Effects of N\textsubscript{2}-O\textsubscript{2} and CO\textsubscript{2}-O\textsubscript{2} Tensions on Growth of Fungi Isolated from Damaged Flue-Cured Tobacco\textsuperscript{1}

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Ten fungi, Aspergillus niger, A. flavus, A. ochraceus, A. ruber, A. repens, A. amstelodami, Alternaria tenuis, Penicillium brevi-compactum, Cladosporium herbarum, and Chaetomium dolicotrichum, were isolated from moldy flue-cured tobacco and grown in various mixtures of N\textsubscript{2}-O\textsubscript{2} or CO\textsubscript{2}-O\textsubscript{2}. A 1 to 5% concentration of O\textsubscript{2} in an N\textsubscript{2} atmosphere caused the greatest change in growth of the nine species, and a 10 to 20% concentration of O\textsubscript{2} for A. flavus. All species, except A. amstelodami and A. ruber, grew faster in air than in mixtures containing 10% O\textsubscript{2}. High O\textsubscript{2} concentrations generally inhibited furrow production in the mycelial mats. In an atmosphere of 5 to 40% O\textsubscript{2} in the N\textsubscript{2} atmosphere, furrows formed in mycelial mats between 5 and 40% O\textsubscript{2} in the species except for A. ruber, A. repens, and A. amstelodami, which produced none in any concentration. As O\textsubscript{2} decreased below 20%, spore production was progressively decreased, colony color faded to white, and cleistothecia formation was suppressed. In CO\textsubscript{2}-O\textsubscript{2} mixtures radial growth of all species increased with each quantitative decrease of CO\textsubscript{2}. All species except A. niger grew faster in air than in 10% CO\textsubscript{2}. In contrast to N\textsubscript{2}-O\textsubscript{2} mixtures, the fungi formed furrows, sporulation and cleistothecial formation were suppressed, and colony color changed to white in higher O\textsubscript{2} concentrations.

Fungi deteriorate stored agricultural products. The storage fungi, mostly species of Aspergillus and Penicillium, invade seeds of corn and wheat when the moisture content is above approximately 12%. These fungi may decrease germination, discolor embryos, increase fatty acids, produce toxins, and cause heating of stored grains (1). According to Johnson (4), Suchsland in 1891 isolated bacteria from sweating tobacco and inoculated them back to tobacco. Other reports prior to 1950 of molds of stored tobacco are reviewed by Wolf (13), Lucas (5) and Lukic (6). Welty and co-workers (10–12) reported 73% of the fungi isolated from moldy flue-cured tobacco to be Aspergillus and Penicillium, whereas species of Alternaria, Cladosporium, Fusarium, and Rhizopus were isolated more frequently from undamaged tobacco.

Immediately after redrying, tobacco is pressed into bales or hogheads and stored up to two years. Because of the moisture and air conditions in storage, considerable interest is directed towards the possible effects of ambient gas composition on the stored tobacco leaves. Particular attention is directed towards possible effects of low oxygen and high carbon dioxide concentrations on the growth of fungal species that commonly infest flue-cured tobacco leaves (9).

The objectives of this investigation were to measure the effects of atmospheres containing various ratios of N\textsubscript{2} to O\textsubscript{2} and CO\textsubscript{2} to O\textsubscript{2} in a closed system on the radial growth and colony characteristics of 10 fungal species isolated from moldy flue-cured tobacco.

**MATERIALS AND METHODS**

The fungi, isolated from moldy flue-cured tobacco (10), were identified as Aspergillus amstelodami (Majin) Thom and Church, A. repens (Corda) de Bary, A. ruber (S and B.) Thom and Church, A. niger Van Tiegham, A. flavus Link, A. ochraceus Wilhelm, Alternaria tenuis Nees, Chaetomium dolicotrichum Ames, Cladosporium herbarum (Pres.) Link, and Penicillium brevi-compactum Dierkx. Stock cultures were maintained in perfume bottles on sterilized wheat grains with the exception of A. tenuis and C. dolico-

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which were maintained on modified Czapek's medium.

Petri dishes of modified Czapek's agar were seeded with several infested grains and grown for 1 week in the laboratory. Two 5-mm discs were cut from each sporulating colony and placed in 5 ml of sterile water in a test tube, the tube was shaken, and the spore suspension was poured into the petri dishes. Melted Czapek's medium reinforced with 34 g of agar per liter was added to these dishes to a depth of approximately 5 mm and mixed with the spore suspension. After 12 hr of growth at room temperature, 5-mm discs were cut from these plates and one disc was transferred to the center of each test plate, with the disc surface bearing germinated spores placed upon the agar surface. Mycelial discs of A. tenuis and C. dolici-trichum were cut from the colonies growing on Czapek's medium. One plate of each of the 10 fungi was placed at random in a stack of 10 plates, taped together, placed in a 9.5-liter desiccator, and sealed.

In studies with 5% or more of O₂ in the N₂ atmosphere, the desiccators were filled with the desired gas mixtures by evacuating them repeatedly and flushing with either N₂ or O₂ depending upon which gas was in greater concentration in the mixture. In the 50:50 mixture, N₂ was used. During the final evacuation, the pressure was decreased with N₂ or O₂, allowing 0.76 cm on a mercury column for 1% of the gas desired. The other gas was then added until the mixture reached atmospheric pressure. Mixtures containing 5% O₂ or less in N₂ were commercially mixed and analyzed. In the 100% N₂ treatment, 40 ml of alkaline pyrogallol was placed in the desiccator to absorb any trace of remaining O₂. A beaker containing 30 ml of 20% KOH was placed in the desiccator to absorb evolved CO₂. In the closed-system air control, the desiccator was similarly evacuated and filled with air. Each gas mixture was replicated in five desiccators, and the closed-system air control was replicated in three desiccators. Two gas mixtures and one air control consisting of 13 desiccators composed a test. To measure growth when O₂ and CO₂ were not limiting, the fungi were grown in air flowing at 1 liter per hr.

The organisms were grown at 25 C in continuous white fluorescent light. After 1 week of growth, two horizontal diameters of each colony were measured in millimeters. These values were averaged and analyzed statistically to determine significant growth differences. The initial and final compositions of various mixtures in two desiccators were analyzed with an Orsat-type gas analyzer (Fisher Scientific Co., Pittsburgh, Pa.).

RESULTS

Influence of N₂-O₂ mixtures on fungal growth. The comparative growth of the 10 fungi in atmospheres of N₂-O₂ is summarized in Fig. 1. Aspergillus flavus, A. niger, A. ochraceus, Cladosporium herbarum, and Penicillium brevicompactum did not show any significant increase in growth above 40% O₂. A. amstelodami reached maximal growth in 30% O₂. Alternaria tenuis reached maximal growth at 50% O₂ and decreased in higher concentrations.

Below 30% O₂ all fungi, except A. niger at 20% O₂, showed significant decreases in growth with each decrease in O₂ concentration and none grew in the absence of O₂. Nine species showed the greatest change in growth in the nitrogen atmosphere containing 1 to 5% O₂ and A. flavus at 10 to 20% O₂. The 10 species can be divided into two groups on the basis of their growth at 0.5% O₂: (i) Alternaria tenuis, Aspergillus flavus, A. niger, A. ochraceus, and Penicillium brevicompactum, which grew in 0.5% O₂; (ii) and A. amstelodami, A. repens, A. ruber, and Cladosporium herbarum, which did not grow in 0.5% O₂ and Chaetomium dolici-trichum, which was inhibited in 1% O₂. All species except A. niger grew more extensively in 30% O₂ and above than their corresponding closed-system air control. However, when the growth of the 10 species was compared with that of the continuous-airflow control, growth of the control was equal.

![Fig. 1. Growth of fungi after 7 days in various mixtures of nitrogen and oxygen at 25 C.](image-url)
| Fungus                  | Color in air                | Furrowing of mat | Sporulation                      | Color change                                | Cleistothecia formation |
|------------------------|----------------------------|------------------|----------------------------------|---------------------------------------------|-------------------------|
| Aspergillus niger      | Black                      | 5–40             | Sparse, 1.0                      | Black and white, 5–20                       | Absent, 1.0             |
| A. flavus              | Green and white            | 5–40             | Sparse, 1.0                      | White, 1.0 and 0.5                          | Absent, 1.0–10          |
| A. ochraceus           | Orange-yellow and white    | 5–20             | Sparse, 1.0                      | Green and white, 50–100                     | Absent, 1.0             |
| A. ruber               | Brown and yellow           |                  | Absent, 1.0                      | Green, 30 and 40                            | Absent, 1.0             |
| A. repens              | Green-yellow and translucent white |              | Absent, 1.0                      | Green and white, 5–20                       | Absent, 1.0             |
| A. amstelodami         | Blue-green                 |                  | Mature conidial heads present in all concentrations | Yellow and black-brown, 5–10 | Absent, 1.0 and 5 |
| Penicillium brevico-compactum | Blue-green and white    | Abundant, 5–40   | Absent                           | White, 0.5                                  |                         |
| Alternaria tenuis      | Gray                       | 5–40             | Mature conidial heads present in all concentrations | No color change                           |                         |
| Cladosporium herbarum  | Black                      | Abundant, 50–100 | Grayish black, 1.0               |                                             |                         |
| Chaetomium doliocriticum | White                  | Abundant, 20     |                                 |                                             |                         |

* Abundant vegetative mycelium above 40% O₂.
to the maximal growth of the fungi in the most favorable $N_2-O_2$ mixtures.

**Influence of $N_2-O_2$ mixtures on fungal morphology.** In addition to differences in growth, the fungi exhibited changes in colony morphology in the $N_2-O_2$ mixtures. In general, furrows in the mycelial mats were produced in the presence of 5 to 40% $O_2$. Spore production was progressively reduced when the $O_2$ concentration was 20% or less and ceased below 1.0% $O_2$ concentration, except for *A. amstelodami* and *Cladosporium herbarum*, which produced mature spores in all mixtures. The colony color generally faded to white as $O_2$ concentration decreased except for *Alternaria tenuis* and *Chaetomium dolicochicum*, in which no color change occurred. Cleistothecial formation also was suppressed in $O_2$ concentrations below 10% in the three species—*Aspergillus ruber*, *A. repens*, and *A. amstelodami*—which produced cleistothecia.

**Influence of $CO_2-O_2$ mixtures on fungal growth.** The comparative growth of the 10 fungi in atmospheres of $CO_2-O_2$ is summarized in Fig. 2. In general, radial mycelial growth of all species increased with each quantitative decrease of $CO_2$. At $CO_2$ concentrations higher than 50%, the mycelial growth of all the fungi fell into two general patterns. *Chaetomium dolicochicum*, *A. amstelodami*, *A. ruber*, *A. repens*, and *Cladosporium herbarum* were $CO_2$-sensitive, whereas *A. niger*, *A. flavus*, *A. ochraceus*, *Alternaria tenuis*, and *Penicillium brevi-compactum* were $CO_2$-tolerant. The radial mycelial growth in air in closed systems was faster than in $CO_2$ concentrations of 10% or higher, with the exception of *A. niger* whose mycelial growth in 10% $CO_2$ equaled that in air. In 100% $O_2$ these fungi grew faster than their corresponding closed-system air controls. The mycelial growth in flowing air exceeded that in all gas mixtures with the exception of *A. niger*, *A. flavus*, and *A. ochraceus* in pure $O_2$.

**Influence of $CO_2-O_2$ mixtures on fungal morphology.** The different $CO_2-O_2$ mixtures also affected colony morphology. In contrast to the effect in $N_2-O_2$ mixtures, furrowing generally occurred in the $CO_2$ atmosphere at higher $O_2$ concentrations; sporulation was suppressed, e.g., *A. ochraceus* in 50%, *A. ruber* and *A. repens* in 30%; colony color faded to white; and cleistothecial formation was suppressed in *A. ruber*, *A. repens*, and *A. amstelodami*.

**DISCUSSION**

The above results agree with those of Peterson et al. (7), who investigated the influence of $O_2$ and $CO_2$ concentrations on stored grain and found that the fungal growth and germ damage gradually decreased as the $O_2$ concentration was lowered. Stotzky and Goos (8) reported that $CO_2$ and $N_2$ completely inhibited the growth of soil microbiota as well as *A. niger*, *A. flavus*, *Alternaria* species, and *Penicillium* species and that 95% $CO_2$ completely inhibited growth of these fungi in soil, whereas in the present study growth occurred in this mixture. As in the present study, the inoculum was allowed to germinate before the test was started; this initial advantage in growth, in conjunction with availability of nutrients in soil vs. Czapek's medium, may be responsible for the differences between the two investigations.
### Table 2. Concentrations of O<sub>3</sub> in CO<sub>2</sub> that bring changes in colony morphology of 10 fungi

| Fungus               | Color in air                  | Furrowing of mat | Sporulation       | Color change                          | Cleistotheca formation |
|----------------------|-------------------------------|------------------|-------------------|---------------------------------------|------------------------|
| *Aspergillus niger*  | Black                         | 60 and 70        | Sparse, 10-30     | Black and brown, 70 and 80            | Absent, 30-60          |
| *A. flavus*          | Green and white               | 40-80            | Sparse, 10        | Brown, 40-60                          | Absent, 5              |
| *A. ochraceus*       | Orange-yellow and white       | 60 and 70        | Absent, 5         | Yellow-green, 20 and 30               | Absent, 5              |
| *A. ruber*           | Brown and yellow              |                  | Absent, 5-50      | White, 5 and 10                       | Absent, 10             |
| *A. repens*          | Green-yellow and translucent white |              | Sparse, 40        | Yellow-white, 60                      | Absent, 30-60          |
| *A. amstelodami*     | Blue-green                    |                  | Absent, 30        | Brown and orange, 70                  | Absent, 30-60          |
| *Penicillium brevi-compactum* | Blue-green and white | Abundant, 50-70 | Absent, 40        | White and yellow, 50 and 60           | Absent, 30-60          |
| *Alternaria tenuis*  | Gray                          | None produced in any mixtures | Absent, 5     | White, 30 and 40                      | Absent, 30-60          |
| *Cladosporium herbarum* | Black                        | Trace, 100       | Absent, 20 and 30 | Black and blue-green, 30 and 40       | Absent, 40-80          |
| *Chaetomium dolichothrum* | White                       | Trace, 100       | No color change   | White-blue-green, 10 and 20           | No color change        |
A. repens, A. ruber, and A. amstelodami, which belong to the A. glaucus group, showed similar growth responses under different mixtures of CO₂-O₂ and N₂-O₂ and when the O₂ level fell below that of air.

In analyzing the CO₂-O₂ test data, it is difficult to determine whether increasing CO₂ or decreasing O₂ of the initial mixture altered the growth of these fungi. However, when these data are compared with the N₂-O₂ data, there is little doubt that the CO₂ is the important inhibitor of growth. Although oxygen is essential for fungus growth, the minimum concentration for satisfactory growth is rather low. In general, only O₂ concentrations below 20% inhibited growth of the fungi. All species that were inhibited with high CO₂ and low O₂ mixtures resumed normal growth after exposure to air. Thus, high concentrations of CO₂ and low concentrations of O₂ are only fungistatic.

O₂ and CO₂ altered furrow development in 7 of the 10 fungi. In the N₂-O₂ test, high O₂ reduced or eliminated formation of straight furrows. The disappearance of the furrows at the lower O₂ levels was associated with reduced growth. In the CO₂-O₂ test, furrow formation was affected by both O₂ and CO₂. The concentration of CO₂ had a marked effect on furrow formation and overcame the inhibiting effect of O₂.

The colony color of the eight species that produced spores was directly related with the amount of colored spores, pigmented mycelium, and cleistothecia. These characteristics were in turn influenced by different levels of O₂ and CO₂. In the N₂-O₂ mixture, when O₂ became a limiting factor, some of the conidial heads that were formed were devoid of color. In general, the growth was composed entirely of vegetative mycelium at the lowest O₂ concentrations permitting growth, as Follstad (2) reported for his isolates of A. tenuis and Cladosporium herbarum. Aspergillus flavus produced extensive vegetative mycelium at high O₂ levels, which may be a result of an early physiological maturity of the culture. In the CO₂-O₂ tests, A. ochraceus cultures became vegetative at 60% CO₂ and the others before their growth was inhibited. Both low O₂ and high CO₂ tensions, therefore, affect conidial head formation and its coloration. The effect of the 80% N₂-20% O₂ mixture was similar to the effect of air. There were more colorless conidial heads in the A. niger and A. flavus cultures at 80% N₂-20% O₂ than were present in the corresponding air control.

Tobacco leaves are a natural substrate for many microorganisms and are exposed to soil and air contaminants from the time the seeds germinate until the cured leaves are made into cigarettes. Few, if any, tobacco leaves are devoid of leaf spots at the time of harvest. In addition, cured tobacco leaves are highly hygroscopic, and the moisture content of tobacco therefore is often favorable for the growth of contaminating fungi and bacteria. Many of these organisms are killed by heat during flue-curing but, inevitably, as the tobacco is handled, the leaves become recontaminated.

If tobacco is maintained at moisture contents below approximately 12%, there is little danger of the tobacco becoming moldy. Since flue-cured tobacco, as it is offered for sale today, may range in moisture content from 12% to 25% or higher, it must be redried to below 12% moisture to prevent or reduce fungal growth in storage. However, if such levels of moisture cannot be maintained during storage, the results from the present study indicate that tobacco might be stored under high CO₂ or low O₂ mixture of gases that preclude growth of fungus contaminants that frequently damage the stored leaf. This practice is used successfully in Argentina and France where huge bulks of grain are stored in CO₂ (3).

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