Growth of Erwinia carotovora, E. atroseptica and Pseudomonas fluorescens in Low Oxygen and High Carbon Dioxide Atmospheres

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ABSTRACT

Growth of six isolates of *Erwinia carotovora, E. astroseptica,* and *Pseudomonas fluorescens* on a buffered asparagine-yeast extract broth at 21 C in atmospheres of 21, 3, 1,0.50, 0.25, and 0% O_2 + 3% CO_2 decreased linearly with the logarithm of decreasing O_2 concn. Mean percent growth at 3% O_2 ranged between 54 and 64% of that in air, and at 0% O_2 growth was between 3 and 12.5% of that in air. In the absence of CO_2, *Erwinia* spp. did not grow at any concn of O_2 within a 24 h experimental period, and the growth of four of six isolates of *P. fluorescens* at 1% O_2 or below was approximately half of that in the presence of 3% CO_2.

In atmospheres containing different concns of CO_2 and either 3 or 21% O_2, the growth of *Erwinia* spp. and *P. fluorescens* was inhibited by CO_2 concns in excess of 10%. Growth in 30% CO_2 was 15 to 34% of that in air.

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Additional key words: Modified atmospheres, controlled atmospheres.

The *Erwinia* and *Pseudomonas* genera of bacteria include many species that are pathogenic to plants. These species commonly occur in the soil, and many are associated with postharvest diseases of agricultural commodities. *Erwinia carotovora* (Jones) Holland and *E. atroseptica* (Van Hall) Jennison cause soft rot of lettuce, potatoes, tomatoes, and other vegetables (1). *Pseudomonas fluorescens* Migula has been associated with pink eye disease of potato tubers (5), and its metabolic and cultural characteristics are closely related to those of *P. marginalis* Brown, the bacterium that causes a marginal leaf blight on lettuce (11).

Postharvest losses of some vegetables, due to soft-rotting bacteria, may be reduced when the commodity is stored in low oxygen (O_2) and high carbon dioxide (CO_2) atmospheres, Lipton (8) observed a significant decrease in bacterial soft rot on asparagus spears stored for 17 days at 6 C in an atmosphere of 1% O_2 compared to air. Incidence of bacterial soft rot of lettuce after 1 mo at 16.2 C (41 F) was reduced by 50% in an atmosphere of 3% O_2, and was negligible in an atmosphere of 3% O_2 + 4% CO_2 (9). Parsons and Spalding (10) noted similar effects on decay of tomatoes inoculated with *E. carotovora* and then stored in 3% O_2 and 5% CO_2.

Little is known of the effects of lowered oxygen concns on the growth of the plant pathogenic species of *Pseudomonas* and *Erwinia,* but work has been done with other genera of bacteria. Harrison and Pirt (6), using different concns of air mixed with nitrogen, found that the growth of *Klebsiella aerogenes* (Kruse) Beijernick was reduced under anaerobic conditions to 40% of that in air, and that growth increased linearly with increasing concns of O_2 up to 8% (partial pressure of 60 mm Hg in the gas phase). Dry cell weights of the highly aerobic *Serratia marcescens* Bizio vary directly with the degree of aeration in the medium (14). In the absence of O_2, aerobic bacteria may germinate, but do not grow (13). *Pseudomonas* and *Erwinia,* however are facultative

anaerobes (2) and grow without O_2 under some conditions.

Carbon dioxide has long been known to be a growth requirement for many bacteria (12), and high concns of CO_2 can be either stimulatory or inhibitory to the growth of microorganisms. King (7) reported that gas mixtures of 50% air and 50% CO_2 were stimulatory to the growth of, and decreased the generation time of *Pseudomonas aeruginosa* (Schroeter) Migula by a factor of 2 to 3. With organisms such as *Euglena* (15) and some fungi (16), however, such concns of CO_2 are inhibitory to growth. There is no information available on the effects of high concns of CO_2 on plant pathogenic bacteria.

This study describes the growth of three plant pathogenic bacteria in a medium under low concns of O_2, high concns of CO_2, and a combination of low O_2 and high CO_2 concns.

MATERIALS AND METHODS.—Cultures of *Erwinia carotovora*, isolates C14S, C9, C7, C3, E30, and E33, and of *E. atroseptica* isolates E25S, E8, E1S, E28, E27, and E29, were obtained from the collection (3) of W. L. Smith, Jr., at the USDA, Beltsville, Md. *Pseudomonas fluorescens*, isolates 13, 24, 25, 26, 31, and 32, were obtained from the collection (undescribed) of M. Huether through G. McIntyre, Univ. of Maine, Orono, Me. The bacteria were maintained in test tubes at 5 C on nutrient agar slants prepared with 5 g peptone, 3 g beef extract, and 15 g agar per liter. Actively growing starter cultures, used as inoculum for growth tubes, were initiated prior to each test by transferring with an inoculating needle bacteria from agar slants to flasks containing 10 ml nutrient broth composed of 5 g peptone and 3 g beef extract per liter. Starter cultures of *E. carotovora* and *P. fluorescens* were then incubated for 16 h at 21 C, and those of *E. atroseptica* for 24 h, at which times optical densities (OD) were between 1.2 and 2.0 units at 550 nm, and growth was near the end of the logarithmic phase. Growth tubes contained 9 ml of medium prepared with 2.25 g asparagine, 0.123 g $MgSO_4 \cdot 7H_2O$, and 3.0 g Difco yeast extract per liter of 0.02 M potassium phosphate

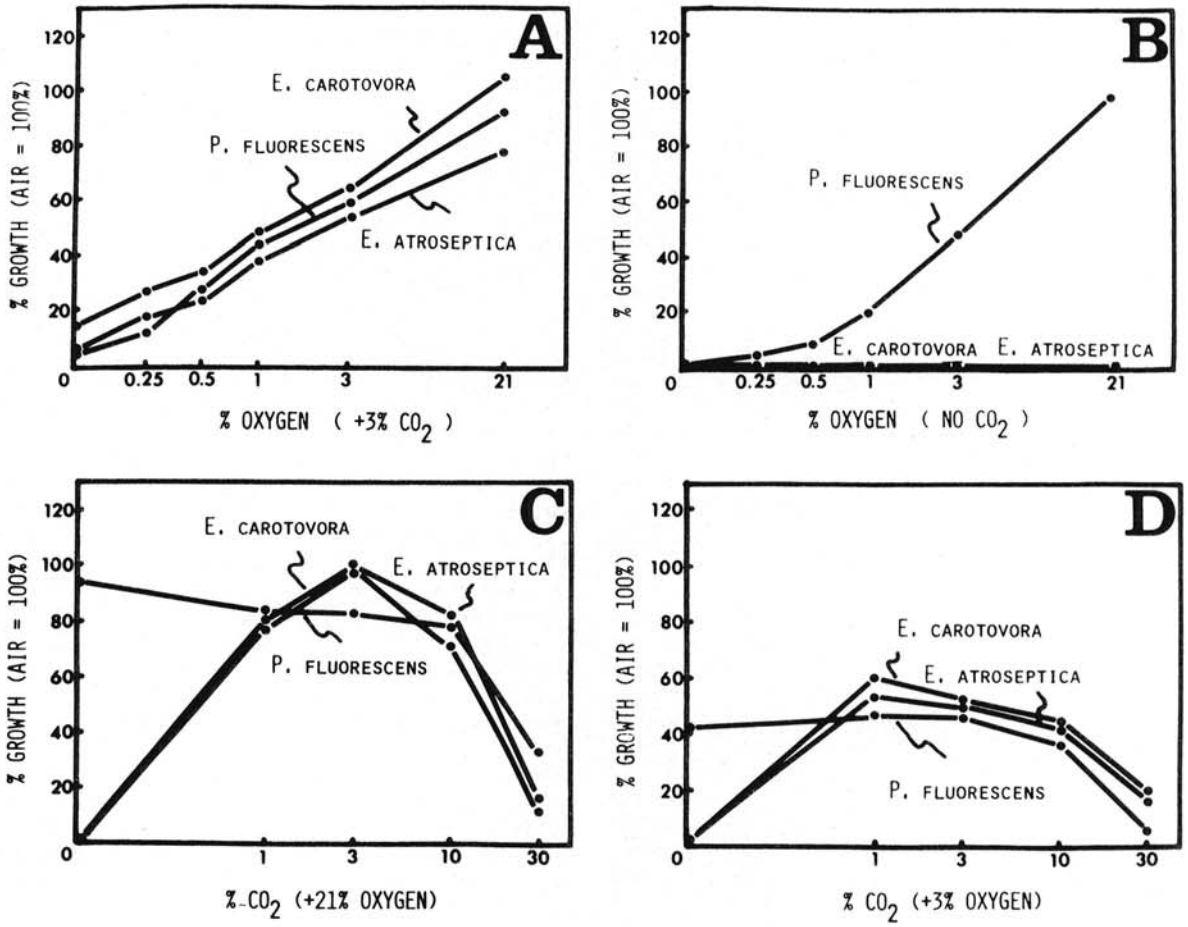

Fig. 1-(A-D). Mean percent growth, compared to air of *Erwinia carotovora*, *E. atroseptica*, and *Pseudomonas fluorescens* in **A)** different concns of $O_2 + 3\%$ CO_2; **B)** different concns of $O_2 + 0\%$ CO_2 **C)** different concns of $CO_2 + 21\%$ O_2; and **D)** different concns of $CO_2 + 3\%$ O_2. Growth was in the dark at 21 C on a buffered asparagine-yeast extract broth for 16 h for *E. carotovora*, 20 h for *P. fluorescens*, and 24 h for *E. atroseptica*. Each point represents growth averages of six isolates of each organism replicated three times, with the exception of Fig. 1-B where only four of the six isolates of *P. fluorescens* are included in the data.

buffer, pH 7.0. The medium was autoclaved in flasks or tubes for 15 min at 121 C at 1.05 kg-force/cm^2 (15 psi). Tubes were inoculated with a standard 3-mm loop-full of bacteria from starter cultures, and sealed with two-hole sterile rubber stoppers fitted with a disposable micropipette as a gas inlet and with a gas outlet. Tubing was plugged with cotton to maintain a contamination-free system. Tubes were then connected to Tygon lines through which flowed streams of the desired atmospheres. Gas streams were bubbled through the medium, at 8.3 ml per min, which was sufficient to maintain a constant level of O_2 in the medium. Atmospheres were 0, 0.25, 0.50, 1, 3, and 21% oxygen, mixed without CO_2 or with 3% CO_2; and 0, 3, 10, and 30% CO_2 mixed with 3% or with 21% O_2. Gases were premixed with high-purity N_2 as the filler gas, and compositions were checked with an Orsat-type analyzer accurate to ±0.1%. Concentrations of dissolved O_2 in the medium before and during growth were checked with an oxygen electrode. CO_2-free gases were purged through a 25 × 1.9 cm Lithasorb (Fisher Scientific Co., Fairlawn, N.J.) column to insure removal of trace levels of CO_2.

Bacterial cultures were grown in modified atmospheres in the dark at 21 C until the air checks neared the end of the logarithmic phase of growth, which occurred at 16 h for *E. carotovora*, 20 h for *P. fluorescens*, and 24 h for *E. atroseptica*. In some experiments, growth was observed for periods as long as 155 h. Growth was measured by OD readings at 550 nm. In a separate test, the pH levels of bacterial cultures were monitored and found to remain at 7.0 or above during the 24 h experimental period. Growth was expressed as percent OD of the normal atmosphere (air) checks. Data presented in this paper are means of three replications, each with six strains of each organism unless otherwise noted.

RESULTS.—*Low-O_2 atmospheres.*—In the presence of 3% CO_2, the growth of *E. carotovora*, *E. atroseptica*, and *P. fluorescens* decreased with the decreasing oxygen concn. At 3% O_2 (+3% CO_2) growth of the three organisms ranged between 54 and 64% of that in normal atmospheres (Fig. 1-A). At 0% O_2 (+3% CO_2) growth was slight; only 5% of that in air for *E. atroseptica*, 3% for *P. fluorescens*, and 12.5% for *E. carotovora*. Throughout the range of oxygen used in these tests, percent growth varied directly with the log of the oxygen concn.

Bacterial growth patterns were considerably different in the absence of CO_2 compared to those in the presence of CO_2. In an atmosphere of 21% O_2 + 0% CO_2, there was no growth in any of the isolates of *E. atroseptica* after 24 h; only trace levels of growth in isolate C3 of *E. carotovora* after 24 h; only trace levels of growth in isolate C3 of *E. carotovora* after 16 h; and growth in only isolates 24, 25, 26, and 32 of *P. fluorescens* (Fig. 1-B). Average growth of the four isolates of *P. fluorescens* in low O_2 atmospheres without CO_2 was considerably less than the average growth of the six isolates in the corresponding atmospheres with CO_2. Growth at 1% O_2 + 0% CO_2 was 20.2% of that in air, approximately half of that which occurred in the presence of 3% CO_2. At 0% O_2 there was no growth of *P. fluorescens*, while in the presence of CO_2 average growth in 0% O_2 was 3% of that in air (Fig. 1-A).

Prolonged incubation was necessary for the growth of the CO_2-requiring isolates of *E. carotovora* and *P. fluorescens* in an atmosphere of 21% O_2 + 0% CO_2. Isolate C3 of *E. carotovora* approached the end of the logarithmic phase of growth after 36 h, strain C7 after 70 h, strain E30 after 93 h, and E33 after 110 h. There was no growth of isolates C14S and C9 by 134 h. Isolate 13 and 31 of *P. fluorescens* terminated logarithmic phase of growth after 40 h. In the absence of CO_2 there was no growth in any of the isolate of *E. atroseptica* by 155 h.

High-CO_2 atmospheres.—In an atmosphere of 21% O_2, *E. carotovora*, and *E. atroseptica* were inhibited both by the absence of CO_2 and by the presence of excessive concns of CO_2. At 0% CO_2 there was no growth, and at 30% CO_2 growth was approximately 15% of that in air (Fig. 1-C). At 3% CO_2 growth was equal to that in air.

The growth of *P. fluorescens*, was not significantly affected by CO_2 concns below 10%. At 30% CO_2 (+21% O_2) growth was 34% of that in air (Fig. 1-C).

In atmospheres with 3% O_2, where oxygen was limiting to bacterial growth, the relative effect of different concns of CO_2 approximated that in atmospheres with 21% O_2. Growth rates of *Erwinia* spp. were inhibited both by the absence of CO_2 and by concns of CO_2 in excess of 10%. Maximum growth responses in 3% O_2, however, occurred at 1% CO_2 rather than at 3% CO_2 (Fig. 1-D).

Relative growth of *P. fluorescens* in atmospheres containing different concns of CO_2 was similar in oxygen-limited atmospheres as in those containing 21% O_2. Inhibition of growth occurred only when CO_2 concns exceeded 10%.

DISCUSSION.—In vitro growth of decay-causing bacteria was sensitive to the concn of O_2 in the atmosphere. Significant reductions of growth resulted from lowering the O_2 concn to between 1 and 3%, a range tolerated by many agricultural commodities in storage. The medium used in these tests does not approximate the natural substrate of stored produce. Therefore, caution should be exercised in the application of in vitro results to the inhibition of bacterial decay on stored agricultural commodities. Nevertheless, there is good agreement with the in vivo inhibition of bacterial decay on asparagus (8) and lettuce (9). A greater degree of inhibition may be possible with *Erwinia* spp. by the purging of the atmosphere of CO_2. Concentrations of CO_2 well above 10% (which are not recommended for the storage of most commodities) are necessary for significant inhibition of both *Erwinia* and *Pseudomonas*.

In this study it was assumed that the gas concns available to the bacteria in the liquid medium were the same as the concns in the gaseous phase. Previous studies with submerged growth of fungi in liquid media (16), and with surface growth on agar plates (4) under similar atmospheric conditions, indicate that growth in submerged cultures is equally if not more sensitive to changes in O_2 and CO_2 concns than surface growth.

Normal atmosphere (air) is composed of approximately 78% nitrogen, 21% O_2, 1% A, 0.03% CO_2, 0.01% H_2, and trace gases. In these tests, only the concns of nitrogen, O_2, and CO_2 were regulated. Effects of the other atmospheric components are unknown. There may be evidence in these tests, however, that their effects on bacterial growth is significant. Growth in the gas mixture 21% O_2 + 3% CO_2 most closely approximated the growth response of *Erwinia* spp. in air. A decrease in the CO_2

concn resulted in a decrease in growth. At 21% O_2 + 3% CO_2 growth was 80% of that in air, and at 0% CO_2 growth was inhibited. Normal atmosphere (100% growth response) contains only 0.03% CO_2. Thus, not only a closer examination is needed of the effects of trace levels of CO_2, but also of possible effects or interactions due to other gaseous components of the atmosphere.

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