Germination and Growth of Five Fungi in Low-Oxygen and High-Carbon Dioxide Atmospheres

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ABSTRACT

Mycelial growth of *Alternaria tenuis, Fusarium roseum, Botrytis cinerea, Cladosporium herbarum,* and *Rhizopus stolonifer* on a liquid glucose-salt medium at 19 C in atmospheres of 21, 4, 2, 1, ½, ¼, and 0% oxygen decreased linearly with decreasing O_2 concentrations below 4%. Mean per cents growth of the respective organisms at 4% oxygen, as compared to growth in air, were 31, 38, 45, 50, and 85%; at 0% oxygen, only *Rhizopus* grew significantly.

Growth of *A. tenuis, B. cinerea, R. stolonifer,* and *C. herbarum* in atmospheres of 10, 20, 30, and 45% CO_2 plus 21% O_2 decreased linearly with increasing CO_2 concentrations, and was inhibited about 50% in an atmosphere of 20% CO_2. Growth of *F. roseum,* however, was stimulated at 10% CO_2, and inhibited 50% at 45% CO_2. When the O_2 concentration was 2% and thereby limiting to growth, CO_2 at the lower levels tested stimulated growth of all the fungi except *R. stolonifer.*

Low oxygen atmospheres inhibited germination of all fungi tested. Responses to high CO_2 atmospheres, however, varied. At 16% CO_2, the germination of *R. stolonifer, B. cinerea,* and *C. herbarum* was inhibited 90%. *A. tenuis* was inhibited only at CO_2 levels higher than 32%, and *F. roseum* was stimulated by concentrations of CO_2 as high as 16%. When the oxygen concentration was 1% and thereby limiting, CO_2 at the lower levels tested stimulated the germination of all fungi except *A. tenuis.* Phytopathology 60:50-53.

Postharvest decays of perishable fruits and vegetables can be reduced by the use of low oxygen (O_2) or high carbon dioxide (CO_2) atmospheres during storage and transit (4, 7). Research in this area has emphasized decay reduction, and few studies have dealt with the effects of modified concentrations of O_2 or CO_2 in the atmosphere on the growth response of the decay-causing organisms.

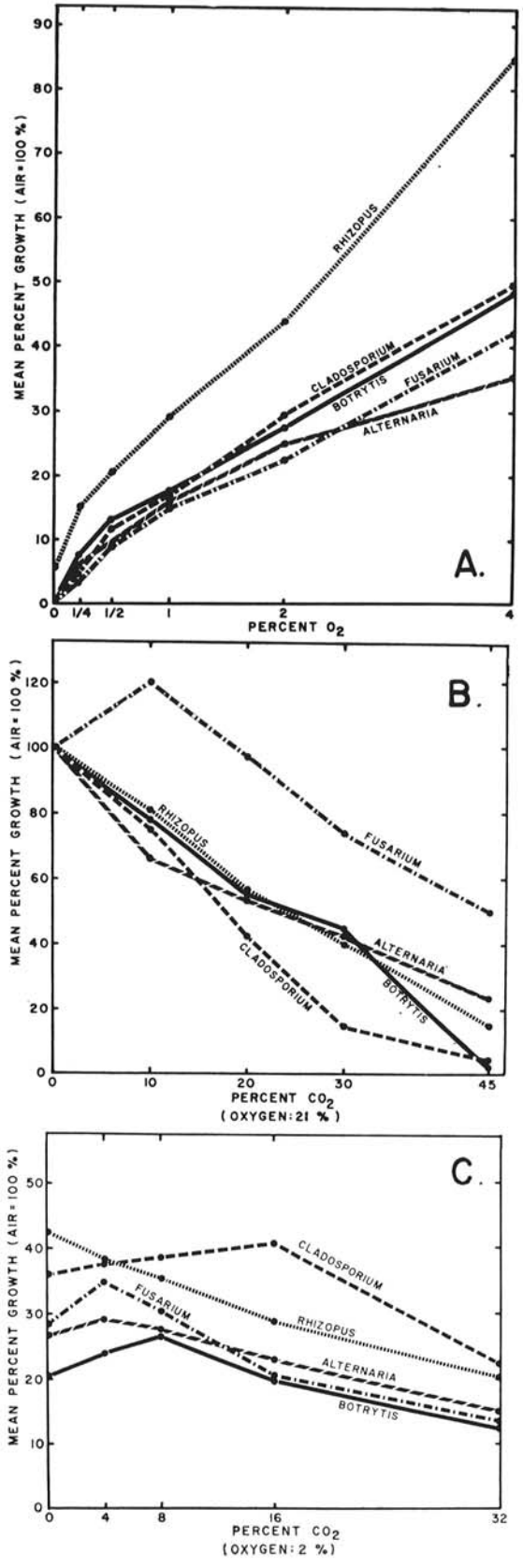
The early studies of Brown (2) and Brooks et al. (1) demonstrated the inhibitory effect of high-CO_2 atmospheres on the surface mycelial growth of *Botrytis cinerea, Rhizopus nigricans,* and other fungi. Littlefield et al. (8) observed reduction of the surface growth of three decay fungi growing in atmospheres of 2% O_2 plus 10% CO_2. Follstad (6) found that colony diam of four decay fungi cultured in vitro under low-oxygen atmospheres decreased linearly with decreasing oxygen concentrations of less than 1%. Colony diam on solid media, however, are not entirely accurate as a measurement of growth, nor do small differences in the composition of the atmosphere necessarily result in observable effects on the diffuse surface growth of the mycelia of fungi cultured on a solid medium. A similar conclusion was reached by Cochrane (3) in review of the effects of oxygen deficits on *Ophiobolus oryzae* and *Aspergillus oryzae,* and also by Fellows (5) reporting the effects of modified atmospheres on *Ophiobolus graminis.* Therefore, the increase in mass of mycelia grown in a liquid medium under continuous agitation was utilized in this study as an index of growth.

This report examines the effects of modified atmospheres on both the germination and the mycelial growth of five common decay-causing fungi.

MATERIALS AND METHODS.—The fungi selected for this study were *Alternaria tenuis* Auct. isolated from grape (*Vitis vinifer* L.) berries; *Fusarium roseum* (Lk.) emend. Snyd. & Hans. isolated from cataloupe (*Cucumis melo* L.) fruit; and *Botrytis cinerea* Pers. ex Fr., *Cladosporium herbarium* Lk. ex Fr., and *Rhizopus stolonifer* (Ehr. ex Fr.) Lind isolated from strawberry (*Fragaria chiloensis* Duchesne var. *ananássa* Bailey) fruit. The fungi were cultured on potato-dextrose agar (PDA) slants by mass spore transfer and were stored at 4 C.

Fungi were grown in the dark at 19 C in an artificial medium under continuous agitation. Each liter of medium contained the following: glucose, 20.0 g; NH_4NO_3, 1.0 g; KH_2PO_4, 2.04 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; $CaCl_2$, 0.11 g; and 2 ml each of 0.001 M $FeSO_4$, 0.001 M $ZnSO_4$, 0.0015 M Na_2MoO_4, 0.003 M $MnSO_4$, and 0.003 M $CuSO_4$. The pH was adjusted to 5.5 prior to sterilization. Fifty ml of the medium were dispensed into 200-ml Erlenmeyer flasks equipped with a rubber stopper having a gas inlet and outlet. The medium was autoclaved for 15 min at 121 C at 15-lb. pressure, after which the flasks were inoculated with 0.2 ml of a standardized spore suspension containing approximately 5×10^5 spores/ml, as estimated with a hemocytometer. Under normal aeration at 19 C, cultures of *R. stolonifer* were incubated for 24 hr; cultures of the other fungi were incubated for 48 hr. At the end of these periods, cultures were in early linear-phase growth, and the flasks were connected to lines through which flowed gas streams of the desired atmospheres.

The streams were adjusted to a flow rate of 150 ml/min for a 1-hr purging period, after which they were readjusted to 25 ml/min for the remainder of the experiments. Atmospheres were 0, ¼, ½, 1, 2, and 4% oxygen; and 0, 10, 20, 30, and 45% CO_2 plus 21% oxygen. A second series of high CO_2 atmospheres were used containing 0, 4, 8, 16, and 32% CO_2 plus 1% oxygen; and 0, 4, 8, 16, and 32% CO_2 plus 21% oxygen. The gases were premixed by the method of Uota & Garazsi (11), using oxygen and CO_2 with high-purity



nitrogen as the filler gas. Gas compositions were checked with an Orsat-type analyzer accurate to ±0.1% and the effluent gases from the culture flasks were periodically analyzed by gas chromatography to determine the concentration of oxygen and CO_2. The flow rate of the gas streams through the flasks was sufficient to maintain constant level of oxygen and CO_2.

Cultures of *R. stolonifer*, *B. cinerea*, and *F. roseum* were maintained in controlled atmospheres at 19 C for 24 hr, and the cultures of *A. tenuis* and *C. herbarum* for 48 hr. Mycelial pads were then collected on tared Whatman No. 1 filter paper discs, washed with 100 ml distilled water, oven-dried at 90 C for 24 hr, and weighed.

Germination of the fungi in modified atmospheres was studied by pipetting 0.5 ml of the standardized spore suspensions into petri dishes 55 mm in diam containing 4% water agar. The seeded dishes were immediately placed into a one-liter chamber connected to gas lines. Gas flow rates were 300 ml/min for a half-hr purging period, and 25 ml/min for the remainder of the experiment. The gases were humidified by bubbling through water prior to entering the chamber. After a modified atmosphere treatment of 16 hr in the dark at 19 C, the dishes were removed from the chambers and the spores killed and stained with 0.1% aniline blue in lactophenol diluted with an equal volume of distilled water. Germination was determined by microscopic examination of at least 250 spores in each dish. Spores were considered germinated when the germ tube equaled or exceeded spore diameter or width. Only spores that were separated from others by a distance greater than the spore diameter or length were counted.

The growth data presented in this paper are means of five replicated experiments. Standard deviations from the mean and linear regressions with respect to decreasing O_2 and increasing CO_2 concentrations were tested for significance. Germination data are means of three replicated experiments.

RESULTS.—*Growth studies.*—Mycelial growth of the fungi tested decreased linearly with decreasing oxygen concentrations below 4% (Fig. 1-A). Mean growth values at 4% oxygen, compared with growth in air, were 31% for *A. tenuis*, 38% for *F. roseum*, 45% for *B. cinerea*, 50% for *C. herbarum*, and 85% for *R. stolonifer*. At 0% oxygen, there was significant growth of *R. stolonifer* but not of the other fungi. Linear regressions of mean per cent growth values with respect to oxygen concentrations below 4% were significant at the 95% level of confidence. The regression coefficient

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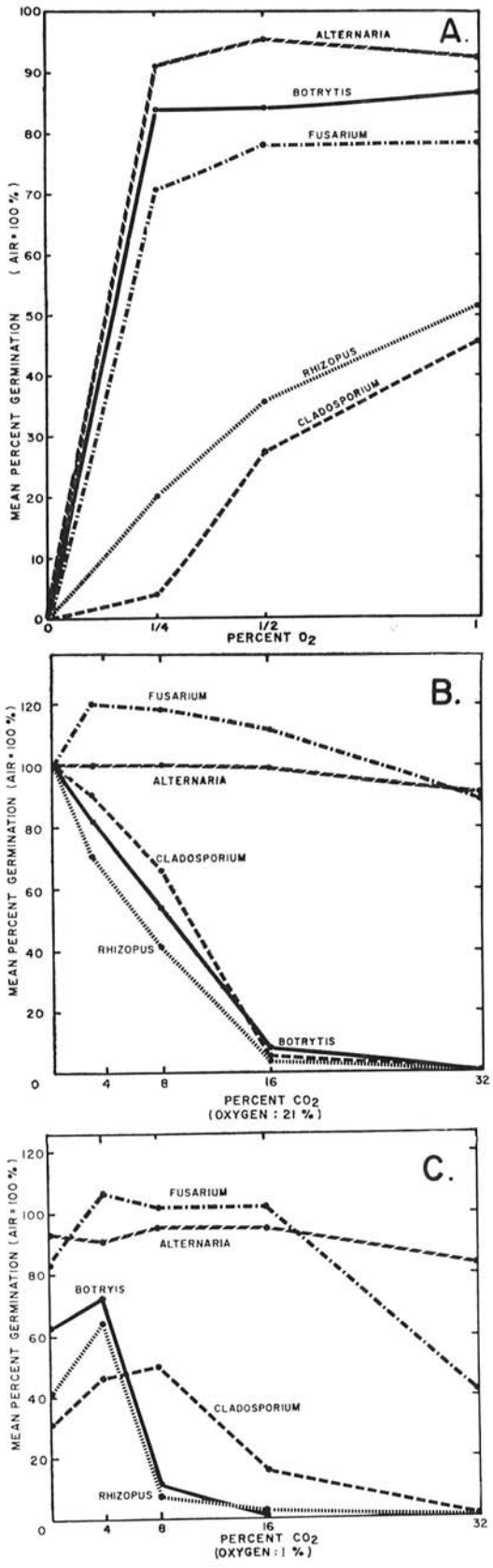
Fig. 1. Growth of *Alternaria tenuis, Botrytis cinerea, Cladosporium herbarum, Fusarium roseum,* and *Rhizopus stolonifer* in atmospheres containing **A)** different concentrations of oxygen; **B)** 21% oxygen with different concentrations of CO_2; and **C)** 2% oxygen with different concentrations of CO_2. Data are shown as mean per cents of control cultures grown in air. Fungi were cultured at 19 C on artificial liquid medium and were maintained under modified atmospheres during linear-phase growth. Growth was measured by dry wt of mycelia. Points represent means for five replicated experiments. Linear regressions for growth in low-oxygen and high-CO_2 atmospheres (oxygen: 21%) are significant at the 5% level.



for *R. stolonifer* was 4.7006 and was significantly different from those of *B. cinerea* (2.8123), *A. tenuis* (2.0691), *C. herbarum* (2.8223), and *F. roseum* (2.5626).

Higher than normal concentrations of carbon dioxide greatly inhibited mycelial growth when the oxygen level was 21% (Fig. 1-B). Growth of *A. tenuis, B. cinerea, C. herbarum,* and *R. stolonifer* decreased linearly with increasing CO_2 concentrations. Growth of *F. roseum,* however, was stimulated at 10% CO_2. Linear regressions of mean per cent growth values with respect to CO_2 concentrations above 10% were significant at the 95% level of confidence.

When the oxygen concentration was 2%, and thereby limiting to mycelial growth, CO_2 at the lower concentrations tested stimulated growth of all fungi except *R. stolonifer* (Fig. 1-C). Increases of growth peaked at 4% CO_2 for *F. roseum* and *A. tenuis,* at 8% CO_2 for *B. cinerea,* and at 16% CO_2 for *C. herbarum.* High concentrations suppressed growth. Growth of *R. stolonifer* was inhibited at all concentrations of CO_2 tested.

Germination studies.—Germination of *R. stolonifer* and *C. herbarum* in 1% oxygen was about 50% of that in air, and decreased linearly with decreasing oxygen concentrations below 1% (Fig. 2-A). *A tenuis, B. cinerea,* and *F. roseum,* however, were not greatly affected until the concentration of oxygen was ¼% or below. Mean per cent germination values at 1% oxygen were 77% for *F. roseum,* 87% for *B. cinerea,* and 93% for *A. tenuis.* At 0% oxygen, the germination of all fungi was completely inhibited.

Germination of *R. stolonifer, C. herbarum,* and *B. cinerea* decreased linearly with increasing CO_2 concentrations, and at 16% CO_2, inhibition was over 90% (Fig. 2-B). Germination of *A. tenuis* and *F. roseum* was significantly reduced by CO_2 concentrations greater than 32%. At CO_2 concentrations, less than 32% germination of *F. roseum* was stimulated.

When the accompanying oxygen level was 1% and thereby limiting, CO_2 stimulated the germination of all fungi tested except *A. tenuis* (Fig. 2-C). Stimulation peaked at 4% CO_2 for *F. roseum, R. stolonifer,* and *B. cinerea,* and at 8% CO_2 for *C. herbarum.*

Quantitative differences in germination responses to modified atmospheres were observed with different strains of *R. stolonifer, B. cinerea,* and *C. herbarum.* However, CO_2-induced stimulation occurred in all cases when oxygen was a limiting factor.

Discussion.—As measured by the increase in mass of mycelia, growth of *A. tenuis, B. cinerea, C. herbarum,* and *F. roseum* was inhibited more than 50% in 4% oxygen. *R. stolonifer* was inhibited 50% in 2% oxygen. Reports of similar reductions in mycelial growth

Fig. 2. Germination of *Alternaria tenuis, Botrytis cinerea, Cladosporium herbarum, Fusarium roseum,* and *Rhizopus stolonifer* in atmospheres containing **A)** different concentrations of oxygen; **B)** 21% oxygen and different concentrations of CO_2; and **C)** 1% oxygen with different concentrations of CO_2. Data are mean per cents of controls germinated in air. Spores were maintained in modified atmospheres 16 hr at 19 C. Points represent means for three replicated experiments.

in O_2 concentrations below 1% were based on observations of cultures grown on solid media (6, 13). Thus, the mycelial growth of fungi may well be more sensitive to low concentrations of oxygen than earlier studies suggested.

The growth response of *R. stolonifer* at all levels of oxygen tested, and its regression coefficient with respect to O_2 concentration, differed significantly from those of the four other fungi studied. These results substantiated the conclusions of Wood-Baker (13) and Wells (12) that *R. stolonifer* is capable of growth on artificial medium at lower oxygen tensions than other fungi thus far tested.

High concentrations of carbon dioxide also supressed mycelial growth. For *A. tenuis, B. cinerea, R. stolonifer,* and *C. herbarum,* growth was inhibited about 50% in an atmosphere of 20% CO_2 and 21% O_2. Equivalent inhibition of *F. herbarum* occurred in an atmosphere of 45% CO_2 and 21% O_2.

When oxygen was limiting, concentrations of CO_2 slightly higher than normal caused an increase in the growth responses of all the fungi tested except *R. stolonifer,* and in the germination of fungi except *A. tenuis.* When the oxygen level was normal, CO_2-induced stimulation of growth or germination was apparent only with *F. roseum.* These results agree with the reports of Stover & Freiberg (9) and of Toler et al. (10) regarding stimulation of the growth of *Fusarium* spp. by increases in the CO_2 concentration of the atmosphere.

It is possible that the growth of other fungi are slightly stimulated by CO_2 concentration lower than the tested minimums of 10%. When 4% CO_2 was tested with 2% oxygen, growth stimulation of *A. tenuis* and *F. roseum* was observed which would have been overlooked had the minimum CO_2 concentration been 10%. Thus, more information is needed on the effects of slight increases of CO_2 on growth.

Carbon dioxide stimulation of the growth of fungi has been attributed to CO_2 fixation (3). CO_2 enters by fixation into lactic, fumaric, citric, and other acids of the Krebs cycle which are later utilized for energy and growth. This type of energy utilization may be significant to some species of fungi when oxygen is limiting to growth, and when the CO_2 concentrations are not in themselves inhibitory.

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