Relation of Leaf Age to the Reaction of Tobacco to Alternaria alternata

J. R. Stavely and L. J. Slana

Research Plant Pathologist and Research Assistant, Plant Pathology, respectively, Crops Research Division, ARS, USDA, Beltsville, Maryland 20705.

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ABSTRACT

The reaction of tobacco leaves to infection by *Alternaria alternata* was governed by age. The fungus directly penetrated all inoculated leaves within about 48 hours after inoculation, caused necrosis of epidermal cells, and began intercellular invasion of the mesophyll. In the 2 to 4 youngest inoculated leaves, resulting necrotic lesions were usually less than 1 mm in diameter, but in older leaves, lesions enlarged to average about 8 mm in diameter by 14 days after inoculation. Expansion of heavily inoculated leaves that had reached less than 75% of their potential size on the day of inoculation was severely inhibited following inoculation. A cicatrice of densely packed, angular cells developed around infections on these leaves, stopping further advancement of the fungus. A similar response occurred around mechanical wounds on young leaves. Both cell division and inhibition of expansion apparently had roles in cicatrization. No walling-off response occurred around lesions incited by *A. alternata* or mechanical wounding in older tobacco leaves. Walls of cicatrice cells had greater staining affinity than walls of other host cells. Chloroplasts were absent from cells in the walling-off layers and cells in chlorotic halos around lesions in older leaves. Phytopathology 61:73-78.

Additional key words: Nicotiana tabacum, leaf growth, brown spot.

Brown spot, incited by *Alternaria alternata* (Fr.) Keissl. (*A. tenuis Nees*), is the most important leaf disease of flue-cured tobacco, *Nicotiana tabacum* L., in the United States. The disease is usually found on older leaves in the field, and was considered a disease of senescence (4) before Ramm & Lucas (7) confirmed the report of Riley (8) that plants are susceptible at all ages and stages of growth. They noted that infections on young leaves resulted in small, pinpoint lesions. A pictured leaf showed that marked distortion occurred when immature leaves were heavily infected (7). From histological investigations of developing lesions, Ramm (6) concluded that healthy tissue surrounding leaf spots develops no defense reaction, such as formation of a cicatrice or cork layer. Thus, the reaction of tobacco to *A. alternata* was placed in the same category as the leaf spot reactions of tomato and potato (6) according to Cunningham's classification of host reactions to leaf spotting fungi (3). Stavely & Main (11) reported that expansion of the youngest inoculated leaves is markedly inhibited. The pinpoint lesions that develop on the young leaves are much smaller and have a more restricted halo of chlorotic tissue than the lesions that develop on older leaves.

The research reported here was initiated to (i) determine the effect of leaf age on infection and symptom development; (ii) measure the effect of infection on leaf expansion; and (iii) compare histologically postinfection events in leaves of varying ages. Some of our results have been published (12).

MATERIALS AND METHODS.—Inoculum methods were similar to those previously described (11), except that conidial concn/ml was increased from 3 to 12×10^4. Highly pathogenic *A. alternata* isolates A3 and A5, reisolated from inoculated plants to restore virulence, and nonpathogenic isolate A37 were used for inoculum. All leaves were inoculated on both the lower and upper surfaces. Inoculated plants and noninoculated controls were incubated at 20 ± 2 C for 7 days after inoculation, then placed in a greenhouse at 27 ± 3 C. Greenhouse grown plants, 2 to 3 months old, were used in all comparisons.

Cultivars Coker 187 Hicks (C187H), very sensitive; NC95, moderately tolerant; and PD121, tolerant to the disease (11), were used in studies of the effect of infection on leaf expansion. In each test, five plants of each cultivar were inoculated with a pathogenic isolate, and five plants of each cultivar were inoculated with a nonpathogenic isolate. Leaf widths and lengths were measured on the day of inoculation and 7, 14, and 21 days later. Results were based on the average leaf width and length measurements from three different tests.

The effect of infection was compared with the effect of mechanical wounding on C187H tobacco plants. Half of the plants were inoculated, and small puncture wounds were made 3 to 4 mm apart over the leaf surfaces of the other half.

For histological studies, samples were taken from leaves of C187H tobacco plants at 24-hr intervals from 24 to 168 hr and on the 10th, 14th, and 54th day after inoculation. Leaf samples were placed for 24 to 72 hr in a fixative containing 40% Formalin, propionic acid, 95% ethanol, and distilled water in a 10:5:50:35 ratio. Air was removed from the tissues by applying a partial vacuum for 5 to 15 min. The tissues were dehydrated in a tertiary butyl alcohol series, passed through paraffin oil, and infiltrated with Fisher Tissuemat (mp 56.6 C). Embedded tissues were sectioned with a rotary microtome at thicknesses of 10 or 15 μ. Sections were affixed to slides, deparaffinized, and stained with safranin-fast green (10).

RESULTS.—*Macroscopic effects.*—On the 2nd day

after inoculation, minute yellow spots began to appear on the two to four youngest inoculated leaves. Within these spots, a smaller necrotic central area usually developed within the next 24 hr. By the 4th day after inoculation, many small spots were present, and they had reached their max size. They rarely exceeded 1 mm in diam (Fig. 1). Expansion of those leaves that were less than 75% of their potential size at the time of inoculation was noticeably inhibited. Irregular distribution of inoculum on the surfaces of these leaves often resulted in marked leaf distortion due to the greater inhibition of expansion in heavily infected than in lightly infected areas.

The first symptom of infection on older leaves was the appearance of small, water-soaked areas on the 3rd or 4th day after inoculation. Within 24 hr, the water-soaked areas developed into necrotic lesions. The lesions on mature leaves expanded and increased in number during the following week until they averaged about 8 mm in diam with a range of 1 to 30 mm (Fig. 2). These lesions were usually surrounded by a halo of chlorotic tissue having a radius of up to 10 mm (Fig. 2).

Although individual lesions on immature leaves were 1 mm or less in diam, some larger spots occurred on these leaves due to coalescence of concn of small lesions. Often, various sized spots occurred on the third or fourth leaf below the youngest inoculated leaf. In these cases, the smaller lesions were largely restricted to the physiologically younger leaf base and the larger lesions to the physiologically older tip (Fig. 3).

Expansion of the youngest completely inoculated leaf was so inhibited that by 14 days after inoculation this leaf was smaller than the next younger, more recently produced leaf on the same plant (Fig. 2). By 19 days, the youngest fully inoculated leaf often had one-half the area of the leaf at the next higher stalk position (Fig. 3). Dimensions of the youngest fully infected leaves on NC95 (Table 1), C187H, and PD121 were consistently smaller, to a similar degree, than those of leaves of comparable age on noninfected plants. Measurements of these leaves, infected at the stage of rapid expansion, were more variable than those of comparable leaves on noninfected plants and those of all mature leaves. This greater variability was due to irregular infection patterns on the affected leaves. A similar effect on expanding leaves occurred on nearly all *Nicotiana* species when inoculated with *A. alternata* (J. R. Stavely, *unpublished data*). When the inoculum concn was reduced or conditions for infection were less favorable, the number of small lesions on the young leaves and consequent reduction in leaf expansion were less on tolerant cultivars than on sensitive cultivars.

TABLE 1. Effect of infection by *Alternaria alternata* on average leaf dimensions of tobacco cv. NC95 at 21 days after inoculation[a]

Leaf position[b]	Leaf dimensions (width × length)[c]		
	Noninfected	Infected	Difference
	mm	*mm*	*mm*
−6	100 × 195	107 × 195	− 7 × 0
−5	120 × 223	120 × 227	0 × −4
−4	125 × 240	122 × 234	3 × 6
−3	121 × 246	122 × 242	− 1 × 4
−2	111 × 244	90 × 189	21 × 55
−1	108 × 230	77 × 172	31 × 58
0	115 × 236	111 × 190	4 × 46
+1	111 × 245	121 × 232	−10 × 13

[a] Inoculum consisted of 120,000 *A. alternata* conidia/ml. Each dimension is the average from 15 plants.

[b] Leaves at position 0 measured 8 ± 2 × 26 ± 5 mm on the day of inoculation. Minus leaf positions are progressively lower (older) leaves on the stem; plus leaf position is a progressively higher (younger) leaf.

[c] Noninfected plants were inoculated with nonpathogenic *A. alternata* isolate A37; infected plants were inoculated with pathogenic A3.

The restricted size of the lesions on the youngest inoculated leaves was permanent. They did not enlarge on any inoculated plants, even after resubmitting the plants to the postinoculation incubation conditions (11) on days 21-28 following inoculation. By 21 days after inoculation, the leaves having restricted lesions were as old as leaves that developed more extensive, typical brown spot lesions in the initial 10 days after inoculation.

Microscopic effects.—The fungus invaded all leaves by direct penetration. We found several cases of guard cell penetration (Fig. 4), but only one case of stomatal penetration and formation of the microsclerotiumlike growth described by Ramm (6). Early postpenetration events were as described by Ramm (6). Epidermal cells collapsed 2 to 4 days after inoculation, and the fungus began advancing by intercellular penetration (Fig. 5). At this stage, the age of leaf tissue had a marked effect on subsequent events.

1) *Older leaves.*—In leaves that had reached 75% or more of their potential size on the day of inoculation, the fungus continued advancing intercellularly through the mesophyll. The invaded area soon collapsed. The spongy mesophyll collapsed more rapidly than the palisade mesophyll. The concentric rings characteristic of the macroscopic lesion were apparently due to different degrees of palisade mesophyll collapse when the leaves were alternatively wet and

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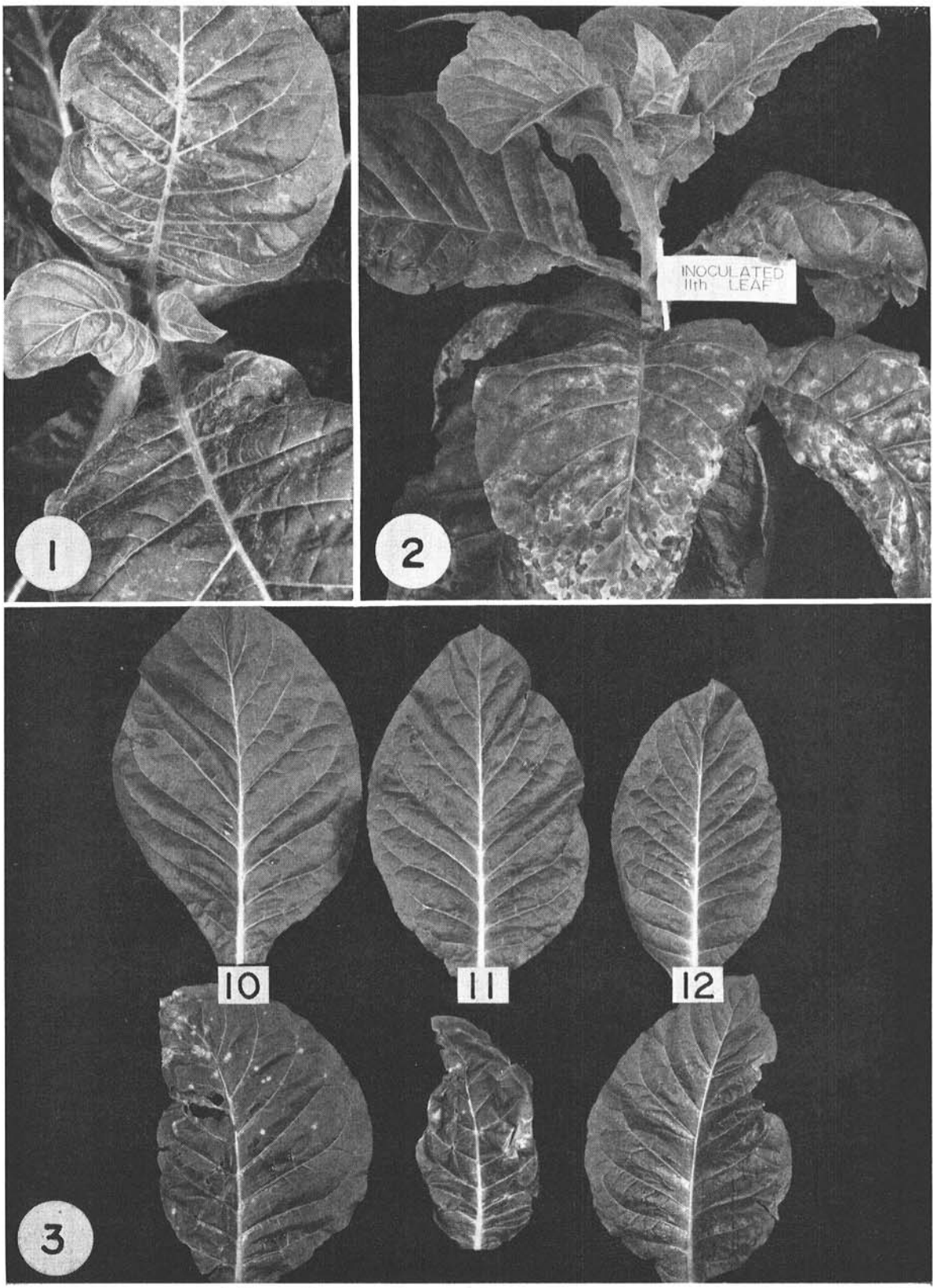
Fig. 1-3. Stages in the development of symptoms incited by *Alternaria alternata* on a Coker 187 Hicks tobacco plant. **1)** Small lesions on the youngest inoculated leaves 4 days after inoculation. Note the distortion and crinkling of the youngest leaves. **2)** Development of the disease on the same plant 14 days after inoculation. Note the large, necrotic lesions on the older leaves; the lack of the large lesions on the youngest inoculated 10th, 11th, and 12th leaves from the base; and the small size of the youngest completely inoculated 11th leaf (immediately above the label). **3)** The 10th, 11th, and 12th leaves from the same inoculated plant (bottom row), and from a noninoculated plant (top row) 19 days after inoculation. Note the severe size reduction of the inoculated 11th leaf, the occurrence of larger lesions on the tip but not the base of the 10th leaf, and the retarded growth on the heavily inoculated right half of the outer margin of the 12th leaf.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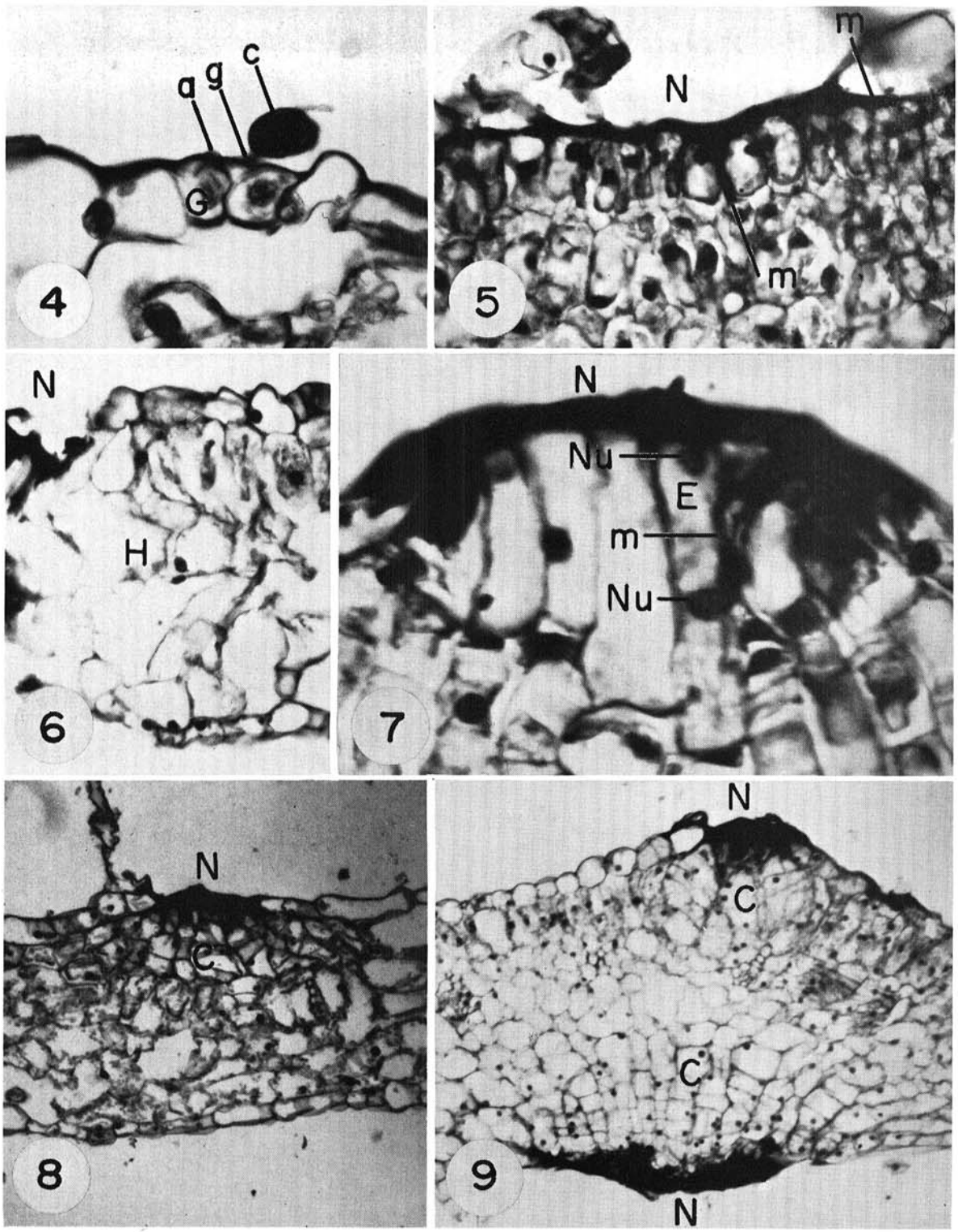

Fig. 4-9. Invasion of Coker 187 Hicks tobacco leaves by *Alternaria alternata* and subsequent developments. a = Appressorium; C = cicatrice; c = conidium; E = elongated host cell; G = guard cell; g = germ tube; H = halo area; m = intercellular mycelium; N = necrotic host cells; Nu = host nucleus. Magnifications are approximate. **4)** Germ tube passing over stomatal opening, and appressorium on penetrated guard cell of an older leaf 72 hr after inoculation. (×2,800) **5)** A developing infection on an intermediate leaf 96 hr after inoculation showing collapsed epidermal cells

dry; during the last 4 days of the period the plants were kept at 20 ± 2 C.

Fully developed lesions on mature leaves at 14 days after inoculation are characterized by a necrotic central area surrounded by chlorotic halo and green prehalo zones (5). The necrotic central zone was composed of fungal mycelium and collapsed host cells. Mycelium extended no more than 1 mm beyond the necrotic area into the halo. Arrangement of host cells throughout the halo, prehalo, and more distal areas surrounding the necrotic zone in older leaves was similar to that in noninvaded areas of the leaf or in noninfected leaves (Fig. 6). The spongy mesophyll cells in the halo zone were loosely arranged, with abundant intercellular space. The palisade mesophyll contained less intercellular space, but was organized the same in the halo and prehalo as it was in the more distal areas. Chloroplasts degenerated in the prehalo area and could not be detected by the time the halo had become yellow (Fig. 6). Nuclei remained identifiable, but became granular with no distinguishable nucleolus.

2) *Younger leaves.*—A radically different sequence of events occurred in leaves that were less than 75% of their potential size at inoculation. As soon as necrosis developed in epidermal cells and when intercellular invasion of the mesophyll began, cells surrounding the infected, necrotic area began elongating and dividing. The long axis of the elongated cells radiated out from the infected area. This orientation apparently developed while the leaf was growing by expansion. Around the typical small necrotic spot on the youngest leaf, cells also elongated perpendicular to the epidermis of the leaf, causing the spots to become slightly raised and the leaf to be somewhat thicker at these points (Fig. 7). With the low magnification of a dissecting microscope, the surface of these small spots appeared raised above the level of the noninfected epidermis. Most of the elongated cells divided into ca. 2 to 4 cells by 24 hr after the cell-elongation phase (Fig. 7, 8). The occasional detection of mitotic nuclei, the presence of elongated cells with two nuclei (Fig. 7), and the increased number of cell layers provided evidence that the elongated cells divided into two or more daughter cells. By 7 days after inoculation, a well-defined cicatrice of compactly arranged cells had reached full development (Fig. 8). Formation of this layer was similar whether infection occurred through the upper or lower epidermis (Fig. 9). When infection occurred through the lower epidermis, the resulting elongate cells often caused the affected area to more closely resemble the upper palisade layer than the lower spongy mesophyll of noninfected leaves. The fungus sometimes advanced far enough to cause necrosis through the thickness of slightly older leaves, 50 to 75% of their potential size at inoculation, before a cicatrice developed around the resulting bifacial lesions. These intermediate lesions were more common and larger in the physiologically older tip region than in the younger basal portion of the leaf (Fig. 3, leaf 10).

Cells constituting the cicatrice differed markedly from cells in tissue distal from the infection (Fig. 8, 9). The cicatrice cells were devoid of chloroplasts and were vacuolate. As in the halo area on older leaves, the nuclei took on a more granular appearance and nucleoli could not be detected. Cell walls were more intensely stained in these cells than in more distal cells (Fig. 9). The cicatrice cells tended to be more angular or square shaped, whereas nonaffected mesophyll cells had a more circular profile.

Cicatrice development on the edge of mechanical wounds on young leaves was similar in time sequence, leaf age relationship, and anatomical appearance to cicatrice development around *A. alternata* infections on young leaves.

DISCUSSION.—Our results suggest an explanation for the inconsistency between reports that leaf age has little relation to susceptibility to *A. alternata* infection (7, 8) and the general belief that brown spot is a disease of senescence (4). We found that leaves are susceptible to invasion by *A. alternata* at any age. On leaves still young enough for potential meristematic activity, production of a cicatrice of host cells around the infection quickly and permanently stops the fungus from advancing to produce the large, conspicuous lesions that occur on older leaves. Consequently, the lesions on young leaves are so small that, under field levels of inoculum, conspicuous symptoms of the disease are rare. In older leaves, lacking the capacity to wall off invasion, the fungus continues to advance, under favorable conditions, to produce conspicuous necrotic spots.

The time we found required for penetration of tobacco leaves by *A. alternata* and for development of brown spot symptoms is considerably less than that reported by Riley (8). He concluded that more than 48 hr was required for penetration and that 8 to 9 days was required for water-soaked spots to appear. He incubated inoculated plants at 30 C, however, 10 C above the opt temp for infection (11) and he may not have used highly pathogenic isolates of the fungus (8).

The stage of leaf development at which the cicatrice response occurs extends little, if any, beyond the stage at which cell division ceases in the developing tobacco leaf (2). Normal division stops first in the epidermis when the leaf is less than 16-20% of its potential size.

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and initiation of intercellular invasion. (×1,200) **6)** Outer margin of a necrotic lesion on an older leaf and the adjacent, chlorotic halo of largely noninfected host tissue 54 days after inoculation. Note the lack of a cicatrice in the host and absence of chloroplasts in the host cells. (×1,300) **7)** A small lesion on a young, inoculated leaf 96 hr after inoculation. Note the elongation of cells proximal to the infection, an elongated cell with two nuclei, absence of chloroplasts in these cells, and elevation of the lesion. (×4,800) **8)** A lesion on a young leaf surrounded by a cicatrice of closely packed, angular cells with well-stained cell walls 14 days after inoculation. (×600) **9)** Appearance of lesions at 54 days after inoculation of a young leaf. Invasion has occurred from both surfaces. (×600)

Division next ceases in the spongy mesophyll and finally in the palisade mesophyll before the leaf is 25-33% of its potential size. Subsequent growth is by cell and tissue expansion (2). Provascular strands, however, arise in the ontogenically younger central and basal portions of the lamina as long as that particular part of the leaf is undergoing marked expansion. Apparently a portion of the spongy mesophyll remains meristematic for a longer period of time than any other leaf tissue. These progressive events are completed more rapidly in the tip than in the basal portion of the leaf (2). We found that cicatrice layers develop around infections in leaves that are less than 75% of their potential size on the day of inoculation. In the younger leaf tissue, the fungus causes death of only a few cells, predominantly epidermal, before cicatrice formation occurs. In the oldest leaf tissue exhibiting a cicatrice reaction, however, the fungus often causes necrosis through the thickness of the leaf, with cell division occurring at a later stage, around the outer margin of this somewhat larger lesion. The degree of fungal advancement in this oldest reacting leaf is proportional to the physiological age of the tissue, being greater in the tip than in the base.

The cicatrization response to *A. alternata* infection is similar to the response to mechanical wounding. Apparently, both mechanical wounds and wounds caused by *A. alternata* infection can initiate a cell division response as long as any meristematic potential remains in leaf cells.

Cellular orientation during development of the walling-off reaction suggests that during leaf expansion the parts of surrounding cells proximal to the infected area are held at the position occupied at the time of infection. No intercellular space develops among the cells proximal to the infection. This inhibition of the expansion of the more densely packed cells in the young leaf (2) apparently is a contributing factor to formation of the cicatrice. Cells that are killed as a result of infection lose their capacity for expansion. In heavy inoculations, the severe reduction in leaf size apparently results from inhibition of expansion in both the cicatrice and necrotic areas.

Histological studies have been conducted on a wide range of leaf diseases and associated defense reactions (1), but the only known reported case of an effect of leaf age somewhat similar to that found by us involved *Cladosporium carpophilum*-infected leaves of *Prunus amygdalus* (9). Cicatrice layers were formed around all *C. carpophilum* lesions, but the reaction was quicker and closer to the point of invasion in young leaves than in older leaves. In the young leaves, an abscission layer developed resulting in a shothole effect, but no such layer occurred on older leaves. We found no abscission layer or shotholes in *A. alternata*-infected tobacco leaves. Cunningham (3) reported that the major determining factor for cicatrice formation around lesions incited by leaf-spotting fungi is the host species. On some hosts, all lesions were surrounded by these walling-off cells; on others, no such reaction was detected. A cicatrice was found in *Nicotiana glutinosa* leaves around local lesions incited by tobacco mosaic virus, but, as in many other cases, no mention was made of an effect of leaf age (13).

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