Effects of Oxygen and Carbon Dioxide Tensions on Growth of Several Species of Phytophthora

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ABSTRACT

Growth of *Phytophthora capsici, P. citrophthora,* and *P. palmivora* was more rapid on sucrose-asparagine agar at 5% O_2 than at the normal atmospheric O_2 concentration; maximum radial growth of *P. parasitica* occurred in air. Growth of all species on the solid medium was reduced at O_2 levels below 5%. With the exception of *P. palmivora,* the growth of the other *Phytophthora* species in a sucrose-asparagine liquid medium decreased with decreasing O_2 concentrations below that of air.

Growth on the solid medium was stimulated by the addition of 5% CO_2 when the O_2 level was 1%, but growth of most isolates in liquid or solid media was reduced by CO_2 concentrations above 5% in comparison to growth at the same O_2 level without added CO_2. At 15% CO_2 plus 20% O_2, growth in liquid or solid media was reduced to two-thirds of the control. Phytopathology 61:787-791.

The O_2 and CO_2 concentrations in living plant tissues or in plant debris in soil in which species of *Phytophthora* may grow or survive usually differ significantly from those of the normal atmosphere of air. Since soil or plant tissues such as wood or fruits may contain concentrations of O_2 as low as 1% and CO_2 concentrations in excess of 10 to 20% (2, 3, 17, 19, 20, 21, 22, 26), it is of interest to determine the effects of similar concentrations of O_2 and CO_2 upon growth of *Phytophthora* spp.

While considerable research has been conducted on the influence of O_2 on growth and pathogenicity of several species of *Phytophthora* (6, 7, 8, 15, 23, 24, 29), little work has been reported on the effects of CO_2 on the growth of these plant pathogens (8, 9). Spence (22) observed that the growth of *P. palmivora* on carrot agar was not significantly affected by O_2 concentrations ranging from 2 to 80%. While the growth of three isolates of *P. cactorum* in potato-dextrose broth diminished with decreased O_2 concentrations, the growth at 5 to 10% O_2 was similar to that under normal atmospheric O_2 (6). Klotz et al. (15) reported that growth of *P. citrophthora* and *P. parasitica* in potato-dextrose yeast-extract broth was directly proportional to the concentrations of O_2 supplied, and good growth occurred even under 1.6% O_2. In contrast to the observations of Covey (6) and Klotz et al. (15), Dukes & Apple (8) reported that *P. parasitica* var. *nicotianae,* the causal organism of black shank of tobacco (*Nicotiana tabacum*), grew more rapidly on oatmeal agar under 6% O_2 than under air. When compared to the air control, growth was not significantly reduced in CO_2 concentrations below 15%, but little growth occurred under 99% CO_2 or under 0.1% O_2. Durbin (9) noted that in 20% CO_2 the linear growth of *P. cactorum* was inhibited by 25%, while that of *P. cinnamomi* was not inhibited.

The ability of species of *Phytophthora* to attack roots in deep or poorly aerated soil has been partially attributed to their tolerance to soil atm containing low O_2 and high CO_2 concentrations. Concentrations of CO_2 in excess of 15% and of O_2 lower than 5% in the gaseous phase of tobacco soils for a period of 5 weeks did not significantly alter the inoculum potential of *P. parasitica* var. *nicotianae* as compared with the inoculum potential in soils with an atm of normal air (8). *Phytophthora cinnamomi* was able to attack equally well roots of pine (*Pinus echinata* and *P. taeda*) seedlings grown in containers of soil kept at soil atm of normal air or 1.5% O_2 plus 10.1% CO_2 (28). Stolzy et al. (23) demonstrated that root decay of *Citrus sinensis* caused by *P. parasitica* or *P. citrophthora* was most severe in soils with limited oxygen supplies. Curtis & Zentmyer (7) found that *P. cinnamomi* was able to attack the roots of avocado (*Persea americana*) seedlings in nutrient solutions containing an O_2 concentration as low as 0.05 to 0.5 ppm. Stolzy et al. (24) also observed that *P. cinnamomi* was able to infect avocado seedlings in soil containing low O_2 concentrations. These studies on aeration effects upon the growth and development of selected species of *Phytophthora* indicate that these fungi are capable of growing, surviving, and causing disease in environments with relatively low O_2 and high CO_2. The objective of this investigation was to determine the effects in vitro of various concentrations of O_2 and CO_2 on the growth of several species of *Phytophthora.*

MATERIALS AND METHODS.—The cultures studied were obtained from the collection of *Phytophthora* species of the Department of Plant Pathology, University of California, Riverside, and were isolates P-504 and P-505 of *P. capsici* Leonian isolated from pepper (*Capsicum annuum* L.), P-479 of *P. citrophthora* (R. E. Sm. & Br.) Leonian isolated from lemon (*Citrus limon* [L.] Burm.), P-253 and P-255 of *P. palmivora* (Butl.) Butl. isolated from cacao (*Theobroma cacao* L.), and T-131 of *P. parasitica* Dast. isolated from sweet orange (*Citrus sinensis* [L.] Osbeck).

The synthetic medium used for growth studies was modified from that of Erwin & Katznelson (10) and contained per liter: L-asparagine, 2.0 g; sucrose, 15 g; β-sitosterol, 0.03 g; 2-(N-morpholino)-ethanesulfonic acid (MES) as the buffer (12), 5.3 g; thiamine hydrochloride, 0.001 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $FeSO_4 \cdot 7H_2O$, 0.001 g; KH_2PO_4, 0.5 g; $CaCl_2 \cdot 2H_2O$, 0.01 g; and 1 ml of a minor element mixture which provided, in the final solution, 1 ppm of Zn ($ZnSO_4 \cdot 7H_2O$) and 0.02 ppm each of Cu ($CuSO_4 \cdot 5H_2O$), Mo ($NaMoO_4 \cdot 2H_2O$), and Mn ($MnCl_2 \cdot 4H_2O$). All ingredients except sucrose and β-sitosterol were dissolved in 775 ml of deionized water, the pH adjusted to 6.2 by titration with 6 N KOH, and the final volume brought to 800 ml. Beta-sitosterol was dissolved in 40 ml of dichloromethane, which evaporated upon autoclaving, and added to the medium. Sucrose was added to 200 ml of deionized water in a separate flask, and the components of the liquid medium (LM) were mixed after autoclaving at 121 C at 15-lb. pressure for 12 min. For linear growth studies on solid medium (SM), Difco purified agar (15 g/liter) was added to the medium prior to autoclaving. The single zoospore stock cultures were maintained on SM.

For studies on growth in stationary liquid medium, 15 ml of LM were dispensed into a 250-ml Erlenmeyer flask equipped with a rubber stopper having a gas inlet and outlet. Each of three flasks, joined by amber latex tubing, was inoculated with a 7-mm plug from the margin of a 7-day-old culture. A line through which flowed a stream of gas of the desired atm at 20 ml/min was connected to each of six series of three flasks. The effects of atm containing different concentrations of O_2 and CO_2 were evaluated by comparison to air (20.9% O_2, 0.03% CO_2, and 78.1% N_2) at the same flow rate. All flasks were kept at room temperature (22-25 C) in constant fluorescent light (200 ft-c at the level of the cultures) for 6 days. Mycelial mats were collected on tared Whatman No. 42 filter paper discs, washed with 100 ml of deionized water, oven-dried at 60 C for 24 hr, and weighed.

For radial growth studies, a 7-mm plug of inoculum was placed in a 100 × 15 mm petri dish containing 15 ml of SM. The lids were removed from the dishes and the open dishes were aseptically placed in a sterile 24.5 liter plexiglass chamber fitted with glass wool filters at the inlet and outlet. The chambers were aerated with the desired atm at 100 ml/min. After 4 days of incubation at 25 C in constant fluorescent light, growth was assessed by measuring the diam of the colonies.

Atmospheres used for studies on growth in LM contained 0, 5, 15, 30, or 45% CO_2 plus 1, 5, or 20% O_2. For studies on radial growth, the fungi were exposed to atm of 0, 5, 15, 45, or 80% CO_2 plus 1, 5, or 20% O_2. Gases were either purchased as mixtures of O_2, CO_2, and N_2 from the Matheson Co., or were premixed from O_2, CO_2, and high purity N_2 with a series of microflow valves and flowmeters (19). Constant gas flows of 20 ml/min for studies on growth in LM were obtained by using a capillary flowmeter apparatus (19) similar to the manifold developed for respiration studies on fruits by Claypool & Keefer (4). Effluent gases from the culture flasks or plexiglass chambers were periodically analyzed by gas chromatography to determine the concentrations of O_2, CO_2, and N_2. The flow rates of the gas streams through the flasks or chambers were sufficient to maintain constant levels of O_2 and CO_2. The data presented in this paper are means of three replicated experiments.

RESULTS.—Dry weights of *P. parasitica* (T-131), *P. citrophthora* (P-479), and *P. capsici* (P-504 and P-505) decreased as the O_2 concentration in O_2-N_2 atm in equilibrium with LM decreased from that in air (Fig. 1-A, B, C). Growth of *P. palmivora* (P-253 and P-255) in LM was as great at 5% O_2, and growth of P-255, but not of P-253, was greater at 1% O_2 than at 20% O_2.

With few exceptions, dry weights of all isolates decreased with increasing CO_2 concentrations. The growth of P-505 in atm containing 1, 5, or 20% O_2 was stimulated by the addition of CO_2; P-505 was more tolerant to high CO_2 levels than were the other isolates (Fig. 1-A, B, C). The addition of 5% CO_2 allowed as good or better growth of P-504 at 1% O_2 or 20% O_2, and of T-131 and P-479 at 5% O_2 as in the corresponding atm without added CO_2. All isolates were inhibited 50% or more at 1% O_2 plus 15% CO_2. The final pH of the culture filtrates after 6 days of growth varied less than 0.5 unit from the original pH of 6.2.

Areas of growth of all of the isolates on SM at 1% O_2 without added CO_2 were at least 50% of the air controls (Fig. 1-D, E, F). Isolates P-255 and P-505 grew as rapidly in an atm containing 1% O_2 as in air. All of the isolates except T-131 grew more rapidly at 5% O_2 without added CO_2 than at the atmospheric O_2 concentration.

Stimulation of growth by low concentrations of CO_2 was greater on SM than on LM. Growth of all isolates on SM was greater at 1% O_2 plus 5% CO_2 than at 1% O_2 without CO_2 (Fig. 1-D). Although growth was not as compact in an atm containing 1% O_2 and 5% CO_2 as in air, the areas covered by all isolates except T-131 and P-253 were greater at 1% O_2 plus 5% CO_2 than in the controls (Fig. 1-D). Isolates P-504 and P-505 of *P. capsici* grew more rapidly in atm containing 1% O_2 plus up to 30% CO_2 than they did in air. While growth at 5% O_2 typically decreased with increasing concentrations of CO_2, areas of growth of all isolates except T-131 were greater at 5% O_2 plus 5% CO_2 than in air (Fig. 1-E). The addition of 5% CO_2 to 20% O_2 stimulated the growth of P-255, P-504, and P-505 (Fig. 1-F). The final pH of the cultures of SM varied less than 0.3 unit from the original pH of 6.2 after 4 days.

DISCUSSION.—While the growth of *Phytophthora* species in liquid and agar media is reduced by low O_2 tensions, growth is maintained in an atm containing O_2 concentrations as low as 1%. Thus, the O_2 levels in environments such as plant tissues and plant debris in water-logged soils, in which these fungi may be found, would rarely reach levels at which growth would be completely inhibited.

Recently, Covey (6) discussed the differences between the effects of O_2 on the growth of *Phytophthora*

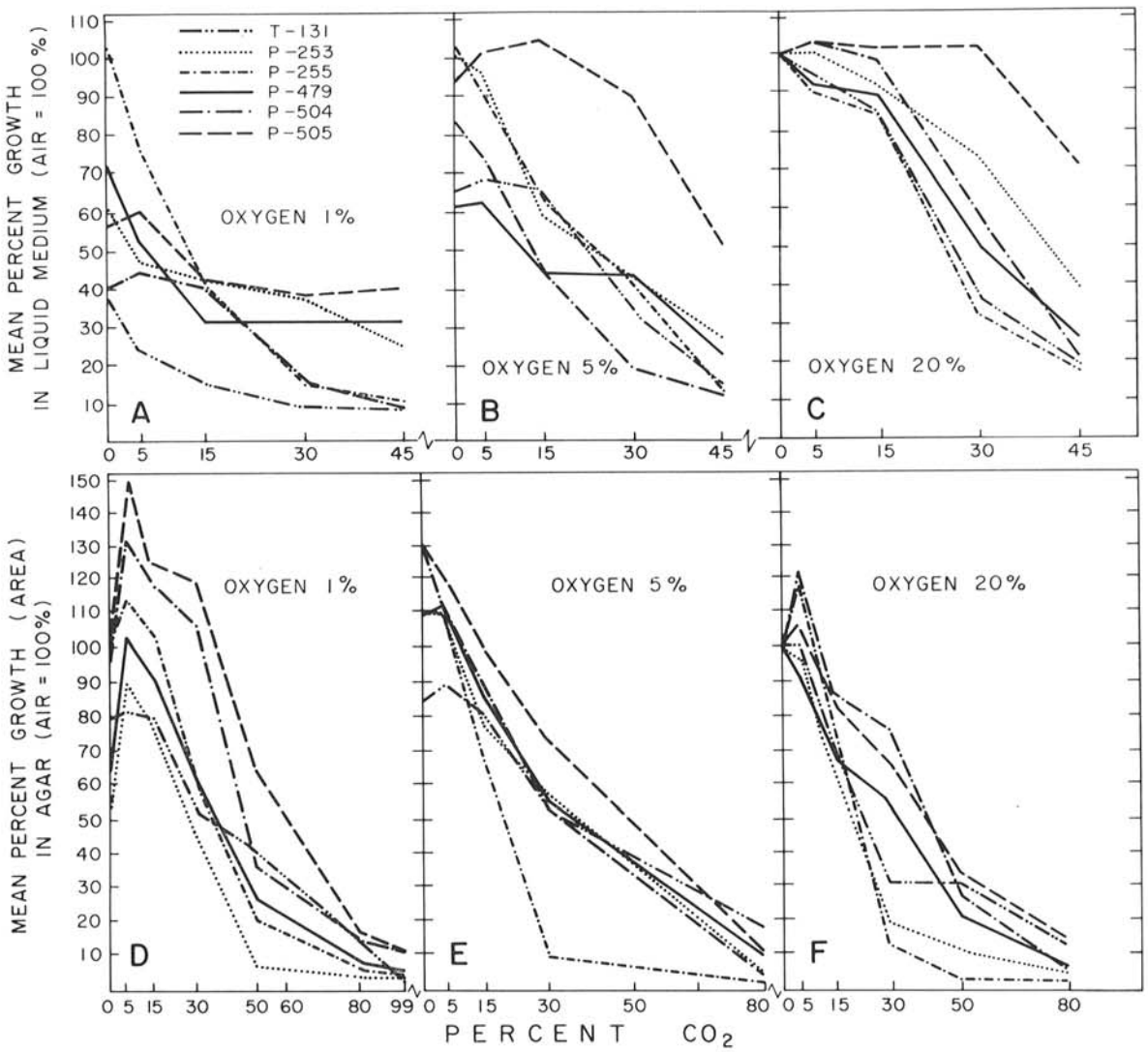

Fig. 1. A, B, C) Growth (dry wt) in sucrose-asparagine liquid medium as mean per cent of control cultures grown in air of *Phytophthora capsici* (P-504 and P-505), *P. citrophthora* (P-479), *P. palmivora* (P-253 and P-255), and *P. parasitica* (T-131) in atm containing **A)** 1% O_2 with different concentrations of CO_2; **B)** 5% O_2 with different concentrations of CO_2; and **C)** 20% O_2 with different concentrations of CO_2. **D, E, F)** Growth (area) on sucrose-asparagine agar as mean per cent of control cultures grown in air of *Phytophthora capsici* (P-504 and P-505), *P. citrophthora* (P-479), *P. palmivora* (P-253 and P-255), and *P. parasitica* (T-131) in atm containing **D)** 1% O_2 with different concentrations of CO_2; **E)** 5% O_2 with different concentrations of CO_2; and **F)** 20% O_2 with different concentrations of CO_2.

species as reported by Dukes & Apple (8), who observed maximum growth of *P. parasitica* var. *nicotianae* at 5% O_2, and as reported by Klotz et al. (15), who noted that the growth of *P. citrophthora* and *P. parasitica* was maximum at 21% O_2. Covey (6), whose work with *P. cactorum* supported that of Klotz et al. (15), speculated that perhaps the difference in growth patterns was the result of the utilization of static conditions by Dukes & Apple (8) as compared to the constant flow systems employed by Klotz et al. (15) and by himself. The question posed by Covey (6) as to whether the differences in growth patterns observed in these different studies were due to differences in techniques or to difference in the organisms tested can be partially answered by the results of this study. Since the same isolates of *Phytophthora* species were tested in two media which differed only in the addition of agar for radial growth experiments, the difference in growth patterns in this study, and probably between those discussed by Covey (6), can be attributed to the use of agar or liquid media and not to the difference in organisms or to the use of static or constant-flow conditions.

It is not known why *Phytophthora* species usually have opt growth rates at 5% O_2 when grown on agar, but have opt growth rates at higher O_2 tensions in liquid medium. When grown on agar, mycelium is more exposed to the atm than is the submerged colony in the

liquid medium. However, since only 15 ml of liquid medium per 250-ml flask were used in this study, the upper surface of the mat was always exposed to the atm. Another consideration in relation to differential growth patterns in liquid or solid substrates is that perhaps inhibitory compounds accumulate at decreasing O_2 levels and that these compounds cannot diffuse as rapidly through agar as through liquid.

The only report which compares the effects of different concentrations of CO_2 on the growth of a species of *Phytophthora* was supplied by Dukes & Apple (8), who observed growth of *P. parasitica* var. *nicotianae* under five different combinations of O_2, CO_2, and N_2. Since no combinations of O_2 and CO_2 with less than 50% CO_2 added to 1 to 10% O_2 were tested, the results cannot be discussed in relation to the stimulation of growth observed with low CO_2 levels combined with 1 or 5% O_2 in this study. Their work (8), however, showed that CO_2 concentrations of 5% or more in combination with 16% or less O_2 were inhibitory to the fungus growing on oatmeal agar. Linear growth of colonies, as compared with the air control, was reduced by one-third after 7 days at 16% O_2 plus 15% CO_2, and by nearly two-thirds at 10% O_2 plus 50% CO_2. Durbin (9) observed that when compared to growth in air, the linear growth of *P. cinnamomi* was not inhibited, but that linear growth of *P. cactorum* was inhibited 25% on Czapek's agar in equilibrium with an atm containing 20% O_2 and 20% CO_2. Since thiamine, which is required for growth of *Phytophthora* spp., was not included in the medium, the growth rates of *P. cactorum* and *P. cinnamomi* observed by Durbin (9) may have been greatly affected by poor growth of these fungi even in air. On SM in equilibrium with a gaseous phase containing 20% O_2 and 15% CO_2, the areas covered by the isolates examined in this study were reduced in comparison to the air checks as follows: T-131 of *P. parasitica,* 31%; P-253 of *P. palmivora,* 39%; P-255 of *P. palmivora,* 28%; P-479 of *P. citrophthora,* 32%; P-504 of *P. capsici,* 14%; and P-505 of *P. capsici,* 17%. Thus, the few studies that have dealt with the inhibitory effects of CO_2 on linear growth have shown that CO_2 concentrations of 15 to 20% in atm containing O_2 levels similar to that of air do not reduce the growth of any species of *Phytophthora* by much more than one-third. *Phytophthora capsici* in this study and *P. cinnamomi* in Durbin's (9) work were more tolerant of these levels of O_2 and CO_2 than were the other species tested. None of the species in this study was inhibited more than 20% at 15% CO_2 plus 20% O_2 when grown in LM.

While the stimulative effect of low levels of CO_2, especially in combinations with O_2 concentrations lower than that of air, on the growth of *Phytophthora* species on agar has not been previously reported, at least one study pointed to a stimulative role for a gas that may have been CO_2. The stimulation of growth of *P. citrophthora* on Czapek's agar or water agar that Bittancourt (1) attributed to a volatile produced by *Mucor spinosus* in a sealed desiccator system could have been caused by a reduction in O_2, an increase in CO_2, or a combination of these gas changes in the desiccators. Radial growth of *P. citrophthora* in the present study was more rapid on SM agar at 5% O_2 plus 5% CO_2 than it was in air.

The most pronounced stimulation of growth of *Phytophthora* species by CO_2 occurred on SM in an atm containing 1% O_2 plus 5% CO_2. Growth of all of the isolates was greater at 1% O_2 plus 5% CO_2 than at 1% O_2 without added CO_2; *P. capsici, P. citrophthora,* and P-255 of *P. palmivora* grew faster at 1% O_2 plus 5% CO_2 than in air. Both isolates of *P. capsici* were stimulated by high CO_2 levels in atmospheres containing 1% O_2; P-504 and P-505 grew faster at 1% O_2 plus 30% CO_2 than in air. The growth of other fungi has been reported to be stimulated by low levels of CO_2 (2, 5, 13, 16, 18, 25, 26, 27).

When the same isolates tested on SM were grown in LM in equilibrium with gaseous phases containing different concentrations of CO_2 plus 1, 5, or 20% O_2, most of the isolates were inhibited with increasing CO_2 concentration. Isolate P-505 of *P. capsici,* however, was slightly stimulated by the addition of 5% CO_2 to atm containing 1, 5, or 20% O_2 in comparison to growth at the corresponding O_2 levels without CO_2. Other workers have also observed differences in CO_2 stimulation of growth when fungi are grown at different CO_2 tensions on agar or in liquid media (2, 11, 14, 25).

While dry weight is a better estimate of total growth, the importance of linear growth as an evaluation of the ability of fungi to traverse distances should not be overlooked. The ability of P-505, for instance, to grow more rapidly at 5% O_2 or at 5% O_2 plus 5% CO_2 than in air or in an atm containing 1% O_2 plus 5% CO_2 than in any other gas mixture tested indicates that, despite an actual decrease in total mass of growth at the lower O_2 and higher CO_2 levels, the pathogen is able to grow over a greater area because of more diffuse growth under adverse conditions. Thus, the growth of species of *Phytophthora* into healthy tissue from infected tissue may be stimulated by higher CO_2 and lower O_2 concentrations in the infected tissue as compared to the healthy tissue. While evidence to support this possibility has been provided by work that has demonstrated that infections may be more extensive in plant organs held in low O_2 atm than in air (22, 28), the effects of CO_2 accumulation or O_2 depletion in plant infection sites upon the growth of the pathogen and development of the infection are poorly understood.

The utilization of MES in the media provided a buffer that effectively stabilized the pH at a concentration of the dipolar ionic amino acid that was not toxic to the species of *Phytophthora* tested. Since the pH of the cultures was stable, the suppression of growth by CO_2 was due to a direct effect on the fungi and not to an increase in the hydrogen ion concentration.

Some of the differences between isolates in relation to high CO_2 or low O_2 levels may relate to the use of constant temperature and nutritional conditions. For a more thorough examination of the effects of O_2 and CO_2 upon growth, each isolate should be incubated at its opt temperature in the most favorable medium for growth.

LITERATURE CITED

1. Bittancourt, A. A. 1951. Stimulation of growth of Phytophthora citrophthora by a gas produced by Mucor spinosus. Science 113:531.
2. Burges, A., & E. Fenton. 1953. The effect of carbon dioxide on the growth of certain soil fungi. Brit. Mycol. Soc., Trans. 49:387-396.
3. Chase, W. W. 1934. The composition, quantity and physiological significance of gases in tree stems. Minn. Agr. Exp. Sta. Tech. Bull. 99. 51 p.
4. Claypool, L. L., & R. M. Keefer. 1942. A colorimetric method for CO_2 determination in respiration studies. Amer. Soc. Hort. Sci. Proc. 40:177-186.
5. Cochrane, V. W. 1958. Physiology of fungi. John Wiley & Sons, Inc. N.Y. 524 p.
6. Covey, R. P. 1970. Effect of oxygen tension on the growth of Phytophthora cactorum. Phytopathology 60:358-359.
7. Curtis, D. S., & G. A. Zentmyer. 1949. Effect of oxygen supply on Phytophthora root rot of avocado in nutrient solution. Amer. J. Bot. 36:471-474.
8. Dukes, P. D., & J. L. Apple. 1965. Effect of oxygen and carbon dioxide tensions on growth and inoculum potential of Phytophthora parasitica var. nicotianae. Phytopathology 55:666-669.
9. Durbin, R. D. 1959. Factors affecting the vertical distribution of Rhizoctonia solani with special reference to CO_2 concentration. Amer. J. Bot. 46:22-25.
10. Erwin, D. C., & H. Katznelson. 1961. Studies on the nutrition of Phytophthora cryptogea. Can. J. Microbiol. 7:15-25.
11. Fellows, H. 1928. The influence of oxygen and carbon dioxide on the growth of Ophiobolus graminis in pure culture. J. Agr. Res. 37:349-355.
12. Good, N. E., D. Winget, W. Winter, T. N. Connolly, S. Izawas, & R.M.M. Singh. 1966. Hydrogen ion buffers for biological research. Biochemistry 5:467-477.
13. Hollis, J. P. 1948. Oxygen and carbon dioxide relations of Fusarium oxysporum Schlecht and Fusarium eumartii Carp. Phytopathology 38:761-775.
14. Jensen, K. P. 1967. Oxygen and carbon dioxide affect the growth of wood-decaying fungi. Forest Sci. 13: 384-389.
15. Klotz, L. J., L. H. Stolzy, & T. A. Dewolfe. 1963. Oxygen requirement of three root-rotting fungi in a liquid media. Plant Dis. Reptr. 46:606-608.
16. Lockhart, C. L. 1968. Influence of various carbon dioxide and oxygen concentrations on the growth of Fusarium oxysporum in vitro. Can. J. Plant Sci. 48:451-453.
17. Macauley, B. J., & D. M. Griffin. 1969. Effects of carbon dioxide and oxygen on the activity of some soil fungi. Brit. Mycol. Soc. Trans. 53:53-62.
18. Macauley, B. J., & D. M. Griffin. 1969. Effect of carbon dioxide and the bicarbonate ion on the growth of some soil fungi. Brit. Mycol. Soc. Trans. 53:223-228.
19. Mitchell, D. J. 1970. The effects of oxygen and carbon dioxide on growth and sporulation of several species of Phytophthora. Ph.D. Thesis, Univ. Calif., Riverside. 79 p.
20. Magness, J. R. 1920. Composition of gases in intercellular spaces of apples and potatoes. Bot. Gaz. 70:308-316.
21. Russell, E. W. 1961. Soil conditions and plant growth [9th ed.]. Longmans, London. 635 p.
22. Spence, J. A. 1961. Black pod diseases of cacao. II. A study in host-parasite relations. Ann. Appl. Biol. 49:723-724.
23. Stolzy, L. H., J. Letey, L. J. Klotz, & C. K. Labanauskas. 1965. Water and aeration as factors in root decay of Citrus sinensis. Phytopathology 55:270-275.
24. Stolzy, L. H., G. A. Zentmyer, L. J. Klotz, & C. K. Labanauskas. 1967. Oxygen diffusion, water, and Phytophthora cinnamomi in root decay and nutrition of avocados. Amer. Soc. Hort. Sci. Proc. 90:67-76.
25. Tabak, H. H., & W. B. Cooke. 1968. The effects of gaseous environments on the growth and metabolism of fungi. Bot. Rev. 34:126-252.
26. Thacker, D. G., & H. M. Good. 1952. The composition of air in trunks of sugar maple in relation to decay. Can. J. Bot. 30:475-485.
27. Wells, J. M., & M. Uota. 1970. Germination and growth of five fungi in low-oxygen and high carbon-dioxide atmospheres. Phytopathology 60:50-53.
28. Wilson, E. M. 1960. Physiology of an isolate of Fusarium oxysporum f. cubense. Phytopathology 50: 607-612.
29. Zak, B. 1961. Aeration and other soil factors affecting southern pines as related to littleleaf disease. USDA Forest Service Tech. Bull. 1248. 30 p.