Development of a Method to Evaluate Tobacco Genotypes for Resistance to Angular Leaf Spot in the Greenhouse

MARY J. WANNAMAKER, Graduate Research Assistant, Department of Crop Science, and REBECA C. RUFTY, Assistant Professor, Departments of Crop Science and Plant Pathology, North Carolina State University, Raleigh 27695-7620

ABSTRACT

Wannamaker, M. J., and Rufty, R. C. 1989. Development of a method to evaluate tobacco genotypes for resistance to angular leaf spot in the greenhouse. Plant Disease 73:964-968.

A greenhouse technique was developed to evaluate tobacco genotypes for resistance to angular leaf spot. An artist's airbrush was the most effective method of spraying inoculum uniformly on the abaxial surface of the two oldest leaves of plants at the four- to five-leaf stage. A suspension of *Pseudomonas syringae* pv. *tabaci* containing 10^7 cfu/ml produced symptoms typical of those observed in the field. A preinoculation mist period of 0–18 hr did not increase the amount of disease. Postinoculation mist periods of 24, 36, and 48 hr significantly increased the amount of disease. A quantitative disease assessment scale was developed and used successfully to classify genotypes for disease resistance. Lesions observed in the greenhouse were similar in size, shape, and color to those observed in field plant beds. In addition, the rank order of genotypes in greenhouse and field plant beds was the same.

Angular leaf spot of tobacco (*Nicotiana tabacum* L.) is caused by certain strains of the bacterium *Pseudomonas syringae* pv. *tabaci* (Wolf and Foster) Young, Dye, and Wilkie, formerly classified as *Pseudomonas angulata*. In 1976, the revised International Code of Nomenclature of Bacteria grouped *P. angulata* with *P. s.* pv. *tabaci*, the causal agent of tobacco wildfire (15). The main difference between the two organisms is in the ability of the wildfire bacterium to produce a chlorolytic toxin called tabtoxin that the angular leaf spot bacterium either does not produce or produces in very small quantities. The relationship between these two organisms has been debated for many years (1,5,12,13).

Paper No. 11976 of the Journal Series of the North Carolina Agricultural Research Service, Raleigh.

The use of trade names does not imply endorsement by the North Carolina Agricultural Research Service of the products named nor criticism of similar ones not mentioned.

Accepted for publication 23 June 1989 (submitted for electronic processing).

Regardless of whether the angular leaf spot and wildfire bacteria respond similarly to standard biochemical tests, symptoms and host reaction of the two organisms differ under field conditions. Initial symptoms of angular leaf spot consist of water-soaked and slightly angular lesions that later turn tan, brown, or black, become distinctly angular, and range from 1 to 8 mm in diameter. In the late stages of disease development, spots may coalesce and tissue in the center of large lesions may drop out (10). Angular leaf spot is most prevalent in the field plant bed and develops on young plants under conditions of high relative humidity. Infection is enhanced when plant tissue is water-soaked by driving rains or wounded by sandblasting (3). Tobacco is most vulnerable to the disease when high plant populations in the seedbed form a complete canopy over the soil. Factors that increase plant succulence also increase susceptibility to this disease (10). Wildfire symptoms begin as circular, yellowish green areas 3–6 mm in diameter. Within 24 hr, minute necrotic specks appear in the centers of the lesions and yellow-green halos become prominent. As wildfire progresses, the necrotic

center and the halo increase in size to form a lesion 12–25 mm in diameter. The conspicuous halo is caused by the toxin diffusing ahead of the multiplying bacterial cells into adjacent living cells and destroying the chlorophyll (14). Similar to angular leaf spot, infection is enhanced by factors that produce water-soaking of tissue. In contrast to angular leaf spot, wildfire is seldom, if ever, uniformly distributed over the plant bed.

Resistance to wildfire was transferred from *N. longiflora* Cav. to *N. tabacum* (4), and the first wildfire-resistant burley tobacco cultivar, Burley 21, was released in 1955 (6). Most contemporary burley tobacco cultivars possess wildfire resistance (10). Cultivars of tobacco resistant to wildfire are not necessarily resistant to angular leaf spot (*unpublished*). This distinction is important in the development of cultivars resistant to both diseases. There has been no directed effort to develop genotypes resistant to angular leaf spot because of the assumption that cultivars resistant to wildfire are also resistant to angular leaf spot.

The principal objective of this study was to develop a procedure for identification of angular leaf spot resistance in tobacco. The effects of bacterial concentration and of duration of mist period before and after inoculation and the interactions of these factors on the development of angular leaf spot were evaluated in the greenhouse to characterize conditions necessary for disease development. An experiment also was conducted in a field plant bed to ascertain if greenhouse responses corresponded to those recorded under field conditions.

MATERIALS AND METHODS

Burley tobacco cultivars Judy's Pride, male sterile Kentucky (MS Ky) 14 × L8, and Burley 21 and dark air-cured tobacco cultivar Goat's Creek were selected because they differ in reaction to the angular leaf spot and wildfire strains of *P. s.* pv. *tabaci*. Burley 21, MS Ky 14 × L8, and Goat's Creek are resistant to wildfire and Judy's Pride is susceptible. Burley 21 is resistant to angular leaf spot, Goat's Creek shows an intermediate reaction, and MS Ky 14 × L8 and Judy's Pride are susceptible. These cultivars were used in all greenhouse experiments with the exception of the preinoculation mist experiment, in which only Judy's Pride and Burley 21 were included. Seeds were sown in plastic flats (20 × 15 × 5.5 cm) containing Metro Mix 220 (Grace Horticultural Products, Cambridge, MA) and were watered with one-half strength Hoagland's solution (7) as needed. Four-week-old seedlings were transplanted to peat pots (5 × 5 cm) and, upon recovery from transplanting shock, were transferred into 10.2-cm-diameter clay pots containing a 2:1 mixture of Metro Mix 220 and steamed sand; 10 g of 14-14-14 Osmocote (Sierra Chemical Co., Milpitas, CA) was added to each pot.

The presence or absence of host-specific strains of the angular leaf spot bacterium was investigated in preliminary studies to determine which isolate was appropriate to use in this experiment. Ten isolates were inoculated on five burley tobacco cultivars. Results indicated that isolates differed in their general virulence on a given cultivar but did not differ in host specificity. *P. s.* pv. *tabaci* isolate FLA 82-111 (obtained from H. A. Skoog, USDA-ARS, Beltsville, MD) was used in all experiments because it provided a good separation between resistant and susceptible reactions to angular leaf spot. FLA 82-111 was originally isolated in 1978 in Florida from the flue-cured tobacco cultivar Speight G-28. The isolate was stored in refrigerated, sterile, deionized water blanks and recovered as necessary by streaking a loopful of bacterial suspension onto nutrient dextrose agar medium (3 g of beef extract, 5 g of type 1 peptone, 5 g of dextrose, and 15 g of Difco agar per liter of distilled water). The pH of the medium was adjusted to 7.3 before addition of the agar and sterilization. Bacteria were grown for 24 hr, suspended in sterile deionized water, and then adjusted using a standard curve based on spectrophotometric measurement of absorbance (A_{530}) to the particular bacterial concentration being tested. Dilution plating of the bacterial suspensions was performed after inoculation to further substantiate the actual bacterial concentration.

Use of an artist's airbrush as an inoculation tool resulted in the development of symptoms that closely simulated those observed in the field. Approximately 0.5–1 ml of inoculum was applied at a pressure of 4×10^5 Pa to the underside of the two oldest leaves of plants at the four- to five-leaf stage in all experiments. The entire leaf surface was uniformly sprayed from a distance of 8–10 cm. Care was taken to prevent visible water-soaking, since this causes an uncharacteristic collapse of the leaf tissue. Inoculations were conducted routinely between 10:00 a.m. and 2:00 p.m. No supplemental light was supplied. During the summer and fall when these studies were conducted, temperatures in the greenhouse varied from 19 to 27 C.

Standard area diagrams were developed that permitted a more accurate classification of genotypes for disease reaction than would have been possible using a qualitative rating scale. With the aid of diseased leaves and a visual image analyzer (9,11), a set of standards was developed that consisted of artificial leaves having 0–55% of leaf area damaged (% LAD) (Fig. 1). Diseased leaves were then assigned a LAD value by visual comparison of the inoculated leaves with the standards. When symptoms exceeded 55% LAD, a visual estimate of LAD was made without a standard. Individual plants were considered as replications; there were two single leaf subsamples per replication in all greenhouse experiments. Disease symptoms were evaluated 10 days after inoculation in both greenhouse and field experiments.

Effect of bacterial concentration. In a preliminary study, atypical symptoms resulted on three of four cultivars when plants were inoculated with a bacterial concentration greater than 1.0×10^8 cfu/ml and placed in a mist chamber for 24 hr. Atypical symptoms were denoted

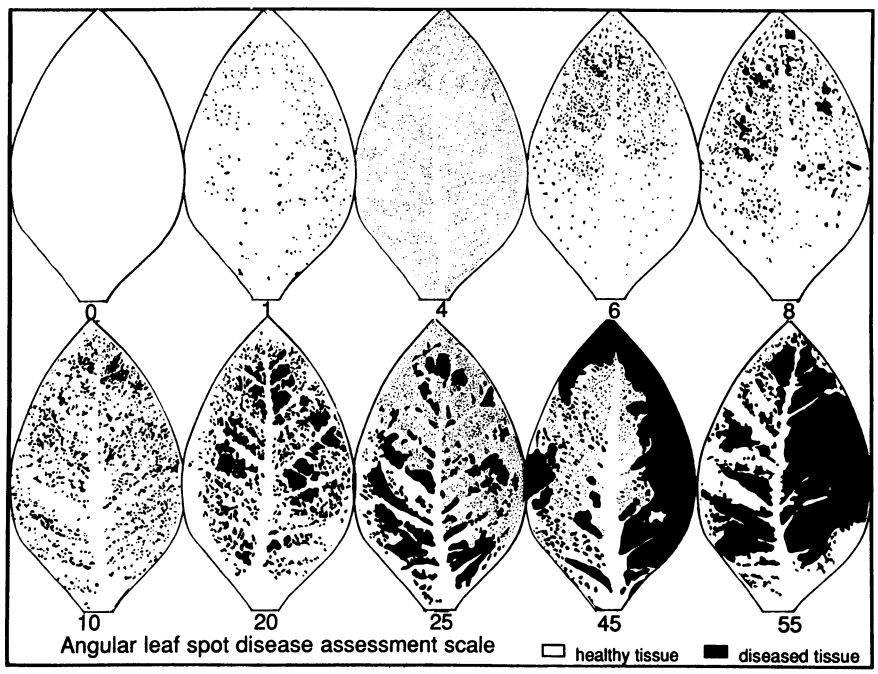

Fig. 1. Disease assessment scale used to classify tobacco genotypes for their reaction to angular leaf spot based on percentage of leaf area damaged that includes chlorotic and necrotic tissue.

by a total collapse of leaf tissue with the absence of any distinct angular lesions. Thus, approximate concentrations of 0.3×10^7, 1.0×10^7, 2.0×10^7, and 3.0×10^7 cfu/ml, plus a water control, were used to inoculate plants of four cultivars using the artist's airbrush method described above. After inoculation, plants were misted for 5 sec every 30 sec for 24 hr and then placed on a greenhouse bench until disease development was recorded. The 20 treatment combinations were replicated eight times in a randomized complete block (RCB) design.

Effect of misting. Two concentrations of bacteria, 2.0×10^7 and 9.0×10^7 cfu/ml, and a water control were used in all misting experiments. Preinoculation treatments consisted of mist periods of 0 (control) and 6, 12, and 18 hr on plants of the cultivars Judy's Pride and Burley 21. After misting, plants were inoculated and the 24 treatment combinations were replicated three times in a RCB design. After inoculation, plants were placed on a greenhouse bench for the 10-day incubation period.

To examine the effects of postinoculation misting periods, plants of the four cultivars were inoculated with the two bacterial concentrations plus a water control and exposed to three mist regimes (24, 36, and 48 hr) subsequent to inoculation. The 36 treatment combinations were replicated three times in a RCB design.

To examine the possible interactions of preinoculation and postinoculation mist treatments, four cultivars were exposed to four mist durations (0, 6, 12, and 18 hr) before inoculation. After inoculation, plants were exposed to mist for 24 or 36 hr. The 96 treatment combinations in this factorial experiment were tested twice, using a RCB design in each run. There were two replications in the first run and four in the second.

Plant bed experiment. A field plant bed experiment was conducted (Mountain Research Station, Waynesville, NC) to determine whether greenhouse reactions corresponded to those observed under field conditions. Foliar inoculation was performed on four cultivars: Judy's Pride, Kentucky 14 (Ky 14), Goat's Creek, and Burley 21. In late March 1987, 100 seeds of each cultivar were seeded in 30.5×30.5 cm plots, with 10 cm between plots, arranged in an RCB design. After seeding, plots were covered with a woven tobacco plant bed cover (Reemay, DuPont, Wilmington, DE) and watered regularly.

Foliar inoculation was performed in mid-May when plants were in the two- to five-leaf stage. The upper surface of leaves in plots receiving the artificial inoculation treatment was sprayed until runoff with a bacterial suspension (concentration = 1.0×10^8 cfu/ml) using an artist's airbrush at a pressure of 4×10^5 Pa. Inoculation was conducted at midday, after which the cover was again placed over plant beds for 4 days.

RESULTS AND DISCUSSION

Effect of bacterial concentration. Differences between resistant, intermediate, and susceptible plants were apparent within 1 wk after inoculation with *P. s.* pv. *tabaci*. Disease levels on intermediate and susceptible plants ranged from 2 to 20% LAD. Lesions were discrete or clustered, with narrow yellow zones surrounding the necrotic angular centers. The resistant reaction ranged from 0 to 5% LAD and consisted of pinpoint-sized light green spots that speckled the entire leaf. Lesions on resistant plants did not progress beyond this initial stage. There was no speckling reaction in the control plants, which eliminated the possibility that the speckling response was mechanical injury caused by the artist's airbrush.

The analysis of variance revealed significant bacterial concentration and cultivar main effects; the concentration × cultivar interaction effect also was significant ($P = 0.05$). When percent leaf area damaged was plotted against bacterial concentration (Fig. 2), the ranking of the cultivars MS Ky 14 × L8 and Goat's Creek changed at the highest concentration, contributing to the significant interaction effect. Analyses of variance were run separately for each cultivar to further elucidate the cause of the significant interaction effect. The concentration main effect was significant for all cultivars, but the variance contributed by Burley 21's concentration main effect was minimal. The other cultivars became more diseased with increasing bacterial concentration. Judy's Pride was the most susceptible cultivar, and Goat's Creek and MS Ky 14 × L8 were intermediate in reaction. Analyses of variance were also performed for each concentration level. Cultivars did not differ significantly for disease reaction when inoculated with the lowest bacterial concentration, 0.3×10^7 cfu/ml, but differences among cultivars were observed at the three higher concentrations. As previously indicated, a bacterial concentration greater than 1.0×10^8 cfu/ml coupled with 24 hr of postinoculation mist produced severe symptoms on three of the four cultivars. We concluded from this study that a successful concentration for separation of tobacco genotypes is 10^7 cfu/ml.

Effect of mist before inoculation. From the analysis of variance, the concentration × cultivar interaction effect and the cultivar main effect were significant ($P = 0.05$). All other effects were nonsignificant. Misting before inoculation had no significant effect on disease development for either cultivar. Water-soaking of leaf tissue was not observed in this experiment. No differences in LAD greater than 5% were observed for either cultivar with the different preinoculation misting durations. There was less disease in this experiment than in the bacterial concentration experiment described above, in which postinoculation mist was supplied for 24 hr. LAD ranged from 0 to 15% in this test, compared with 0 to 35% in the bacterial concentration experiment.

Effect of mist after inoculation. The mist main effect and the concentration × cultivar × mist interaction were significant ($P = 0.05$). Cultivar main effect and the cultivar × mist interaction were highly significant ($P = 0.01$). All other effects were nonsignificant. Results observed at the higher bacterial concentration, 9.0×10^7 cfu/ml, are shown in Figure 3. Similar trends were observed at the lower bacterial concentration (*data not shown*). Varying levels of water-soaking were observed on inoculated leaves of all cultivars except Burley 21 after mist durations of 24, 36, and 48 hr. As duration of postinoculation misting increased, LAD in Judy's Pride, Goat's Creek, and MS Ky 14 × L8 increased, whereas Burley 21 was apparently insensitive to increased duration of the postinoculation mist. Postinoculation mist durations of 36 and 48 hr, coupled with the bacterial concentrations used, caused atypical

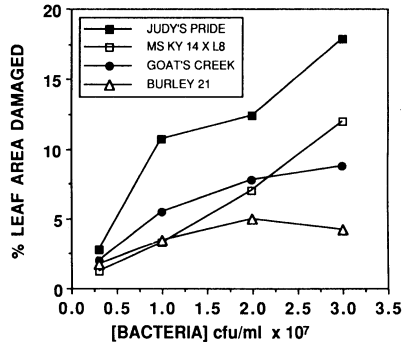

Fig. 2. Effect of bacterial concentration on angular leaf spot development in four tobacco cultivars. Points represent the mean of eight replications for each cultivar.

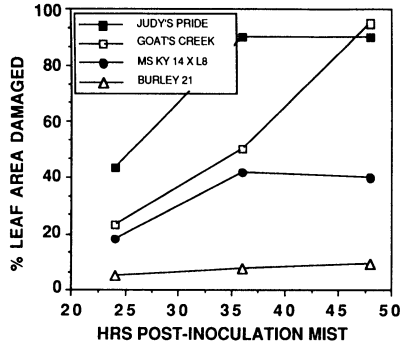

Fig. 3. Effect of duration of mist subsequent to inoculation on angular leaf spot development in four tobacco cultivars. Points represent the mean of four replications. Bacterial concentration was 9.0×10^7 cfu/ml.

angular leaf spot symptoms, with LAD values as high as 90–100%. Such extreme degrees of tissue collapse are rarely seen under field conditions. The postinoculation mist regime of 24 hr resulted in symptoms characteristic of those viewed in the field, with LAD approximately 0% for resistant cultivars and up to 40% for susceptible cultivars.

Interaction of preinoculation and postinoculation mist treatments. Run 1 and run 2 differed significantly, with less disease development in run 1. Although severity ranged from 0 to 20% in run 1 and from 0 to 75% in run 2, trends were similar. Percent LAD observed at the higher bacterial concentration in run 2 is shown in Table 1. Analysis of this subset of the data revealed significant main effects and interaction effects, with the exception of the preinoculation mist main effect, the preinoculation × postinoculation interaction, and the cultivar × preinoculation mist × postinoculation mist interaction effect, which were nonsignificant. The postinoculation mist effect was significant at the 0.10 level. As before, increases in duration of postinoculation mist resulted in increasing amounts of angular leaf spot on all cultivars except Burley 21. Increases in duration of mist periods before inoculation had no effect. Water-soaking of leaf tissues either before or after inoculation has been found to have similar effects on angular leaf spot and wildfire progression (2,3,8). When the water-soaked areas persist for 24 hr or more, severe symptoms of both diseases develop, resulting in total collapse of leaf tissue. However, varying degrees of angular leaf spot and wildfire occur when water-soaked areas disappear after a brief rainstorm or limited exposure to artificial mist (2,3). In our study, exposure of plants to mist for 0–18 hr before inoculation was not sufficient to cause prolonged water-soaking of leaf tissue in the four cultivars tested, and thus preinoculation mist did not have any significant visible effect on development of angular leaf spot. However, mist durations of 24, 36, and 48 hr after inoculation were sufficient to cause varying levels of water-soaking, and their effect on angular leaf spot development was profound. Water-soaking was not observed in leaves of Burley 21 throughout these studies.

Plant bed experiment. Angular leaf spot symptoms were evident on foliar-inoculated plants, and one disease rating was recorded for each plot. Judy's Pride was highly susceptible to angular leaf spot, with 25% LAD; Burley 21 was highly resistant, with 25% LAD; and Goat's Creek showed a moderate reaction, with 12.5% LAD. Despite the low level of disease observed in the field plant bed, the cultivars maintained the same ranking as in the greenhouse. In the field plant bed, Ky 14, which was not tested in a greenhouse experiment, had a 5% LAD rating. These results are consistent with multiple observations (*unpublished*) on these cultivars under plant bed conditions for 5 yr.

It is important to note that cultivar MS Ky 14 × L8 showed a bimodal response to angular leaf spot, i.e., 50% of the plants were highly susceptible and 50% were highly resistant in each of the four greenhouse experiments conducted. Such a bimodal response is uncommon for a F_1 hybrid because hybrids are theoretically uniform and stable for all traits. The bimodal reaction was further investigated. Hybrid seed used in the experiments had been obtained from a private seed company. To determine if the seed were contaminated, the hybrids were manually generated using our own stock of male sterile and fertile sources of Ky 14. Fifty plants of each manually crossed hybrid, MS Ky 14 × L8 and fertile Ky 14 × L8, were inoculated with *P. s.* pv. *tabaci* isolate FLA 82-111, using the greenhouse technique described. Both populations were highly resistant to angular leaf spot, with no suggestion of a bimodal reaction. A second sample of MS Ky 14 × L8 seed was obtained from the same seed company that produced the first seed lot, and 110 plants were tested. The bimodal response was observed but was not as pronounced: 15% of the plants were susceptible, 85% were resistant. The cause of this unique segregation response could be seed contamination during seed cleaning, although such a high degree of contamination is highly improbable. It is unlikely that the bimodal response was due solely to technique, because of the consistency of the data. We do not know the basis for the heterogeneous response of MS Ky 14 × L8, but it may be related to the source of cytoplasmic male sterility used by the commercial seed company.

With the exception of the MS Ky 14 × L8 data, highly reproducible results were obtained by using the greenhouse screening technique reported here. This technique has many advantages over screening for angular leaf spot resistance in the plant bed or field: 1) Environmental conditions and growth of plants are more easily controlled in the greenhouse, 2) more uniform plants can be obtained using the methods described, 3) screening of germ plasm can be conducted throughout the year rather than being limited to the tobacco growing season, 4) there is no danger of contamination of other plant beds because studies can be confined to the greenhouse, and 5) the technique is simple and can be readily incorporated into any tobacco breeding program.

The type of angular leaf spot resistance found in Burley 21, a cultivar also resistant to wildfire, is appealing because of its insensitivity to increasing levels of bacterial concentration with or without increasing levels of preinoculation or postinoculation mist. Should incidence of angular leaf spot continue to increase, it is reasonable to expect that Burley 21 would be able to maintain its level of resistance, provided that new strains of *P. s.* pv. *tabaci* virulent on Burley 21 do not develop.

Table 1. Percent leaf area damaged of four tobacco cultivars inoculated with *Pseudomonas syringae* pv. *tabaci* under various misting regimes before and after inoculation[x]

	Percent leaf area damaged[y]								
	Postinoculation mist (hr)								
	24				36				
	Preinoculation mist (hr)								
Cultivar	0	6	12	18	0	6	12	18	Mean
Judy's Pride	46	30	35	27	70	56	47	47	50 a[z]
Goat's Creek	27	11	14	12	46	34	13	37	24 b
MS Ky 14 × L8	5	3	5	1	10	7	4	27	8 c
Burley 21	1	1	1	1	1	1	1	3	1 d

[x]The undersides of the two oldest leaves of plants at the four- to five-leaf stage were uniformly sprayed in the greenhouse with an artist's airbrush at a pressure of 4×10^5 Pa. Inoculations were conducted between 10:00 a.m. and 2:00 p.m., and temperature ranged from 19 to 27 C. Concentration of bacteria was approximately 9.0×10^7 cfu/ml.

[y]Disease readings made 10 days after inoculation using a quantitative disease assessment scale from 0 to 55%. Value is average of four replications.

[z]Waller-Duncan comparison of general cultivar means. Means with different letters are significantly different (k-ratio = 100).

ACKNOWLEDGMENTS

We wish to thank William Nesmith for providing Goat's Creek seed, the late H. A. Skoog for the *Pseudomonas syringae* pv. *tabaci* isolate, and the North Carolina Tobacco Foundation, Inc., for financial support.

LITERATURE CITED

1. Braun, A. C. 1937. A comparative study of *Bacterium tabacum* Wolf and Foster and *Bacterium angulatum* Fromme and Murray. Phytopathology 27:283-304.
2. Clayton, E. E. 1936. Watersoaking of leaves in relation to development of the wildfire disease of tobacco. J. Agric. Res. 52:239-269.
3. Clayton, E. E. 1937. Watersoaking of leaves in relation to development of the blackfire disease of tobacco. J. Agric. Res. 55:883-889.
4. Clayton, E. E. 1947. A wildfire resistant tobacco. J. Hered. 38:35-40.
5. Deall, M. W., and Cole, J. S. 1986. A comparative study of the pathogenicity and epidemiology of strains of *Pseudomonas syringae* pv. *tabaci* that cause wildfire and

angular leaf spot diseases of tobacco in Zimbabwe. Plant Pathol. 35:74-81.
6. Heggestad, H. E., Clayton, E. E., Neas, M. W., and Skoog, H. A. 1960. Development of Burley 21, the first wildfire-resistant tobacco variety, including results of variety trials. Tenn. Agric. Exp. Stn. Bull. 321. 51 pp.
7. Hoagland, D. R., and Snyder, W. C. 1933. Nutrition of the strawberry plant under controlled conditions. Proc. Am. Soc. Hortic. Sci. 30:288-294.
8. Johnson, J. 1937. Relation of water-soaked tissues to infection by *Bacterium angulatum* and *Bacterium tabacum* and other organisms. J. Agric. Res. 55:599-618.
9. Lindow, S. E., and Webb, R. R. 1983. Quantification of foliar plant disease symptoms by microcomputer-digitized video image analysis. Phytopathology 73:520-524.
10. Lucas, G. B. 1975. Diseases of Tobacco. 3rd ed. Biological Consulting Associates, Raleigh, NC. 621 pp.
11. Russ, J. C. 1986. Practical Stereology. Plenum Press, New York. 185 pp.
12. Stapp, C. 1930. Bakterielle Tabakkrankheiten und ihre erreger. Angew. Bot. 12:241-274.
13. Valleau, W. D., Johnson, E. M., and Diachun, S. 1943. Angular leaf spot and wildfire of tobacco. Ky. Agric. Exp. Stn. Bull. 454. 60 pp.
14. Wolf, F. A., and Foster, A. C. 1917. Bacterial leaf spot of tobacco. Science 46:361-362.
15. Young, J. M., Dye, D. W., Bradbury, J. F., Panagopoulos, C. G., and Robbs, C. F. 1978. A proposed nomenclature and classification for plant pathogenic bacteria. N.Z. J. Agric. Res. 21:153-177.