Growth of *Aspergillus repens* in Flue-Cured Tobacco

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In laboratory tests, flue-cured tobacco inoculated with *Aspergillus repens* was stored at 75, 80, 85, 87, and 95% relative humidity at 20 and 30°C. Samples were taken weekly for 4 weeks and evaluated for mold growth (colony count) and moisture content (MC). The weekly rate of fungus increase was slower at 20°C than at 30°C. Tobacco at 20°C with MC between 25 to 30% supported a slight to moderate increase in *A. repens* after 3 weeks of storage. However, tobacco at the same MC stored at 30°C was subject to rapid invasion by the fungus in as few as 1 to 2 weeks. Tobacco with MC above 30% stored at either 20 or 30°C became moldy in about 1 week. A mold index is proposed for evaluating populations of *A. repens* in tobacco.

In a survey to determine the fungi associated with damaged flue-cured tobacco, *Nicotiana tabacum* L., species of *Aspergillus* and *Penicillium* were found to be predominant (13). *Aspergillus repens*, the most prevalent, was isolated from 81% of the samples, averaged 12,900,000 colonies per g of tobacco, and ranged from 0 to 120 million colonies per g. The official USDA definition of tobacco damage does not include a qualitative or quantitative determination of the microorganisms. Damage is defined as "the effect of mold, must, rot black or other fungal or bacterial diseases which attack tobacco in its cured state. Tobacco having the odor of mold, must, or rot is considered damaged" (10). Factors that determine whether microorganisms will grow in cured tobacco are probably temperature and moisture content, as has been reported for cereal grains (1, 2), some forest seeds (7), and textiles (6).

Moisture content is important in the handling, storage, marketing, manufacture, and preservation of flue-cured tobacco. Tobacco grades, however, do not reflect moisture content unless the U.S. Government grader believes the tobacco is too moist for safe storage for a few days. Such tobacco is graded "W" (wet), "U" (unsound), or "No-G" (No-grade; reference 10). It is advantageous to the farmer to market tobacco as moist as possible without risking apparent deterioration. Moisture content directly affects gross return to the grower but, above safe levels, also increases the danger of deterioration. Moisture content considered proper by the farmer during marketing differs from that considered proper by the buyer for storage and manufacture. Therefore, tobacco is redried immediately after purchase to about 11% moisture (wet-weight basis); tobacco at or below 12% moisture ages properly and is safe from deterioration by fungi for prolonged periods (4).

After farm curing and until purchased by the consumer as a finished product, tobacco undergoes several changes in moisture content. In a 1967 survey, 100 samples of tobacco from 12 tobacco markets in two tobacco belts had moisture contents ranging from 12.6 to 30.2% (13); the mean was 19.6%. For aging, tobacco is dried to 10 to 11% and stored for 1 to 3 years. During the manufacture of blended cigarettes in the United States, tobacco is remoistened to 16 to 20% for cutting and redried to 12 to 15% for machine manufacture into cigarettes. Most American cigarettes are marketed with 12 to 13% moisture.

*A. repens*, the predominant fungus isolated from damaged tobacco, was also isolated from nondamaged flue-cured tobacco in storage and at the market (12, 13). The fungus was not isolated from green tobacco leaves before curing or from dried leaves immediately after flue-curing (14). Green plants sprayed in the field did not become infected by *A. repens* (11). These data indicate that the association between *A. repens* and tobacco is saprophytic and, when moisture content is sufficient for fungal invasion, some time between on-farm curing and marketing, *A. repens* becomes associated with the tobacco leaf.

To determine the conditions for and the rate

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at which *A. repens* grows in tobacco at different moisture contents and to use this information to relate to tobacco in storage, inoculated flue-cured tobacco was stored in the laboratory at different relative humidities and temperatures. Fungus increase and tobacco moisture content were measured weekly. A preliminary report has been published (R. E. Welty, Phytopathology 60:1319).

**MATERIALS AND METHODS**

**Fungus source.** The strain of *A. repens* (MQF-1) used was isolated from nondamaged tobacco offered for sale in a tobacco warehouse at Durham, N.C., in 1967.

**Tobacco source.** Field grown tobacco with relatively little brown-spot disease (caused by *Alternaria tenuis* Nees) was harvested and flue-cured in the usual manner (4) at the Border Belt Tobacco Research Station, Whiteville, N.C. The cured leaves were selected at random from all stalk positions, compressed, shredded into 1-mm-wide ribbons of various lengths, and stored at 15.8 to 17.5% moisture content at room temperature (23 to 28 C) until used for the experiments.

**Test unit.** For measuring fungus increase, 40 tobacco samples (one for each of five relative humidities × two temperatures × four sample intervals) of 10 g each were weighed in 9-cm diameter petri dishes, sterilized in an unpressurized steam chamber, inoculated, and placed in desiccators above saturated salt solutions. For moisture content determinations, 40 10-g samples of tobacco were weighed in tared stainless-steel dishes, sterilized, inoculated, and also placed in the desiccators. Weekly for 4 weeks a glass and a metal dish of tobacco were removed from each desiccator and evaluated for growth of *A. repens* and for moisture content.

**Inoculation.** A 1-ml amount of an aqueous spore suspension of *A. repens* containing 1 million spores/ml was atomized onto the tobacco in all dishes. This resulted in a calculated initial inoculum of 100,000 spores per g of tobacco. The spore suspension was made by washing, with 10 ml of sterile distilled water (plus 0.06 ml of Tween 80), the surface of a 10-day-old test tube culture of *A. repens* on potato dextrose-agar. The spores were counted in a hemocytometer, and the suspension was diluted with sterile distilled water to obtain the standard spore suspension. The addition of the aqueous spore suspension to the tobacco increased the calculated moisture content to 25.8 to 27.4%.

Storage relative humidities (RH) were maintained with aqueous saturated salt solutions with an excess of salt, in closed 9.5-liter desiccators. Saturated solutions of the following salts maintained the indicated RH at 20 and 30°C, respectively: (i) sodium chloride, 76.0 and 75.5% RH; (ii) ammonium sulfate, 80.5 and 80.0% RH; (iii) potassium chloride, 85.0 and 84.5% RH; (iv) potassium sodium tartrate, 87.0 and 87.0% RH; and (v) lead nitrate, 97.0 and 95.0% RH (16). These salt solutions were previously found to produce moisture contents in the range encountered by flue-cured tobacco during marketing (15). Inoculated tobacco in glass and steel dishes was placed over each of the five saturated salt solutions and incubated at 20 and 30°C.

**Evaluation.** Growth of *A. repens* and tobacco moisture content were determined after 1 week of storage and subsequently at three weekly intervals for each storage condition (five RH × two temperatures). The steel dishes were removed from each storage condition at random and dried by heating at 100°C for 16 h in a ventilated oven (8). Moisture content (MC) was calculated from the weight lost and is expressed on a wet-weight basis.

Populations of *A. repens* were determined by a method previously reported (13) but slightly modified. The glass dish containing 10 g of inoculated tobacco was blended in 500 ml of a sterile solution of 0.15% agar for 2 min. Further dilutions were made, and 1 ml of each dilution was placed in each of three petri dishes. Czapek's plus 6% NaCl agar (Cz+6) and weak tomato juice-agar (WTJ), melted and cooled to 52°C, were added to one and two dishes, respectively. The dishes were swirled to distribute the suspension and incubated at room temperature. After 2, 4, and 6 days, young colonies were counted, and, on the sixth day, the dishes were incubated until the fungal colonies could be identified. Colony counts were determined by multiplying the dilution factors 10-4 through 10-8 by the average number of colonies growing in the two petri dishes of WTJ agar determined on the sixth day. Dilutions below 1:105 were not cultured. A single petri dish of Cz+6 was included in the test to confirm culture purity and to facilitate identification of possible contaminating species of other fungi, particularly of the genus *Aspergillus*. For some treatments, *A. repens* colonies become too numerous to count at lower dilutions. As a result, data used for the analysis of variance were based on the counts from paired dilution cultures that averaged 20 to 100 fungal colonies per dish. Fungal increase in this study is based on colonies per gram. This procedure has been used previously (13) to measure the kinds and amounts of fungi associated with tobacco.

**Experimental design and statistical analysis.** The experiment was set up in a split plot design with the whole plot (RH) arranged as a randomized complete block design. The subplot treatment was storage time, and the experiment was replicated three times. The resulting data for both temperatures were statistically analyzed by using an analysis of variance.

Colony counts were converted to logarithms (base 10) for the analysis. The antilog was used to tabulate the results.

**RESULTS AND DISCUSSION**

When stored tobacco was gaining moisture and an MC suitable for growth of *A. repens* was reached, increased growth of the fungus was directly related to time and temperature (Fig. 1, 2).

It was unfortunate that the tobacco was.
stored in various environments immediately after inoculation as the aqueous spore suspension increased the tobacco MC above the equilibrium MC maintained by some of the saturated salt solutions. This resulted in decreasing tobacco MC in some conditions (solutions removing moisture from the air around the tobacco, which in turn removed moisture from the tobacco) as fungus counts increased. These oppositely changing values make it difficult to locate a minimum MC for *A. repens* growth in stored tobacco.

From the tobacco stored at the five RH levels at 20 C, the minimum MC for *A. repens* growth appears to be near 29% since it was not until this MC was reached that the fungus began to increase. This value probably is not accurate. It seems likely that temperature affected the

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**Fig. 1.** Per cent moisture content and colonies of *Aspergillus repens* per gram of tobacco stored at 20 C and at five relative humidities, measured at four weekly intervals. Each measurement is the average of three replications.

**Fig. 2.** Per cent moisture content and colonies of *Aspergillus repens* per gram of tobacco stored at 30 C and at five relative humidities, measured at four weekly intervals. Each measurement is the average of three replications.
TABLE 1. Weekly colony counts of Aspergillus repens in flue-cured tobacco stored at two temperatures and five relative humidities for 4 weeks

| Relative humidity (%) | Colony count after storage for | Mean count |
|-----------------------|-------------------------------|------------|
|                       | 1 week                        | 2 weeks    | 3 weeks | 4 weeks |          |
| **At 20°C**           |                               |            |         |         |          |
| 75                    | 300 (a)                       | 300 (a)    | 200 (a) | 1 (a)   | 200 (A)  |
| 80                    | 12,000 (a)                    | 3,600 (a)  | 1,600 (a)| 300 (a) | 4,400 (B) |
| 85                    | 7,200 (a)                     | 3,000 (a)  | 6,100 (a)| 431,500 (a) | 112,000 (B) |
| 87                    | 5,800 (a)                     | 816,000 (b)| 93,100,000 (b)| 5,164,000,000 (bc) | 1,314,483,000 (C) |
| 95                    | 8,600 (a)                     | 1,300,000 (b)| 4,159,000,000 (c) | 8,531,000,000 (c) | 3,172,828,200 (C) |
| **At 30°C**           |                               |            |         |         |          |
| 75                    | 300†                          | 12,100     | 16,800  | 48,000  | 19,500   |
| 80                    | 400                           | 303,400    | 1,514,000 | 15,420,000 | 3,855,454,400 |
| 85                    | 300                           | 1,156,000  | 4,207,000,000 | 19,542,000,000 | 1,540,539,100 |
| 87                    | 32,700                        | 216,800,000| 2,018,000,000 | 1,489,000,000 | 930,958,200 |
| 95                    | 31,200                        | 698,200,000| 8,453,000,000 | 14,450,000,000 | 5,900,307,800 |

* Numbers are the log value used in the statistical analysis converted to colony counts per gram from the antilog and are the average for three replications. Numbers with different lower case letters are significantly different (5% level in log form) from each other, between storage times within a relative humidity (horizontal).

† Numbers with different upper case letters are significantly different (5% level in log form) from each other, between relative humidities (vertical).

* Some numbers for 30°C are significantly different from each other only at the 7% level (see text for possible reasons).

The growth of A. repens in tobacco more than MC. Other evidence (2) indicates that A. repens is capable of growth in materials at MC in equilibrium with RH of 71 to 75% and data obtained in this study for 30°C support this contention. Perhaps if the incubation period at 20°C had been longer than 4 weeks, fungus growth in the tobacco at lower RH may have been observed.

Likewise, data obtained at 30°C do not indicate precisely where the minimum MC for fungus growth occurs, but it appears to be between 20 and 25% (see 75% RH, Fig. 2). Despite the decreasing MC from 25.3 to 21.3%, colony counts increased steadily from 293 to 48,750.

The rate A. repens grew in stored tobacco at 30°C with MC between 25 and 30% (see 80% RH) indicated a rapid increase from 358 to over 1 million colonies per g from 1 to 3 weeks. After 3 weeks at 20°C, only a slight-to-moderate increase in the fungus occurred in tobacco in the same MC range (see 85% RH, Fig. 2). At either 20 or 30°C, tobacco with MC above 30% is likely to be thoroughly invaded by A. repens after 1 or 2 weeks.

Statistical analysis of the log of mean colony counts of A. repens per g of tobacco at 20°C showed significant differences at the 0.05 probability level in counts between RH levels and also for storage times within RH (Fig. 1 and Table 1). The mean square value for the analysis of the colony counts at the five RH levels at 30°C (Fig. 2, Table 1) was significant only at the 0.07 level, due probably to considerable variability in the data. The MC of stored tobacco at 75% RH at 30°C was certainly less favorable for A. repens growth than MC at other RH.

The growth of an organism in pure culture frequently follows a sigmoid or S-shaped curve. Although none of the storage conditions with the test intervals used in this study represent the entire typical growth curve, individual storage conditions supply portions of that curve. The maximum stationary phase of the sigmoid curve for log colony count (base 10) appears to be near 10 which would be equivalent to 10 billion colonies per g. As expected, the maximum stationary phase is reached sooner at the higher RH and at 30 instead of 20°C.

If counts of log base 10 are the actual maximum population which A. repens reaches per g of stored tobacco, then count values below these might be used to evaluate tobacco condition in actual storage with respect to the amount of damage caused by A. repens. The log of the colony count (base 10) could be used as a mold index (MI).

Since we usually do not culture dilutions below 10⁻³ in routine tests for the numbers and kinds of fungi in a tobacco sample, the lowest possible mold index would be MI 3. Based on present information, the index for the maximum
population of fungus which might be expected in tobacco would be MI 10 or MI 11.

Applying this scheme to data already published (13) on A. repens in 74 samples of damaged and 26 samples of nondamaged tobacco in storage, damaged tobacco had an average MI of 6 and a range from MI 3 to MI 8; nondamaged tobacco had an average MI of 3 and a range from MI 3 to MI 5. The following data were not presented in that report, but came from that study and are used here to prepare a frequency distribution for the amount of A. repens in each MI category occurring for the samples in each tobacco class.

At MI 3, there were 15 damaged samples and 24 nondamaged samples; MI 4, 6 damaged and 1 nondamaged; MI 5, 12 damaged and 1 nondamaged; MI 6, 18 damaged and 0 nondamaged; MI 7, 15 damaged and 0 nondamaged; MI 8, 10 damaged and 0 nondamaged; MI 9, 0 damaged and 0 nondamaged; and MI 10, 0 damaged and 0 nondamaged.

Although populations of A. repens did not reach MI 10 in any samples of stored damaged tobacco, the system does appear to have some usefulness. The reason MI 10 was not reached is probably because of competition with other storage fungi (in some samples as many as eight) or because the storage period was not long enough.

As more tobacco samples are evaluated for populations of A. repens, they will be assigned an MI by the scheme just described. It is hoped that the system will provide a convenient measure for evaluating fungi in a tobacco sample of unknown storage history and also provide for convenient comparisons between samples.

After 4 weeks of incubation, mold growth in tobacco was not visible at and below 85% RH at 20°C and at 75% RH at 30°C without 25 or 50× magnification. Despite the absence of macroscopically visible mold below 87% RH and 20°C, the dilution culture from 85% RH and 20°C yielded 431,500 colonies of A. repens per g of tobacco (Fig. 1, Table 1). Also, at 75% RH and 30°C, no mold was macroscopically visible and yet the count was 48,800 colonies per g. These examples indicate the subtle nature of growth of the fungus in stored tobacco and support the contention that visual examination of tobacco for mold growth without the aid of magnification is not a reliable indicator of tobacco condition and should not be used to predict storability. By the time fungal growth is evident to the casual observer, it is usually too late to initiate control measures.

These data do not show why fungal populations decrease under certain storage conditions. Some field fungi that invade (9) and some bacteria present on (5) stored barley lose viability rather rapidly if grain is stored at MC just below the limit for their growth. If the seeds are dry, these same organisms will survive for several years (3) and if the seeds are at a high enough MC, these organisms will grow (1). It seems likely, therefore, that the disappearance of A. repens from tobacco in these test conditions might be due to storage at MC near or below their limit for growth and may be similar to the situation with microorganisms in stored barley.

These results might also explain the association of A. repens with the leaf and how tobacco infested with the fungus becomes damaged. Whenever the MC of the cured leaf exceeds a minimum level (perhaps between 21 and 25%) and temperatures approach the optimum for the fungus (in this case near 30°C), populations of the fungus will probably increase and “damage” may occur. If tobacco MC fluctuates around the minimum required for growth, conditions are sometimes favorable for invasion by A. repens but not yet favorable for rapid growth. In this situation A. repens can be isolated from tobacco not yet damaged, as is true of marketed tobacco (13). If tobacco MC is below the minimum for growth of A. repens, as it is immediately after on-farm curing, A. repens cannot invade the tissue (14).

The presence of A. repens in cured tobacco could be interpreted to indicate that at one time or another MC had exceeded the minimum to permit fungus invasion, regardless of the MC when the sample was taken. Christensen and Kaufman (2) found this to be true for stored cereal grain and reported “for each of the common species of storage fungi there is a minimum moisture content in grain below which it cannot grow, these minimum moisture contents have been determined fairly accurately for most of the common storage fungi growing on the starchy cereal seeds and on some of the oils seeds.” Elsewhere (1) they stated “the number and kinds of fungi isolated from a given lot of grain can tell a good deal about how the grain has been stored.” The situation for stored tobacco is likely to be similar to that for stored grain.

Studies are now in progress to establish more precisely the minimum MC required for A. repens to grow in stored flue-cured tobacco alone and in competition with other storage fungi.

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