Relation of Postinoculation Leaf Wetness to Initiation of Tobacco Brown Spot

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ABSTRACT

Leaf wetness was essential for initiation of brown spot on inoculated greenhouse tobacco plants by *Alternaria alternata*. The number of leaf lesions per plant increased significantly as wet periods were lengthened from 8 to 24, 48, 72, and 96 hours. A few lesions developed after as little as 4 hours of wetness. Resistance of cultivars could be differentiated with as little as 48 hours of wetness, but 72 hours was better for this purpose. Total hours of wetness was critical, but the results were the same, whether the exposure was continous or broken by dry intervals. Plants were always allowed to dry after spray inoculation with conidial suspensions, before they were exposed to wetness. Lengthening this dry period from the minimum time to 20 days decreased disease severity, but lengthening it only to 10 or fewer days had little effect. The results suggest that dew may supply enough moisture for disease initiation, and that rain has an additional favorable effect.

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Brown spot, caused by *Alternaria alternata* (Fr.) Keissl. (7), has been the most destructive leaf disease of flue-cured tobacco, *Nicotiana tabacum* L., in the United States for 20 years (6). The disease unfavorably changes levels of chemical constituents important in tobacco quality (8, 10). The relationships between brown spot severity and various chemical, quality, and etiological effects have been quantitated in relation to economic impact (8, 9, 10, 11).

Humidity and temperature are critical environmental factors influencing brown spot development (6, 15, 19). Recently, the optimal temperatures for the brown spot disease and the pathogen, *A. alternata,* have been reported from various studies to be about 20 or 21 C for initiation of infection (14, 16, 19), about the same or slightly higher for germ-tube growth (13), a broad range for conidial germination (13), and about 27 C for colony growth in culture (19).

The relationship of wetness to development of this disease has been less well-defined than that of temperature. Frequent rains and damp weather have been considered favorable or essential for brown spot development (4, 5, 6, 15). In Rhodesia, the disease is most severe in the wettest part of the tobacco-growing area (4). In the United States, its greatest severity has been observed after rainy weather (15). On the other hand, some authors (2, 3, 12, 13) suggest that rainfall and prolonged wetness may not be so critical, but that repetitious, long, nightly dew periods provide enough wetness for infection. Although conidial germination and germ-tube growth were largely restricted to periods of leaf wetness (13), the maximum continuous period of leaf wetness during the weeks before the appearance of brown spot in Malawi was only 48 hours (12).

Brown spot infection has been obtained in the greenhouse when the dried, spray-inoculated plants were incubated in mist chambers for at least 3 days at 20 C (19) or when leaf disks inoculated with drops of conidial suspension were incubated in humid chambers for 5-10 days at 21 C (16). Quantitative study of how duration of leaf wetness affects disease development might reveal that a shorter incubation period is sufficient for good differentiation of levels of resistance in the greenhouse or laboratory. This would facilitate breeding for resistance.

The objectives of this research were: (i) to quantitate the effect of leaf wetness duration and schedule on brown spot development on greenhouse-grown, artificially inoculated tobacco plants, (ii) to determine the duration of postinoculation leaf wetness for best differentiation of levels of resistance, and (iii) to determine the effects on disease development of varying the length of the postinoculation dry period always provided prior to exposure to wetness. Accomplishment of these objectives would furnish information on artificial inoculation technology and contribute to our understanding of the relationship of leaf wetness to field development of brown spot. Some of the results have been reported in an abstract (17).

MATERIALS AND METHODS.—All comparisons were made with greenhouse-grown tobacco plants in 15.2-cm diameter clay pots. The culture and size of plants at the time of inoculation were similar to those previously described (19, 22). Care was exercised to equalize plant sizes for different treatments. In most experiments, only the highly brown spot-susceptible cultivar Coker 187-Hicks (C187H) (9, 10) was used. However, in comparisons of incubation periods for distinguishing levels of resistance, the moderately resistant cultivar NC 95 and resistant cultivar PD 121 were used along with C187H, to test the effect of duration of wetness on expression of resistance (1, 9, 10).

Inoculum concentrations were adjusted, after haemocytometer counts, to 60,000 conidia/ml, for all but one study. Conidia were suspended in water containing Rohm and Haas Triton B-1956 wetting agent (active ingredient, 77% modified phthalic glyceryl alkyd resin). The *A. alternata* isolates we used were our pathogenic A5, A3, and A-H (the latter two of these isolates were mixed

TABLE 1. Effect of duration of continuous postinoculation leaf wetness upon average number of brown spot lesions on *Alternaria alternata*-inoculated Coker 187-Hicks tobacco plants[a]

Time in mist (hours)	Lesions/plant[b] (Avg. no.)
144	375.9 A
96	332.8 A
72	253.5 B
48	142.0 C
24	58.6 D
18	46.4 DE
12	18.9 DE
8	4.3 E
4	3.2 E
0	0 E

[a]All plants were spray-inoculated on both surfaces of all leaves with 60,000 *A. alternata* conidia of isolates A3 and A5/ml of water with Triton wetting agent. Leaves were allowed to dry for 1-3 hours after inoculation, before mist was started.

[b]Any two figures followed by the same letter do not differ significantly, $P = 0.05$, according to Duncan's multipe range test.

and used in all inoculations and have been used in earlier studies (16, 19, 20, 21, 22). The third isolate was used only in combination with the first two in tests of alternating wet and dry periods and prolonged postinoculation dry periods. Conidial suspensions were flooded over V-8 juice agar in petri plates and then incubated in chambers supplying constant cool-white fluorescent light at 27 ± 1 C (19) to produce inoculum. Conidial suspensions for inoculum were prepared by scraping conidia into the water-wetting agent suspension. These suspensions were sprayed to the runoff point with a paint sprayer (19) onto all aboveground surfaces of the plants. The inoculum was allowed to dry onto the plants before the mist exposure started. The normal drying time was 1-3 hours. Because occasional check plants sprayed with water and wetting agent without conidia and kept in the mist chambers for several days produced no symptoms, such checks were not always included.

Inoculated plants were incubated under predesignated time schedules at 20 ± 2 C in Sherer-Gillette CEL 37-14 plant-growth rooms modified to include two uncovered Walton Montclair WF-225 centrifugal atomizing

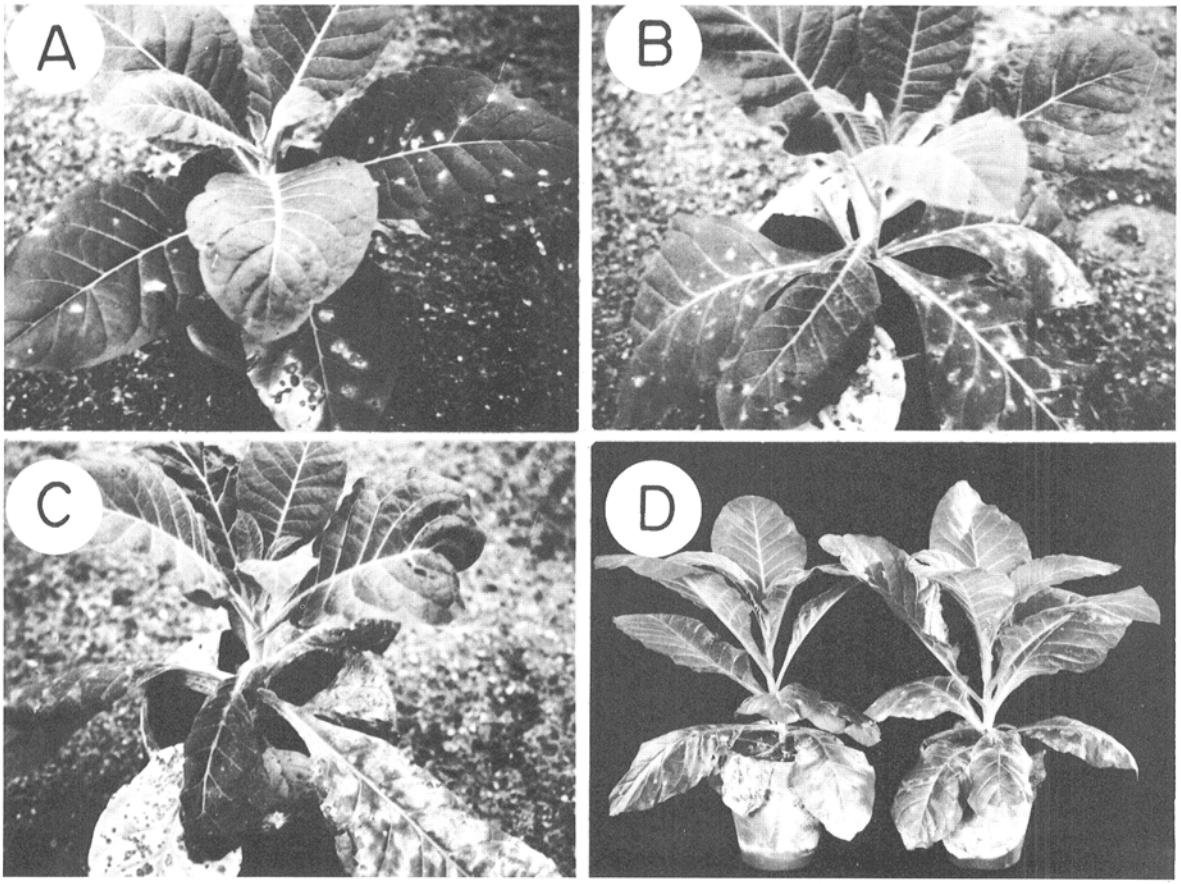

Fig. 1-(A-D). Coker 187-Hicks tobacco plants 16 days after inoculation with *Alternaria alternata*. Plants were exposed after inoculum dried to: **A)** 24 hours continous mist, **B)** 72 hours continuous mist, and **C)** 144 hours continuous mist. **D)** Two plants exposed to 48 hours of mist, the one on the left receiving three 16-hour wet periods interrupted with 8-hour dry periods and the one on the right, 48 hours of continuous wetness. Note increase in symptom severity with longer exposures to moisture and the lack of difference between the two 48-hour treatments.

humidifiers directly under the plant shelves. With this modification, the growth rooms could be programmed to maintain leaf wetness. Light intensity and photoperiod were the same as reported in earlier work (19). Upon completion of the predesignated wet period in the growth rooms, the plants were returned to a greenhouse kept at 24-32 C. The leaves were kept dry for 16-18 days before the numbers of brown spot leaf lesions per plant were counted. Only the older leaf lesions were counted, not the pinpoint lesions on young, expanding leaves (21). These pinpoint lesions were not included, because they are infrequent in the field (21), and their occurrence is generally not well correlated with older leaf resistance (20). All lesions on older leaves were counted.

For determining the effect of the duration of leaf wetness upon the number of lesions per plant, we incubated inoculated C187H plants in the growth rooms with free moisture for periods of none, 4, 8, 12, 18, 24, 48, 72, 96, and 144 hours. Four replicate plants were included per test, and the tests were repeated six times. The final figures were the means from 24 plants per treatment. The data were statistically analyzed by the F-test and Duncan's multiple range test ($P = 0.05$) (23).

A second series of experiments was conducted to determine the optimal duration of wetness for differentiation of resistance levels. All three cultivars were included. They were kept wet for 24, 48, 72, or 96 hours. Each inoculation included three plants per cultivar per treatment, and the inoculations were repeated four times. The data were analyzed statistically by the F-test for significance of wetness duration, cultivar, and the interaction between them and appropriate LSD's were computed ($P = 0.01, 0.05$) (23).

To determine the importance of alternating wet and dry periods, we exposed inoculated C187H plants to 16 hours of wetness and 8 hours of dryness every day for 1, 2, 3, or 4 days. For comparison, other inoculated plants were exposed to 48, 72, or 96 hours of continuous wetness. Two of the growth rooms described above were used, one programmed to keep the leaves wet, and the other kept at $70 \pm 8\%$ relative humidity. The plants were moved to the drier chamber during the predesignated dry periods. To increase lesion numbers, we increased the inoculum concentrations for these tests to 120,000 conidia/ml and included the highly pathogenic A-H isolate. Each inoculation included three plants per treatment, and the tests were repeated four times. The data were analyzed by the F-test and Duncan's multiple range test ($P = 0.05$) (23).

Finally we tried to determine the effect of extending the postinoculation dry period from the usual 1- to 3-hour minimum time for the leaves to become dry to 4, 8, 10, 12, or 20 days. After the desired dry period, the plants were placed in the growth rooms for 96 hours of continuous wetness. This series of tests was repeated only twice on three replicate plants of each of the three cultivars. The results were recorded as a visual estimate of disease severity, rather than as lesion counts.

TABLE 2. Effect of duration of continuous postinoculation leaf wetness on the average number of brown spot lesions occurring on plants of three tobacco cultivars having different levels of resistance to this disease[a]

Cultivar	Time in mist (hours)	Lesions/plant[b] (Avg. no.)
Coker 187-Hicks	96	332.1
	72	264.4
	48	202.0
	24	123.0
NC 95	96	223.2
	72	143.9
	48	133.9
	24	96.0
PD 121	96	73.2
	72	50.0
	48	35.7
	24	13.4
Interaction (time in mist × cultivar)		**[b]
LSD between times in mist on same cultivar $P = 0.05$		51.4
$P = 0.01$		68.7
LDS between cultivars with the same time in mist $P = 0.05$		51.1
$P = 0.01$		67.9

[a]All plants were spray-inoculated on both surfaces of all leaves with 60,000 *Alternaria alternata* conidia of isolates A3 and A5/ml of water containing wetting agent. Leaves were allowed to dry for 1-3 hours after inoculation before mist was started.
[b]** = F-value of the interaction is significant, $P = 0.01$.

TABLE 3. Comparison of the effects of one to four daily 16-hour periods of leaf wetness and three periods of continuous leaf wetness upon the average number of brown spot lesions on *Alternaria alternata*-inoculated Coker 187-Hicks tobacco plants[a]

Mist schedule	Total hours wetness	Lesions/plant[b] (no.)
96 hours continuous	96	839.3 A
72 hours continuous	72	639.9 B
Four 16-hour/24-hour applications	64	598.2 BC
48 hours continuous	48	495.8 CD
Three 16-hour/24-hour applications	48	472.5 CD
Two 16-hour/24-hour applications	32	415.6 D
One 16-hour/24-hour application	16	270.8 E

[a]All plants were spray-inoculated on both surfaces of all leaves with 120,000 *A. alternata* conidia of isolates A3, A5, and A-H/ml of water containing wetting agent. Leaves were allowed to dry for 1-3 hours after inoculation before mist was started.
[b]Any two figures followed by the same letter do not differ significantly, $P = 0.05$, according to Duncan's multipe range test.

RESULTS.—The number of brown spot lesions was directly related to the duration of postinoculation leaf wetness (Table 1). Exposure of inoculated plants to wetness was essential for development of brown spot lesions. No lesions developed on inoculated plants not exposed to wetness. Whereas only a few lesions developed on inoculated plants submitted to 4-8 hours of wetness, the average number of lesions was 18.9 with 12 hours, 46.4 with 18 hours, and continued to increase with longer periods of wetness (Fig. 1-A, B, C). There were

significantly more lesions after a 24-hour exposure than after an 8-hour exposure and further significant increases in number as the time was lengthened to 48, 72, and 96 hours (Table 1). Lesion numbers continued to increase with exposures longer than 96 hours, but the increase was not significant. The pinpoint lesions characteristic of infections on young leaves (22) were not counted due to their large numbers in most treatments, as well as for the reasons given previously. They occurred after moisture exposures as short as 4 hours, and increased in numbers as the duration of leaf wetness increased. Stem lesions were rare with less than 24 hours continuous moisture, few with 24 hours, and more numerous as the wet period was lengthened. The duration of wetness appeared to have no effect on lesion size or general appearance on C187H.

The time in mist × cultivar interaction was significant (Table 2). Whereas the increase in numbers of lesions on C187H was significant with each increase in duration of wetness from 24 to 96 hours, such was true only of the increase between 72 and 96 hours on NC 95, and was not true of any of the 24-hour increments in exposure time of PD 121. These more resistant cultivars had fewer lesions, as well as generally less extensive halos (9) around the lesions. Both cultivars showed a trend towards a direct relationship between duration of wetness and numbers of brown spot lesions. The numbers of lesions per plant per cultivar differed significantly after 48-, 72-, and 96-hour exposures, but after 24 hours, NC 95, and PD 121 did not differ significantly.

Numbers of lesions were not affected by interruption of wet periods with dry intervals (Table 3). The number of lesions on plants given 48 hours of continuous wetness did not differ from the number on plants given three 16-hour wet periods interrupted with 8-hour dry periods (Fig. 1-D). As the number of 16-hour/24-hour wet periods increased from one to four, the number of lesions per plant also increased (Table 3). Stem infections and pinpoint lesions on young leaves also were unaffected by 8-hour dry intervals during the wet period.

Lengthening the postinoculation dry period from 1-3 hours to 4, 8, 10, 12, or 20 days caused a progressive decrease in the number of lesions that developed with a 96-hour wetness exposure. However, the decrease was not as pronounced during the first 4- to 10-day period as later on any of the three cultivars. Even with strict leaf-surface dryness for 20 days after inoculation, some brown spot appeared after the plants were kept wet for 96 hours. The effect on stem infection was similar to that on mature leaves. The most pronounced effect of extending the postinoculation dry period was on the pinpoint lesions usually occurring on the young leaves. When the dry period exceeded 10 days, none of these kinds of lesions appeared, and they were fewer and leaf expansion was less affected with the 4- and 8-day dry periods than with the 1-3 hour period.

To determine if outdoor light quality during a prolonged postinoculation dry period might affect infection differently from greenhouse light quality, we placed two sets of four inoculated plants outside daily for 8 midday hours for 4 or 8 days in late June. An equal number of inoculated plants was kept in the greenhouse, and all leaves on all plants were kept dry for the 4 or 8 days before being exposed to 96 hours of wetness. The severity of symptoms on plants placed outside did not differ from that of those kept in the greenhouse.

DISCUSSION.—The relationship between duration of wetness and numbers of lesions appeared to be linear from the 12- or 18-hour wetness duration through the 96-hour duration. With less than 12 hours, few lesions appeared (Table 1). In none of our tests, including those involving prolonged postinoculation dry periods, did any brown spot lesions occur unless inoculated plants were exposed to wetness. Likewise, Norse (13) found with this same pathogen that conidial germination, germ-tube growth, and host penetration are restricted to periods of wetness. The increase in numbers of lesions between 0 and 12, 18, or 24 hours (Tables 1-3) does not appear to relate linearly to the increase between 12, 18, or 24, and 96 hours. Our experiments were not conducted simultaneously, but in progression and at different seasons of the year. In one experiment (Table 3), an additional fungal isolate was used. Therefore, lesion numbers between tests should not be strictly compared. This is illustrated by the fact that when the inoculum content changed, time of year was different, and inoculum concentration doubled, the number of lesions more than doubled (Tables 1 and 3).

The significant increases in the numbers of lesions with progressively longer wet periods from 8 hours to 1, 2, 3, and 4 days indicates the favorable effect of prolonged wetness on brown spot occurrence. This effect of longer wet periods seems to agree with earlier observations that brown spot is favored by wet, rainy weather (4, 5, 6, 15). However, that lesions appear after 12 hours or less of wetness also suggests that single dewy nights are sufficient to permit some infection to occur. The latter is in agreement with the observations of Norse and Wheeler (14) and Norse (13) that conidia can germinate, germ tubes can grow, and the host can be penetrated in a single wet night. However, our results also indicate that more than a single wet night is required for much infection to occur.

As many lesions resulted when a 48-hour wetness period was continuous as when it was broken into three successive 16-hour/24-hour periods. Thus, a succession of dewy nights would seem to provide sufficient moisture for development of considerable brown spot. The total hours of wetness appeared to be more critical than whether or not the time is broken by dry intervals. These results support other observations (2, 3, 12, 13) that repetitious dewy nights are enough for initiation of some or considerable infection.

Pinpoint lesions on young leaves and a few lesions on older leaves of inoculated plants exposed to wetness for only 4 hours indicate that a small part of the conidial population can complete the infection process in that little time. The increase in number of lesions with longer times suggests that conidial germination and infection vary even under uniform and favorable conditions. All three of our isolates originally were carried through several generations of single conidium reisolation (19) in order to reduce variability. In other inoculations with the individual isolates, there was no indication of differences in responses to mist periods from those reported here.

Our results with the three cultivars indicate that a wet period of about 3 days is needed for good differentiation of resistance levels. Although the cultivars differed

significantly after 48 hours of postinoculation wetness, we believe that another day is needed to insure a difference. Tobacco cultivars and introductions show a gradation of reactions over a wide range to *A. alternata* (9, 10, 18), and conditions for differentiation of host response must be optimized (12).

The lack of pinpoint lesions on plants given more than 10 days of premist postinoculation dryness resulted from growth of the youngest inoculated leaves to a stage beyond which they could restrict lesions by the time of infection (21).

Apparently, relatively long periods of postinoculation dryness are needed to affect brown spot severity adversely. This need could be further evidence that dew is important during low-rainfall periods or in dry climates for providing the conditions necessary for infection. It also indicates that one must avoid accidentally getting inoculum on greenhouse plants before any prolonged exposure to wetness. The results from exposure of plants to outside conditions during 4- and 8-day postinoculation dry periods before wetness exposures suggest that in very dry climates, conidia could lie on leaves of field plants for several days in the absence of dew and initiate infection when sufficient wetness occurs.

We conclude from our study that wetness is needed for brown spot initiation by *A. alternata* and that the longer the wet period, the more lesions result. About 72 hours of moisture is needed to differentiate cultivar resistance reliably in the greenhouse. Prolonged and repeated nightly dew can provide enough moisture for infection, but daytime rain that extends the wetness period should increase disease severity.

LITERATURE CITED

1. CHAPLIN, J. F. 1971. Registration of PD121 tobacco germ plasm. Crop Sci. 15:21-22.
2. DE FLUITER, H. J. 1938. Alternaria longipes (Ell. and Ev.) Mason de verwekker van de bruinvlekziekte in de Besoeki Tabac. Meded. Besoekingsst. Proefst. 59:1-16.
3. GIHA, O. H. 1973. Distribution and morphology of Alternaria tenuis associated with leaf spots in the Sudan. Trans. Br. Mycol. Soc. 61:265-275.
4. HARTILL, W. F. T. 1967. A survey of diseases of tobacco in Rhodesia and Zambia, 1962-64. Rhod. Agric. J. 64:106-111.
5. JONES, A. L. 1968. The seasonal development of tobacco brown spot caused by Alternaria tenuis in relation to the ontogeny of the host. Ph.D. Thesis, N.C. State University, Raleigh. 44 p.
6. LUCAS, G. B. 1965. Diseases of tobacco, 2nd ed. Scarecrow Press, New York. 778 p.
7. LUCAS, G. B. 1971. Alternaria alternata (Fries) Keissler, the correct name for A. tenuis and A. longipes. Tob. Sci. 15:37-42.
8. MAIN, C. E., and J. F. CHAPLIN. 1971. Brown spot damage and flue-cured tobacco quality. II. Alterations within the cured-leaf lesion. Tob. Sci. 15:21-22.
9. MAIN, C. E., and J. F. CHAPLIN. 1972. Brown spot damage and flue-cured tobacco quality. III. Alterations in physical characteristics and color of cured leaf in relation to disease severity. Tob. Sci. 16:26-31.
10. MAIN, C. E., J. F. CHAPLIN, and J. M. SLEGE. 1973. Brown spot damage and flue-cured tobacco quality. IV. A survey of chemical constituents related to disease severity and cultivar resistance. Tob. Sci. 17:19-23.
11. MAIN, C. E., C. J. NUSBAUM, G. B. LUCAS, and J. F. CHAPLIN. 1973. Tobacco quality related to severity of foliage diseases. Abstract No. 0590. *In* Abstracts of Papers, 2nd Int. Congr. Plant Pathol., 5-12 September 1973, Minneapolis, Minnesota (unpaged).
12. NORSE, D. 1971. Lesion and epidemic development of Alternaria longipes (Ell. and Ev.) Mason on tobacco. Ann. Appl. Biol. 69:105-123.
13. NORSE, D. 1973. Some factors influencing spore germination and penetration of Alternaria longipes. Ann. Appl. Biol. 74:297-306.
14. NORSE, D., and B. E. J. WHEELER. 1971. Perennation of Alternaria longipes and the early stages of its development on tobacco. Ann. App. Biol. 67:23-24.
15. RAMM, C. VON, and G. B. LUCAS. 1963. Epiphytology of tobacco brown spot caused by Alternaria longipes. Phytopathology 53:450-455.
16. SPURR, H. W., JR. 1973. An efficient method for producing and studying tobacco brown-spot disease in the laboratory. Tob. Sci. 17:145-148.
17. STAVELY, J. R. 1974. Effects of various schedules of postinoculation leaf wetness on tobacco brown spot development. Annu. Proc. Am. Phytopathol. Soc. for 1974 1:155. (Abstr.).
18. STAVELY, J. R., T. W. GRAHAM, G. R. GWYNN, J. F. CHAPLIN, and C. E. MAIN. 1973. Resistance to Alternaria alternata in Nicotiana tabacum. Phytopathology 63:806 (Abstr.).
19. STAVELY, J. R., and C. E. MAIN. 1970. Influence of temperature and other factors on initiation of tobacco brown spot. Phytopathology 60:1591-1596.
20. STAVELY, J. R., G. W. PITTARELLI, and G. B. LUCAS. 1971. Reaction of Nicotiana species to Alternaria alternata. Phytopathology 61:541-545.
21. STAVELY, J. R., and L. J. SLANA. 1971. Relation of leaf age to the reaction of tobacco to Alternaria alternata. Phytopathology 61:73-78.
22. STAVELY, J. R., and L. J. SLANA. 1973. Comparative effects of Alternaria alternata infection and other leaf injuries on growth of tobacco. Phytopathology 63:495-499.
23. STEEL, R. G. D., and J. H. TORRIE. 1960. Principles and procedures of statistics. McGraw-Hill, New York. 481 p.