adam). Disks also were cut from plants in the same plant bed on 5 June, one week after the last application of ferbam. These seedlings were transplanted into the field in rows of 75 plants each; three rows served as replications. Disks were cut weekly from 1 July to 2 September from leaves of four stalk positions: the lowest four leaves were position 1, the next four leaves position 2, etc. The plants were topped at 16 leaves. Leaves were harvested (removed from the stalk) as they matured. Because leaves of the upper stalk position were undeveloped early in the season, and lower leaves were removed as they matured later in the season, not all stalk positions were sampled at each interval. At each weekly sampling, six discs were randomly cut from four leaves in each available stalk position from 10 plants in each row. No more than two disks were cut from each leaf. Fifty disks from each stalk position were surface-disinfected and cultured on Cz6. During the season, each plant was sampled at least once as evidenced by sample holes, but individual plant or leaves were not repeatedly sampled.

Pathogenicity tests.—Random field isolates of endophytic *Alternaria* from healthy appearing tissues were subcultured on tubes of potato dextrose agar (PDA) and stored at 2-3 C until tested for pathogenicity. An exception to random sampling was in the leaf development study, where one isolate of *Alternaria* for each interval, stalk position, and replication was subcultured and tested for pathogenicity. Pathogenicity was determined on leaf disks, 9-cm diameter, from greenhouse-grown C 298, inoculated with an aqueous spore suspension of *Alternaria* following the procedure described by Spurr (16). After 10 days of incubation, lesions on the leaf disks were rated on a scale of 1-5 representing lesion development (1 = no lesion, 5 = large lesion). Each isolate of *Alternaria* was tested three times.

RESULTS.—*Efficacy of surface disinfection.*—*Alternaria* grew from (i) 100% of the inoculated tobacco leaf and paper disks cultured without

prior treatment, (ii) 90% of the washed tobacco leaf disks and 100% of the washed paper discs, (iii) 17% of the tobacco leaf discs and 0% of the paper discs treated with 0.5% NaClO, and (iv) 10% of the tobacco leaf disks and 0% of the paper disks treated with 1% NaClO. Treatment with 1% NaClO for 120 seconds was only slightly more effective than either 30 or 60 seconds. The frequency of fungal growth from disks cut from field-grown tobacco leaves decreased with increasing time of treatment with 1% NaClO. However, *Alternaria* and *Cladosporium* consistently grew from tissues after 10 minutes of exposure to 1% NaClO. No preference for the proximal or distal portion of the leaf was observed in relation to the population of endophytic fungi.

Immersion of the 9-mm diameter leaf discs in 1% NaClO for 30-60 seconds, followed by three rinses in sterile or running tap water, was adopted as a standard procedure for isolating endophytic fungi of tobacco leaf tissue.

Histological observations of endophytic fungi.—In 1550 cross-sections examined, 917 endophytic fungal colonies were observed. The hyphae were generally stained blue and contrasted with the light green of the plant tissue. The endophytes varied in type, size, and location within the leaf tissues (Fig. 1). Hyphae were the principal fungal structures observed. Smaller hyphae were found under the cuticle and within the epidermal cells. Larger hyphae were found in all tissues including the epidermis, mesophyll (palisade and spongy parenchyma), and vascular tissues. Occasionally, endophytic hyphae

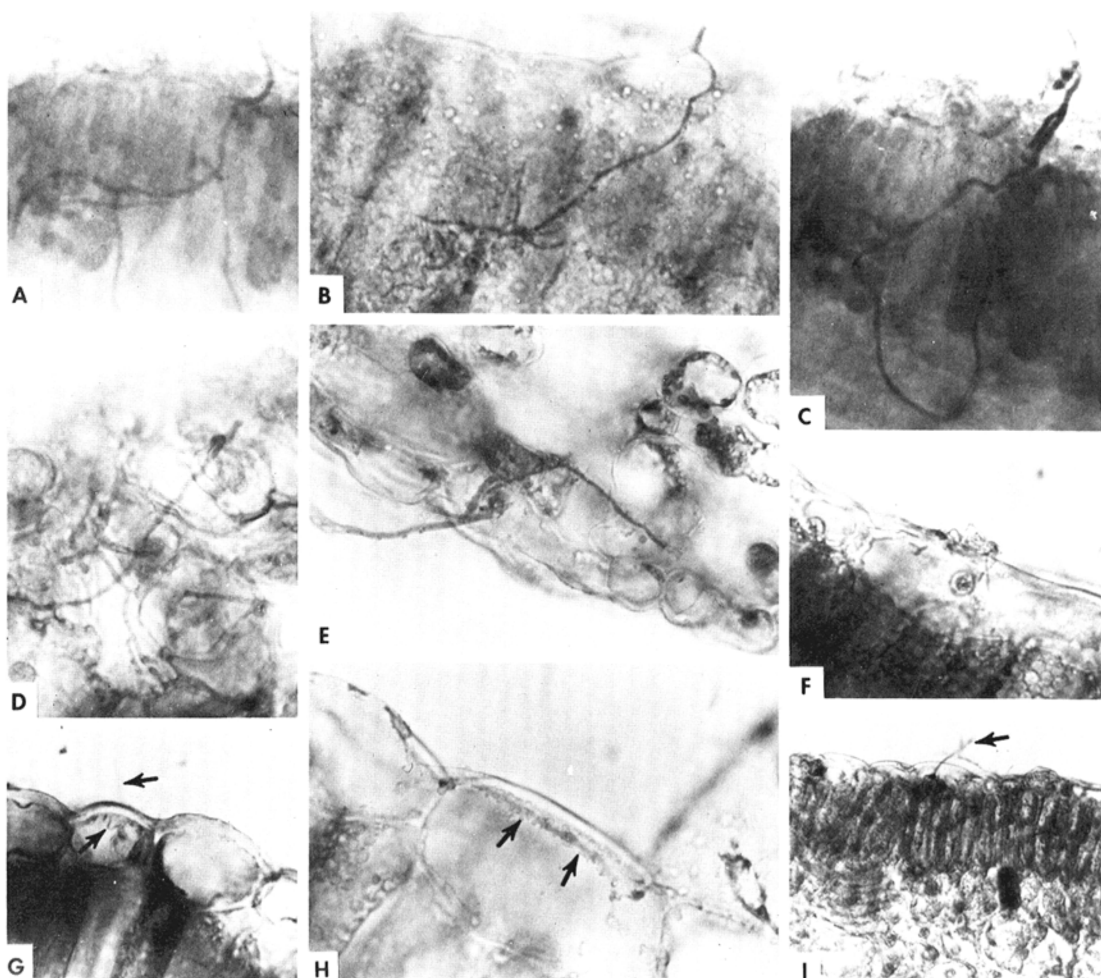

Fig. 1-(A to I). Photomicrographs of endophytic fungi observed in healthy tissues of tobacco leaves. Following killing and fixing, the leaf tissues were sectioned with a Lab-Line/Hooker Plant Microtome and stained with cotton blue in lactophenol. **A,B,C)** Large hyphae often observed growing intercellularly. **C)** An *Alternaria* spore is identifiable on the surface. **D)** Large, endophytic hyphae observed in the mesophyll after stripping the epidermis. **E)** Endophytic hyphae entering lower epidermis and growing in the mesophyll. **F)** Small hyphae entering epidermal cell and growing near the nucleus. **G)** Small epiphytic structure on the leaf surface connected to endophytic hyphae within epidermal cell. **H)** Hyphae growing just under the cuticle by epidermal cells. **I)** Small epiphytic fungal structure penetrating the epidermis to the palisade tissue; the fungal growth present in the palisade tissue is indistinct because it is between two rows of cells in this photomicrograph. A-G (× 640), H (× 1600), I (× 320).

TABLE 1. Mean number of endophytic fungal colonies observed per fifty cross-sections of tissue sampled from tobacco leaves at intervals during their growth and development[a]

Leaf stalk position[b]	Date sampled, July				
	2	8	15	22	29
1	14 (2)	27 (1)	42 (2)		
2	7 (3)	18 (2)	31 (3)	54 (2)	
3		16 (3)	20 (2)	64 (1)	60 (2)
4			14 (2)	30 (2)	40 (3)

[a]The number of replicates per mean is shown in the parentheses (1, 2, or 3), standard deviations for comparing means are dependent upon the number of replications:
mean (1) : mean (1) = 21.8
mean (1) : mean (2) = 18.9
mean (1) : mean (3) = 17.8
mean (2) : mean (2) = 15.4
mean (2) : mean (3) = 14.1
mean (3) : mean (3) = 12.6
[b]Stalk positions 1 = the lowest four leaves of the stalk, 2 = leaves 5-8, 3 = leaves 9-12, 4 = leaves 13-16.

within the tissues could be observed to emerge on the leaf surface where spores and structures of *Alternaria, Cladosporium* or *Penicillium* were identified. The larger hyphae, often found within the palisade and spongy parenchyma, was intercellar and could be followed between the layers of cells by changing the focus of the microscope. These hyphae were also observed in pieces of leaf tissue from which the epidermis had been stripped-off prior to staining. The colonies of large hyphae in the mesophyll were generally restricted in diameter, extending across six or fewer cells in any one plane. No visible disruption or impairment of the plant cells was noted with the fungi considered to be endophytes. This is in marked contrast to the numerous changes in cells and structures which accompany the invasion and development of the brown-spot pathogen, *A. alternata*.

By counting the number of endophytic fungi observed in 50 cross-sections per replicate, a determination of the mean number of endophytes was obtained (Table 1). The data show that emerging leaves are free of endophytes, and that the number of endophytes increases steadily and significantly with leaf growth and development under field conditions.

Endophytic fungi in Nicotiana spp.—Thirteen genera of endophytic fungi were isolated and identified from leaves of field-grown tobaccos (Table 2). The mean percentage frequencies of the endophytes was similar in all *Nicotiana* spp. examined. *Alternaria* spp. were found with almost every leaf disk, including the brown-spot-resistant cultivar, PD 121. *Penicillium* spp. were the next most frequently isolated: Other fungi isolated included species of *Aspergillus, Chaetomium, Cladosporium, Choanephorae, Cytosporella, Epicoccum, Mucor, Nigrospora, Penicillium, Rhizopus, Stemphylium,* and *Trichoderma*. These were not quantified. As in previous studies (17), the data in Table 2 show that more fungal spp. grow from tobacco leaf disks incubated on RB agar than on Cz6. However, the number of leaf disks with *Alternaria* or *Cladosporium* is larger on Cz6 agar.

In the nonreplicated field study with other *Nicotiana* spp. [*N. bonariensis, N. longiflora* (4N), *N. longiflora* var. *breviflora, N. longiflora* var. *grandifolia, N. plumbaginifolia, N. suaveolens,* and *N. velutina*], *Alternaria* grew from nearly 100% of the leaf disks. The percentages of leaf disks with *Alternaria* from other cultivars were: *N. tabacum* (Beinhart 1001) 75%, *N. tabacum* × *N. suaveolens* (4N) 80%, and *N. suaveolens* × *N. tabacum* (4N) 60%. *Cladosporium* grew from 0-50% of the disks, except for Beinhart 1001, which yielded 75%. From all species and cultivars, *Cladosporium* grew from 24% of the leaf disks cultured.

Quantification of endophytic fungi during tobacco leaf growth and development.—No fungi grew from leaf disks cut from seedlings treated with ferbam in the plant beds; one week after the fungicide was discontinued, *Alternaria* grew from 29% of the disks. Following transplanting, the frequency of endophytic *Alternaria* increased as the leaves matured; 3-week-old leaves yielded 64-94%, and 7-week-old (mature) leaves yielded 93-100% (Fig. 2). The frequency of occurrence of *Cladosporium* reached two high points, one in July and one in August, and decreased as leaves matured. This fluctuating pattern with *Cladosporium* was distinctly different from that with *Alternaria*. The frequency patterns determined from the four stalk positions were similar.

TABLE 2. Relative frequency (%) of endophytic fungi isolated from surface-sterilized, 9-mm diameter disks from mature tobacco leaves on Czapek's agar with 6% NaCl (Cz6) and rose bengal streptomycin agar (RB).

	N. tabacum[a]				*Nicotiana* spp.					
	C 298		PD 121		*glutinosa*		*rustica*		*sylvestris*	
Fungi	RB	Cz6	RB	Cz6	RB	Cz6	RB	Cz6	RB	Cz6
Alternaria sp.	81	100	99	100	87	100	93	100	93	100
Aspergillus niger	24	22	4	4	9	3	3	1	9	5
Aspergillus flavus	13	11	5	6	4	2	0	0	3	4
Cytosporella sp.	21	0	29	0	22	0	45	0	57	0
Epicoccum sp.	7	0	18	0	5	0	7	0	5	0
Penicillium sp.	30	3	19	12	34	25	23	3	36	13
Trichoderma sp.	20	0	8	0	9	0	1	0	6	0
Other fungi[b]	57	1	61	0	50	0	63	1	79	3
Unknown fungi[c]	14	29	17	10	3	20	6	29	7	24

[a]Cultivars Coker 298 (brown-spot susceptible) and PD 121 (brown-spot resistant).
[b]Including species of *Chaetomium, Choanephora, Cladosporium, Mucor, Nigrospora, Rhizopus,* and *Stemphylium.*
[c]Including those that did not readily sporulate on these media.

Pathogenicity tests.—None of the *Alternaria* isolates from healthy-appearing tobacco tissue produced brown-spot symptoms when used to inoculate tobacco. Twenty-two isolates from the various *Nicotiana* spp. had an average lesion rating of 1.4, and 27 isolates from each of the 10 sample intervals and four stalk positions averaged 1 (no lesions). A highly virulent isolate of the brown-spot pathogen, A5, was used as a check in each pathogenicity test; it averaged 4.5 (large lesions). These results indicate that endophytic *Alternaria* isolated from healthy appearing tobacco leaves was not the brown-spot pathogen.

DISCUSSION.—Surface sterilization of leaf tissue must inactivate or remove epiphytes if endophytes of healthy tissue are to be identified and quantified. The efficacy of 1% NaClO treatments for the destruction of epiphytes was reaffirmed by the results with inoculated paper and leaf disks. We reexamined the efficacy of this procedure because Norse (8) indicated it might be less effective than other procedures. He used a 2-minute exposure to 0.1% (w/v) $HgCl_2$ in 25% aqueous ethanol followed by three rinses in sterile water, which he indicates may be too harsh because it killed some endophytes. The wetting, penetrating, and killing properties of the NaClO solution are enhanced by the addition of alcohol. Therefore, the solution used for surface sterilization must be adjusted according to the properties of the host tissue. Since surface-disinfection in 1% NaClO for 30-60 seconds consistently reduced epiphytic fungi by 85-95% or more, we chose this as our standard procedure to prepare for the quantitative and qualitative studies of endophytic fungi.

Histological observations of hyphae provide additional evidence of the presence and diversity of endophytic fungi in all tisues of healthy-appearing tobacco leaves. Their numbers increased with the emergence, growth and development of leaves. It is reasonable to expect some of the colonies to die or disappear; i.e., it does not follow that, once established, a colony will persist for the life span of the leaf, although some may do so. Thus, changes in the numbers and types of endophytes with the growth and development of leaves seems normal.

The identification of fungi of 13 genera shows the diverse nature of the endophytic population. Norse (8) conducted a study of endophytic fungi in tobacco leaves at Lilongwe, Malawi, Africa where he identified fungi of 11 genera. As in our study, *Alternaria* and *Cladosporium* were frequently found. However, between locations there were differences in the makeup of the less frequently observed genera. Perhaps the largest difference was the high frequency of *Penicillium* in our study and its complete absence in the African study.

Although the frequency of *Alternaria* was similar in the four *Nicotiana* spp. in the quantitative study, there were indications that host specificity exists for other endophytes; e.g., *Cytosporella* spp. was more frequent in *N. sylvestris* (57%) than *N. tabacum* (21-29%). Additional studies are needed to firmly establish host specificities.

Several investigators (2, 20, 23) suggest that fungal colonization of leaves influences the rate of senescence. Our results indicate that although there may be fluctations in certain endophytes, overall the endophytic population steadily increases with leaf growth and development. Pugh and Buckley (11) found that *Cladosporium* grows and sporulates as soon as leaves are fully expanded. When fungicidal treatment reduced tobacco leaf microflora, senescence was delayed (17). However, it was not clear whether this was the result of a reduction in microflora, chemical stimulation of growth, or both processes.

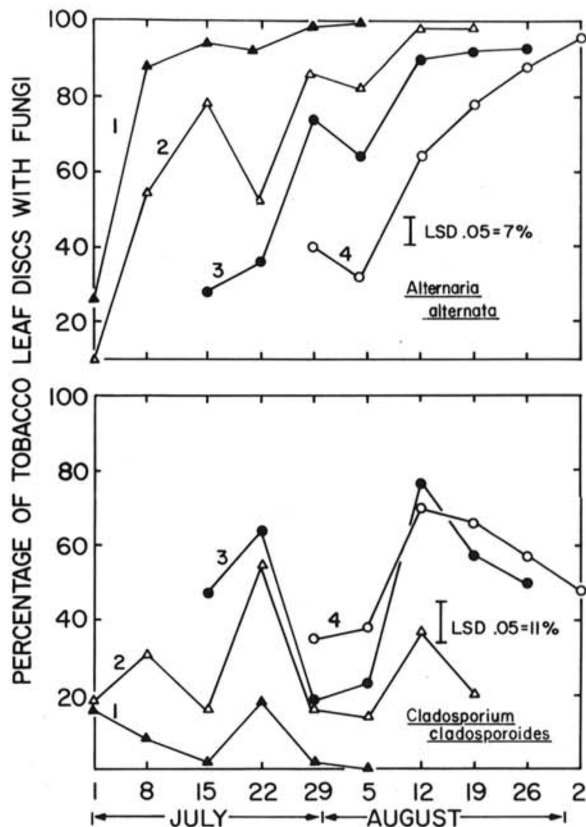

Fig. 2. Quantification of *Alternaria* and *Cladosporium* during tobacco leaf growth and development. Line 1 represents stalk position 1 or the lower four leaves of the plant; stalk position 2 (line 2) represents leaves five-to-eight; position 3 = leaves nine-to-twelve; and position 4 = leaves thirteen-to-sixteen. The plants were topped at 16 leaves. LSD. Figures are for comparing any of the points of one stalk position.

We have been interested for some time in the relationship of the endophytic *Alternaria* to the pathogen *A. alternata,* and to the development of the brown-spot disease. It was clearly indicated by our pathogenicity tests that the majority of the endophytic isolates of *Alternaria* were nonpathogenic; i.e., did not produce symptoms under usual conditions for symptom development. It is difficult to believe these fungi could make a transition from the nonsymptom-producing, endophytic state to the symptom-producing, highly pathogenic state. The presence of endophytic *Alternaria* in similar numbers in both brown-spot-susceptible and resistant cultivars also indicates this was not the brown-spot pathogen. Perhaps endophytic *Alternaria* may influence brown-spot development as nonpathogenic, epiphytic *Alternaria* was

shown to reduce disease (15). Certainly there is ample *Alternaria* present (especially in fully grown, mature leaves) to change leaf quality and colonization by microorganisms. The frequent presence of nonpathogenic *Alternaria* in the leaves may have contributed to the difficulties experienced by some in producing typical brown-spot lesions from cultures isolated from tobacco (12). This could also explain the disagreement among tobacco pathologists regarding conidial measurements and the species name of the *Alternaria* pathogen causing brown-spot disease (6).

In conclusion, tobacco leaves are host to an abundance of endophytic fungi which could influence plant and disease development.

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