Influence of Temperature and Other Factors on Initiation of Tobacco Brown Spot

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ABSTRACT

Infection of tobacco by *Alternaria tenuis,* incitant of brown spot, was most severe at constant 20 C, and was progressively less at 24 and 16 C. Least infection occurred at 28 C or in a cycle of alternating 12-hr periods of 20 and 28 C. Inoculation of the lower leaf surfaces resulted in 2 to 3 times more lesions than when leaves were inoculated on the upper surface. Temperature effects and relative sensitivity of tobacco cultivars to the disease were similar when plants were inoculated with conidia from dead, infected tobacco leaves from the field or from pathogenic single conidium isolates grown on V-8 juice agar. Addition of a carbon source to the inoculum increased the number of brown spot lesions on all cultivars at suboptimal temperatures and on tolerant cultivars at 20 C. Other critical factors for infection included maintenance of moisture on the leaves for at least 3 days following inoculation and use of an inoculum concentration of 30,000 conidia of a proven pathogenic isolate of the fungus/ml inoculum suspension. Relative sensitivity of tobacco cultivars to the disease was the same with controlled inoculations as with natural infection in the field. Optimum temperature for radial growth of five single conidium isolates of the fungus on agar media was 27 C. Phytopathology 60:1591-1596.

Additional key words: *Nicotiana tabacum,* growth of pathogenic and nonpathogenic isolates.

Brown spot has been the most important leaf disease of flue-cured tobacco, *Nicotiana tabacum* L., in the United States since the mid-1950's. The disease has caused estimated losses of 3.9 to 21 million dollars/ year to tobacco growers in North Carolina since 1959 (1, 12). The pathogen that incites the disease was described as *Alternaria longipes* (Ell. & Ev.) Mason in 1931 (11), but variability in conidial morphology has led to adoption of the broader taxon *A. tenuis* Nees by us and others in recent years (1, 9).

All flue-cured tobacco cultivars are susceptible to the disease (12). Breeding for resistance has been inhibited by the lack of a consistently successful technique for artificial inoculation. Tisdale & Wadkins (11) reported variable success in greenhouse inoculations, and concluded that maintenance of an average temp of 26.5-31.0 C and of free moisture on the leaves for 3-5 days were essential to obtain infection. Riley (7) reported that the optimal temp for the disease was about 30 C and that addition of 0.5% glucose to the inoculum favored symptom development. Since publication of these reports, brown spot has been generally considered to be favored by warm temp and high humidity (1, 3). However, Wilson (14) and Ramm & Lucas (6) experienced great difficulty in obtaining infection in the greenhouse. Only by dusting leaves with wheat flour, inoculating with conidia from brown-spot infected, dead tobacco leaves collected from the field in late summer, and keeping inoculated plants in a moist chamber for 8-10 days could Ramm & Lucas (6) obtain significant infection in the greenhouse. In later experiments, they could obtain infection without dusting with wheat flour (Lucas, *personal communication*). They

reported that disease development could occur between 13 and 32 C with the optimal temp near 25 C (5). Ramm & Lucas (6) and Sobers & Doupnik (8) found that many isolates of the fungus were nonpathogenic. The latter workers obtained virulent isolates of the fungus that remained pathogenic even when grown on artificial media (8).

The principal objective of the research reported here was to develop a reliable method for inoculation of greenhouse tobacco plants with *A. tenuis*. Specific objectives were to compare the effects upon infection of (i) postinoculation temp; (ii) inoculating the upper or lower surface of leaves; (iii) several carbon sources used as inoculum supplements; and (iv) controlled inoculation versus natural field infection of tobacco cultivars known to vary in sensitivity to the disease. In addition, the effects of several temp on radial growth of *A. tenuis* were determined in culture. Some of our results have been published (9).

MATERIALS AND METHODS.—*Inoculation experiments.* —*Alternaria tenuis* conidia were obtained from dead, brown spot-infected tobacco leaves collected from sensitive tobacco cultivars in the field in September or from single conidium isolates of the fungus grown on V-8 juice agar (V-8). Dead leaf inoculum was prepared by blending the infected leaves in distilled water with a Waring Blendor and filtering the suspension through cheesecloth to remove most of the leaf debris. From 86 single conidium isolates of *A. tenuis* obtained from brown spot lesions in the field in 1968, the following were selected for use in these studies: isolates A3, highly pathogenic; A5, slightly less pathogenic; A6, less pathogenic than A5; and A31 and A37, nonpatho-

genic. The isolates were maintained on V-8 at 18 C with fresh transfers every 2-4 weeks. Pathogenicity of the isolates was unchanged until A3 became nonpathogenic 15 months after isolation. Conidia for inoculation were produced by flooding V-8 agar in petri plates with conidial suspensions and incubating the plates for 6-9 days at 23-29 C, with other conditions the same as will be described for radial growth studies. Conidia were scraped and washed from the agar surface into distilled water containing 0.01% Rohm and Haas Triton B-1956 wetting agent (active ingredient, 77% modified phthalic glyceryl alkyd resin). The concn of conidia for all inoculations was calibrated with a haemocytometer and adjusted to $30,000 \pm 1,000$ conidia/ml distilled water. Inoculum was sprayed uniformly onto leaves with a Sears-Roebuck Model 30-1502-077 paint sprayer.

The four tobacco cultivars used in inoculation studies were selected to be representative of the range of reaction to *A. tenuis* available in *N. tabacum*. Coker 187 Hicks and Coker 298 are sensitive, NC95 is moderately tolerant, and PD121 has high field tolerance to brown spot (2, 12). The plants were 3-4 months old when inoculated. They were grown in 6-inch clay pots in a greenhouse equipped with carbon filters to eliminate air pollution injury to leaves. One teaspoon of 7-7-7 fertilizer was added to each pot 2 and 5 weeks before the plants were inoculated.

After inoculation, all plants were incubated in Sherer-Gillette CEL 37-14 plant growth rooms with automatic humidity control systems. Free moisture was maintained on the leaves throughout the first 72 hr after inoculation. From 72 to 168 hr after inoculation, the relative humidity at each temp was dropped to $70 \pm 8\%$ during the 12-hr light period, but free moisture was maintained on the leaves during the dark period. An alternating wet-dry cycle was necessary to obtain the typical concentric ring pattern that characterizes the brown spot lesion in the field. A 12-hr light/dark cycle was used for all tests. Light was provided by a combined fluorescent-incandescent source that delivered ca. $3.2-5.5 \times 10^4$ ergs/cm² per sec at the leaf surface.

All inoculated plants were removed from the growth rooms 1 week following inoculation and placed in a greenhouse at 24-32 C. One week later we recorded the number of brown spot lesions on each inoculated leaf.

1) *Effect of postinoculation temperature.*—To determine the effect of postinoculation temp on infection of tobacco and initiation of brown spot by *A. tenuis*, plants of each of the four cultivars were inoculated and one plant of each cultivar was placed at each of several test temp. The three pathogenic and two nonpathogenic single conidium isolates were each tested separately. Inoculations with pathogenic isolates were repeated 4 times, and with nonpathogenic isolates, twice for each temp and isolate. Temperatures tested included constant 16, 20, 24, and 28 C ± 2 C and a 12-hr alternating cycle, 28/20 C ± 2 C, with the 28 C part of the cycle during the light period.

2) *Effect of leaf surface inoculated.*—The effect of inoculating either the lower or upper surface of leaves was compared on plants of sensitive cultivars Coker 187 Hicks and Coker 298. Isolate A3 of *A. tenuis* was used for inoculum and the plants were incubated at 16, 20, 24, or 28 C for 1 week in the growth rooms under the conditions already described.

3) *Effect of inoculum supplements.*—Dusting leaves with wheat flour (6) or addition of glucose to the inoculum suspension (7) has been reported to enhance brown spot infection. At the outset, we tested the effectiveness of several mono- and disaccharides as inoculum supplements. The supplements were added to the distilled water in which the conidia were suspended to give either 0.1 M glucose, xylose, fructose, or galactose or 0.05 M maltose, lactose, or sucrose. Two plants of each of the sensitive varieties, Coker 187 Hicks and Coker 298, were inoculated with each of the supplemented inocula and incubated at 24 C for 1 week using the described humidity and light conditions. In another experiment, 0.01, 0.5, and 1.0 M glucose were compared as inoculum supplements. Finally, the effectiveness of a 0.5-M glucose supplement was compared with unsupplemented distilled water as a suspending medium for conidia with inoculated plants incubated under the same five temp regimes previously tested. Conidia from dead leaves were used in all three tests.

Brown spot reaction of tobacco cultivars in the field. —The same four tobacco cultivars used in the artificial inoculation tests were planted in the field at the Oxford Tobacco Research Station, Oxford, N. C., in 1968 and 1969. The brown spot disease was permitted to develop naturally. As the leaves progressively ripened from the bottom to the top of the plant, they were harvested, and the leaf area (10) and number of lesions per leaf were recorded for each harvested leaf. A total of 20 leaves was harvested (primed) from each plant over eight harvest dates between 15 July and 7 September. Data were recorded from 10 replicate plants/cultivar. From the leaves of each harvest, the average number of lesions/645 cm² (100 in²) of leaf tissue was computed to give an index that eliminated bias due to leaf size and shape. The data were expressed as the total number of lesions per plant of each cultivar, e.g., the sum of the average number of lesions on 645 cm² of each leaf of the 20 leaves/cultivar.

Effect of temp on growth in culture.—The effect of temp on radial growth in culture of the five monoconidial isolates of *A. tenuis* was determined at 15, 18, 20, 23, 25, 27, 29, 31, and 34 ± 1 C. A 3.5-mm disc of inoculum was placed in the center of 20 ml of poured and solidified V-8 or potato-dextrose agar (PDA) in an 8.5-cm plastic petri plate. Ten replicate plates of each medium were incubated at each temp, and radial growth was measured after 7 days. The incubators were provided with constant light from a fluorescent lamp delivering 3×10^3 ergs/cm² per sec light energy at the surface of the medium. Temperatures inside the closed petri dishes did not differ significantly from the ambient temp inside the incubators.

RESULTS.—*Inoculation experiments.*—1) *Effect of postinoculation temp.*—The opt postinoculation incu-
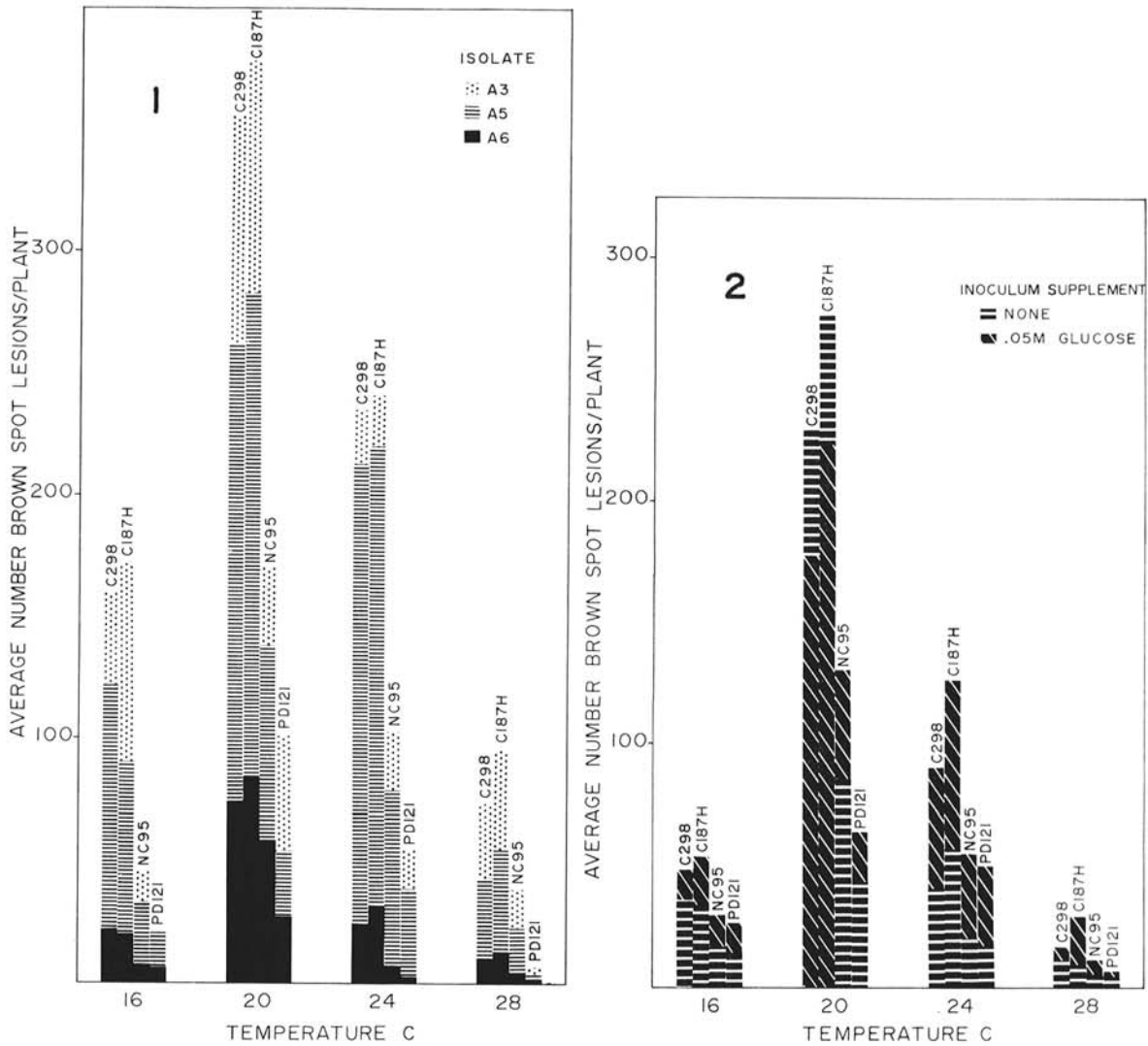
Fig. 1-2. Effects of temp, tobacco cultivar, and other variables on average number of brown spot lesions per plant from four different inoculations involving each variable. **1)** Number of lesions incited by three single conidium isolates of *Alternaria tenuis*. **2)** Effect of presence or absence of 0.05 M glucose in an inoculum suspension of conidia from dead tobacco leaves on number of lesions. C298 = cultivar Coker 298; C187H = cultivar Coker 187 Hicks; NC95 = cultivar North Carolina 95.

bation temp for infection of all cultivars of tobacco by *A. tenuis* was 20 C in all temp comparisons. Least infection consistently occurred on plants exposed to a postinoculation temp of 28 C, even when 28 C was alternated in a 12-hr cycle with 20 C. There was little difference in the level of infection resulting under these two conditions.

The effect of temp on the level of infection produced by the single conidium isolates (Fig. 1) was similar to the effect of temp when conidia from dead leaves were used for inoculum regardless of whether or not inoculum was supplemented with glucose (Fig. 2). No carbon supplement was added to the suspensions when single conidium isolates were used for inoculum. A3 was the most pathogenic isolate on all

cultivars at all temp, except that, on PD121 at 16 C, A3 incited an average of 17 and A5 an average of 20 spots/plant (Fig. 1). Isolates A3, A5, and A6 incited an average of 379, 286, and 84 brown spot lesions, respectively, on Coker 187 Hicks at 20 C. The difference between the number of spots at 20 and 24 C was less marked with A5 than with A3 and A6. In comparative inoculations, although there was some variability, A3 and A5 produced a higher average number of lesions than the conidia from dead leaves, and A6 produced the fewest lesions. Isolates A31 and A37 failed to produce any symptoms on any of the cultivars at the temp tested. In all inoculation experiments and at all temp levels, NC95 had about half or less than half as much infection as Coker 187 Hicks and Coker 298. PD121

consistently had less brown spot than NC95. Coker 187 Hicks had a slightly higher number of lesions than Coker 298 in most inoculation experiments, and a somewhat higher average number of lesions consistently.

Single conidium isolates of *A. tenuis* produced brown spot symptoms at 20 C that were similar to those observed in the field (Fig. 3). The oldest leaves on susceptible varieties were dead from brown spot damage 12-20 days after inoculation. Midplant leaves were heavily infected, and the spots were usually surrounded by a halo of yellow tissue that is characteristic of the disease on sensitive cultivars in the field. Numerous pinpoint lesions 1 mm or less in diam occurred on the leaves that were youngest at the time of inoculation. Expansion of these youngest leaves was inhibited so that 2-3 weeks after inoculation the youngest inoculated leaf was often less than half the size of the next younger leaf that had escaped inoculation by emerging from the bud after the inoculation date.

2) *Effect of leaf surface inoculated.*—When the two sensitive cultivars were inoculated on the lower leaf surface, 2-3 times more brown spot lesions developed than when they were inoculated only on the upper surface (Table 1). The increase in lesion number with lower surface inoculation was not affected by temp. The optimum temp for the disease was 20 C with lower surface inoculation, and least disease developed at 28 C.

Fig. 3. Coker 298 tobacco plant inoculated with 30,000 *Alternaria tenuis* isolate A3 conidia/ml distilled water and incubated at 20 C for 1 week after inoculation. Photographed 2 weeks after inoculation and 1 week after removal from growth room to greenhouse. Note abundant brown spot lesions on all but uppermost leaf, lesions on stem and midrib, and halo of chlorotic tissue surrounding leaf lesions.

TABLE 1. Effect of inoculation with *Alternaria tenuis* of upper or lower leaf surface on average number of brown spot lesions/plant at three temp[a]

| | Temp (C), surface inoculated, and no. lesions/plant[b] | | | | | |
| | 16 | | 20 | | 24 | |
Cultivar	Upper	Lower	Upper	Lower	Upper	Lower
Coker 298	138	264	362	916	225	517
Coker 187 Hicks	149	274	387	1,019	237	549

[a] Inoculum was 30,000 *Alternaria tenuis* isolate A3 conidia/ml distilled water.
[b] Average number of lesions from four different incubations of inoculated plants at each temp.

3) *Effect of inoculum supplements.*—There were no significant differences in the amount of infection with any of the seven tested saccharide inoculum supplements. Only a moderate level of infection developed on the plants which were incubated at 24 C following inoculation. But over twice as many lesions developed with supplemented inoculum as with unsupplemented inoculum at 24 C. As much infection occurred at 24 C with a 0.05 M glucose supplement as with a 1.0 M supplement. Significantly less infection occurred when the glucose concn was reduced to 0.01 M.

When inoculum supplemented with 0.05 M glucose was compared with inoculum having no supplement on inoculated plants incubated at 16, 20, 24, and 28 C, the presence of the glucose resulted in more infection at nonoptimal temp (Fig. 2). More lesions also developed on tolerant NC95 and PD121 at 20 C with supplemented than with unsupplemented inoculum. Somewhat fewer lesions developed on sensitive Coker 187 Hicks and Coker 298 at 20 C when the inoculum contained 0.05 M glucose than when no supplement was added. Relative sensitivity of the four cultivars was the same as in all other inoculation studies.

Brown spot reaction of tobacco cultivars in the field. —The relative sensitivity of the four cultivars in the field (Table 2) was similar to that recorded in the inoculation studies in the growth rooms and greenhouse. PD121 was tolerant; NC95, moderately tolerant; and Coker 187 Hicks and Coker 298 were sensitive

TABLE 2. Number of brown spot lesions recorded on four tobacco cultivars, naturally infected with *Alternaria tenuis*, in the field at Oxford, N.C. in 1968 and 1969

| Cultivar | Tolerance rating | No. lesions/plant[a] | |
		1968	1969
PD121	Tolerant	77	18
NC95	Moderate	148	58
Coker 187 Hicks	Sensitive	251	204
Coker 298	Sensitive	241	331

[a] The number of lesions per plant is the sum of the number of lesions on a 645-cm^2 area of each of 20 leaves/plant. The lesions were counted upon harvest. The leaves were harvested upon ripening at 8 weekly intervals from 15 July through 7 September. Numbers given here are an average from 10 replicate plants/cultivar.

with both natural field infection and artificial inoculation under controlled conditions. The field results also indicate the differences in natural infection rate that occur in different years. In 1968 there was little difference between the two sensitive cultivars, but in 1969 Coker 298 had considerably more infection. Three times more lesions were recorded in 1968 and 18 times more lesions in 1969 on the most sensitive cultivar than on tolerant PD121. As the disease became more severe late in the season, the differences between cultivars became accentuated. The last three lesion counts were responsible for much of the difference between cultivars.

Effect of temp on growth in culture.—The optimum temp for radial growth on V-8 and PDA was 27 C for all five isolates (Fig. 4). Average colony diam on V-8 and PDA were not different at any temp tested. Growth of pathogenic isolates A3, A5, and A6 was more sharply curtailed above ca. 30 C than was growth of the nonpathogenic isolates A31 and A37. Growth of all isolates diminished gradually with decrease in temp below the opt. The nonpathogenic isolates had greater colony diam at all temp than the pathogenic isolates. Aerial mycelium was more abundant on the nonpathogenic than on the pathogenic isolates. Temperature and medium had no affect on sectoring. All cultures sectored frequently.

DISCUSSION.—Our results consistently indicated that 20 C was the optimum temp for infection of tobacco by *A. tenuis* to initiate brown spot. These results were confirmed in a temp-controlled, greenhouse mist cham-

Fig. 4. Effect of temp on colony diam of three pathogenic (A3, A5, and A6) and two nonpathogenic (A31 and A37) isolates of *Alternaria tenuis*. The data are averages of 20 replicates at each temp, including 10 replicates each on V-8 and potato-dextrose agars. All measurements were made 7 days after inoculation of media.

ber, by field observations by the senior author in Beltsville, Md., and by epidemiological data in North Carolina (C. E. Main, *unpublished data*). These results appear to contradict those of Tisdale & Wadkins in 1931 (11), Riley in 1949 (7), Ramm & Lucas in 1962 (5), and Okuura et al. in 1967 (3), who reported optimum temp of 26.5-31.0, about 30, near 25, and 25 C and above, respectively. The data of Okuura et al. (3) were based entirely on field records of temp, rainfall, and lesion development, while those of the other three reports were based on greenhouse studies. We believe that the reported field data (3) could be interpreted as not contradictory to our results if the lag time between infection and symptom development is correlated with the temp records. One might conclude that the optimal temp in Japan was closer to 22 than to 25 C (3). The importance and variability of periods of high humidity complicate such interpretations of field data. Often, but not always, periods of summer rain are accompanied by a drop in temp to around 20 C. We have observed that after such a period inoculated field plants develop abundant brown spot, but that less infection occurs following a warmer wet period. The results of Ramm & Lucas (5) were preliminary and published in a brief report. The results of Tisdale & Wadkins (11) and Riley (7) are difficult to reconcile with our results; however, their conclusions were based on greenhouse temp which were apparently not well controlled (7) or not controlled at all, so that the conclusions had to be drawn from a record of average daily greenhouse temp (11).

We cannot eliminate the possibility that there has been a change in the pathogen since the early work (7, 11), or that the pathogen is different in other areas of the world (3, 7), but we found no indication of differences in disease symptoms or pathogen morphology from those pictured and described previously.

The increased infection resulting from lower leaf surface inoculation may be explainable by the greater sensitivity of the spongy mesophyll than was the palisade mesophyll to invasion and subsequent degeneration by the fungus (4). Differences in light intensity, cuticle characteristics, or substances on the leaf surfaces affecting spore germination are other possible reasons.

We found that supplementing inoculum suspensions with a carbon source increased the number of brown spot lesions under suboptimal conditions. *Alternaria tenuis* is a facultative parasite and is generally considered to be a weak pathogen; however, the organism can penetrate juvenile tissue and incite disease symptoms. The addition of a carbon source to the inoculum probably gives the organism a source of nutrition to better enable it to penetrate and become established under suboptimal conditions.

The consistency in relative sensitivity of cultivars to artificial inoculation as compared to natural infection in the field indicates that the inoculation method described here can be used by plant breeders in testing for brown spot resistance. The method may not be critical enough to detect small differences in disease sensitivity, but appears to be adequate to detect dif-
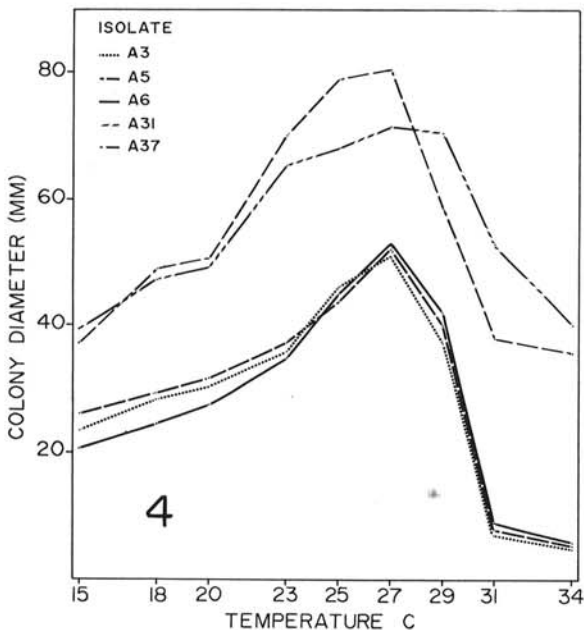
ferences of the magnitude required to screen segregating populations.

The optimum temp for growth of the five single conidium isolates tested was about 27 C. This agrees closely with the previously reported optima of 25-29 (11), 25-30 (7), and 26-28 C (8). Our results indicate that the optimal temp for growth of the organism in culture is about 7 C above the optimal temp for initiation of the disease. The optimal temp for penetration, growth in the host, and parasitism by the fungus may be lower than the opt for growth of the fungus on agar media. Another possibility is that the optimal temp for penetration is lower than that for subsequent growth in the host. Differing optimal temp for various fungal processes have been well documented for *A. solani* (Ell. & G. Martin) Jones & Grout (13). On the other hand, the host may be more susceptible at 20 C than at 27 C, or conditions in both host and fungus may be opt for pathogenicity at 20 C.

We believe that several essential requirements must be met in order to obtain successful infection of tobacco with *A. tenuis*. These include maintaining moisture on the inoculated leaves for a min of 3 days after inoculation, maintaining inoculated plants at 20 C for several days following inoculation, inoculation with conidia from pathogenic isolates of the fungus, adjusting the inoculum concn to about 30,000 conidia/ml, and inclusion of at least one known susceptible cultivar to serve as a check in each inoculation. Additional factors favoring infection are use of plants that have grown beyond the seedling stage, inoculation of the lower surface of the leaves, and the addition of 0.05 M glucose to the inoculum if plants cannot be incubated at 20 C. The disease is probably not serious in the field until the latter part of summer, due to absence of certain of the above requirements and to the rapid increase in secondary inoculum following establishment of primary infections earlier in the summer.

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