Effect of Controlled Oxygen and Carbon Dioxide Atmospheres on Bacterial Growth Rate and Soft Rot of Tomato Fruits Caused by *Pseudomonas marginalis*

S. N. IBE, Department of Biological Sciences, University of Lagos, Akoka, Lagos, Nigeria, and R. G. GROGAN, Professor, Department of Plant Pathology, University of California, Davis 95616

ABSTRACT

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A gas mixture composed of 4% O_2, 2% CO_2, and 94% N_2 (O_2:CO_2 ratio of 4:2) reduced in vitro growth of *Pseudomonas marginalis* during the 36-hr incubation period at 12.5 C to 47% of that in air (21:0.03). At controlled atmospheres (CAs) of 4:0, 4:10, and 1:2, growth was reduced further to 36, 33, and 16% of the control, respectively. With *P. fluorescens*, overall growth was faster but was also reduced at CAs of 4:0, 4:2, 4:10, and 1:2 to 55, 54, 30, and 17% of the control in air. In ripe tomato fruits inoculated with *P. marginalis* and incubated in CAs of 4:0, 4:2, 4:10, and 1:2 and air, only CAs of 4:10 and 1:2 significantly reduced the percentage and severity of decay by the end of a 14-day storage at 12.5 C from that in air, but fruits were softened under these CAs. It is suggested, therefore, that tomato fruits be stored at 12.5 C under a CA of 4% O_2, 0% CO_2, and 96% N_2 to reduce physiological softening and bacterial soft rot by *P. marginalis*.

There are numerous reports on use of controlled atmosphere (CA) combined with low temperature to extend the shelf life of tomatoes (3,4,7,8,10,12–17). Most of the observations have been related to the control of fungal rots of tomatoes. The optimal storage temperature range for tomatoes is 10–13 C (17,19,21), whereas varied optimal O_2 and CO_2 concentrations have been suggested. It has been shown consistently, however, that very low O_2 or high CO_2 concentrations cause injury to tomatoes, observed as brown, scaldlike blemishes (15).

Earlier investigators (10) observed that a CA of 5% O_2, 5% CO_2, and 90% N_2 (5:5) at 12 C was best for retarding ripening and fungal growth. Eaves and Lockhart (4) suggested a CA of 2.5:5 at 12.7 C as best for delaying ripening and 2.5:2.5 as most effective for reducing decay incidence.

Tompkins (19) observed that after storage at temperatures below 10 C and at 15 C under more than 5% CO_2, the skins of glasshouse tomatoes became more susceptible to invasion by *Botrytis*, *Penicillium*, and *Trichothecium*. Parsons et al (15) reported that storing mature green tomatoes at 12.5 C for 6 wk in 3% O_2, 0% CO_2, and 97% N_2 (3:0) reduced decay caused by *Rhizopus* spp. and *Alternaria* spp. better than in air. In a CA of 3:3 or 3:5, however, decay incidence was not changed and CO_2 injury sometimes resulted. Also, oxygen levels of 1% and below produced off-flavors in the fruits.

Dennis et al (3) also stored mature green tomatoes under CAs of 3:3 and 3:5 for 8 wk at 13 C and relative humidity of 93–95% and observed that ripening was retarded and 3–4% of fruits developed rot, caused mainly by *B. cinerea*, under both CAs. The presence of 5% CO_2, however, did not cause injury to the fruits, contrary to Parsons et al (15).

Kader et al (8) studied the use of 4% O_2 at 20 C for a 7-day simulated transit period to retard ripening of tomatoes and observed that ripening and flavor were not affected, as judged after transfer to air. Mansfield et al (14) studied the potential for long-term storage of tomatoes, uninoculated and inoculated with *B. cinerea*, under 4:0 and 4:2 among other CAs at 12.5 C. These workers observed that these CAs had reduced decay significantly at the end of a 7-wk storage period compared with the air control and that storage under these CAs can result in fruit of acceptable visual and taste quality.

Studies on the influence of CA on bacterial soft rot of tomatoes have been carried out by only a few investigators (14,16). Parsons and Spalding (16) observed that when tomato fruits were inoculated with *Erwinia carotovora* and stored at 12.5 C for 6 days in a CA of 3:5, decay incidence was not reduced but the size of decay lesions was decreased. These workers concluded that at 12.5 C, CA was better than air in reducing bacterial soft rot of tomatoes. There is no report on the effect of CA on decay of tomato fruits inoculated with *Pseudomonas marginalis*. This bacterium, a causal agent of marginal leaf blight of lettuce (18), is also capable of inducing soft rot of tomato fruit (1). Bartz (1) reported that this pseudomonad and *P. aeruginosa* were each isolated from about 17% of decay lesions on tomatoes in a commercial Florida shipment rejected because of excessive decay. Although *Erwinia* spp. comprised 66% of the isolates, it was concluded that the pseudomonads could be primary rather than secondary organisms in naturally occurring outbreaks of bacterial rot of tomatoes.

These two pathogenic pseudomonads closely resemble, culturally and metabolically, the versatile saprophytic *P. fluorescens*, a secondary organism often associated with decay of vegetables including tomato (5,18). The effect of CA at 21 C on growth of *P. fluorescens* and *Erwinia* spp. in a buffered asparagine-yeast extract broth was studied by Wells (20). Growth decreased linearly with the logarithm of decreasing O_2 concentrations and was inhibited by CO_2 concentrations higher than 10%. The mean percentage of growth at 3% O_2 ranged between 54 and 64% of that in air. In the absence of CO_2, *Erwinia* spp. did not grow, whereas *P. fluorescens* was not affected. These observations are among the few made on the effect of lowered O_2 and high CO_2 concentrations on the growth of plant-pathogenic species of *Pseudomonas* and *Erwinia*. King (11) reported that an atmosphere of 50% air and 50% CO_2 stimulated growth of *P. aeruginosa*, decreasing the generation time of cells by a factor of 2–3.

In preliminary tests, *P. fluorescens* did not cause soft rot on inoculated breaker (light pink) tomato fruit held in air at 12.5 C. *P. marginalis* caused soft rot on inoculated pink to ripe fruit but not on green fruit at 12.5 C. *P. fluorescens*, however, grew faster than *P. marginalis* in an artificial medium at 12.5 C. This indicated that as a secondary organism, *P. fluorescens* may have the potential to accelerate decay of tomato fruit caused by such primary organisms as *Pseudomonas* spp. or *Erwinia* spp. when fruits are held at 12.5 C.

This study was undertaken to examine the effect of low O_2 and high CO_2 concentrations on in vitro growth of *P. marginalis* and *P. fluorescens* at 12.5 C and on the ability of *P. marginalis* to cause fruit decay of tomato. CAs of 4:0, 4:2, 4:10, and 1:2 were selected on the

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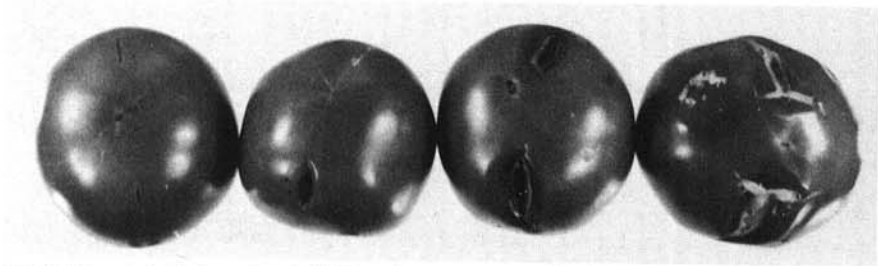

Fig. 1. Tomato fruits inoculated with *Pseudomonas marginalis* showing the various decay severity scores after 14 days at 12.5 C. Lesions were placed in one of four categories: 0 = dry spot (no infection), 1 = wet spot (slight decay), 2 = growth and some white/yellow slime (more decay), and 3 = growth with abundant white/yellow slime (most decay).

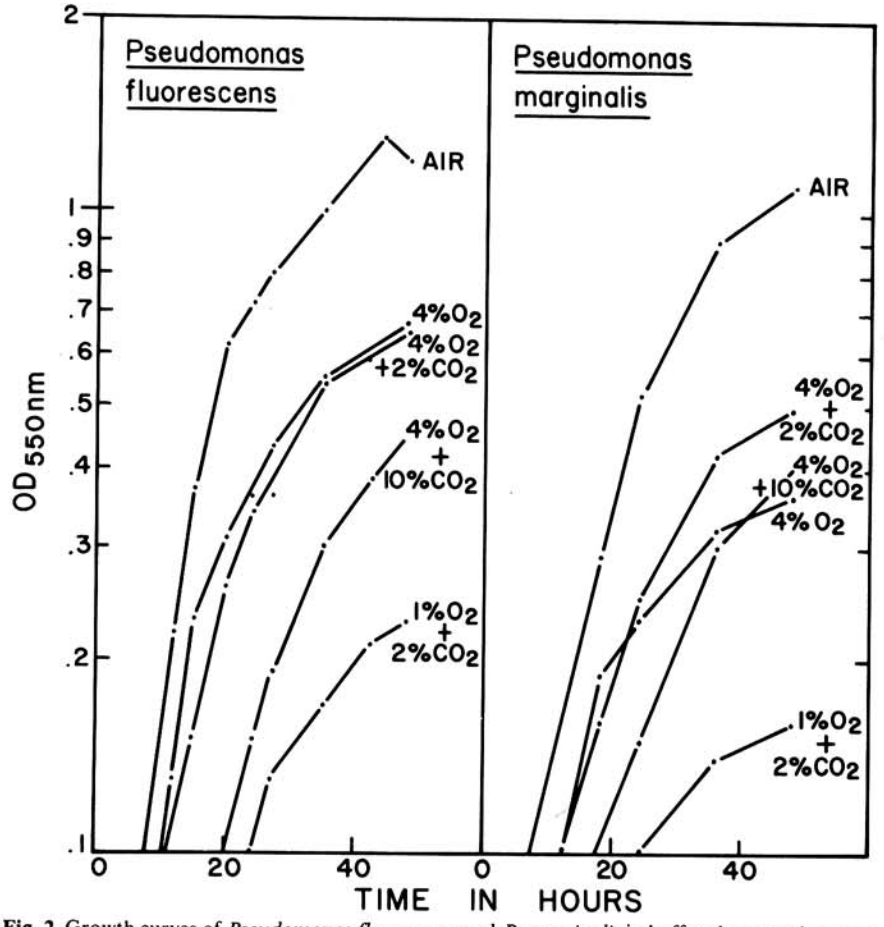

Fig. 2. Growth curves of *Pseudomonas fluorescens* and *P. marginalis* in buffered asparagine-yeast extract broth under different CA treatments at 12.5 C.

basis of observations of previous workers (8,14).

MATERIALS AND METHODS

Inoculum preparation. Cultures of *P. marginalis* (Brown) Stevens strain 8D47 and *P. fluorescens* strain 11D47 (ATCC 13525) were obtained from the collection of C. I. Kado, Department of Plant Pathology, University of California, Davis. Inoculum was obtained from cultures grown on medium 523 (9) and incubated at about 26 C for 24–48 hr. The medium contained 10 g sucrose, 8 g casein hydrolysate (Calbiochem., San Diego, CA 92112), 4 g yeast extract (Difco Laboratories, Detroit, MI 48201), 2 g K_2HPO_4, 0.3 g $MgSO_4 \cdot 7H_2O$, and 15 g Bacto agar in 1 L of water. The final unadjusted pH was within a range of 7.0–7.1. A bacterial suspension was prepared by transferring loopfuls of bacteria into 20 ml of dilute buffered saline, pH 7.0 (0.8 g NaCl, 0.2 g KH_2PO_4, and 0.2 g Na_2HPO_4 added to 1 L of glass-distilled water) (2). The suspension was mixed thoroughly with a Vortex mixer. The optical density (OD) was adjusted to 0.6 units at 600 nm on a Bausch & Lomb Spectronic 20 spectrophotometer. The suspension contained about 1×10^9 cells per milliliter.

In vitro controlled atmosphere growth tests. A buffered asparagine-yeast extract broth (11) used for growth studies contained the following ingredients per liter of 0.02 M potassium phosphate buffer at pH 7.0: 2.25 g asparagine, 0.123 g $MgSO_4 \cdot 7H_2O$, and 3 g yeast extract. One hundred-milliliter portions of broth were measured into 300-ml Erlenmeyer flasks fitted with sidearms. The flasks, sealed with two-hole rubber stoppers fitted with disposable pasteur pipettes that served as inlets and outlets for gas, were sterilized for 15 min at 121 C at 1.05 kg/cm^2. Flasks were inoculated with 1 ml of cell suspension prepared as described before and connected in pairs to polyethylene lines delivering the desired O_2:CO_2 mixtures. The gas mixture was bubbled through the medium. Growth at 12.5 C was measured by taking OD readings at 550 nm at various time intervals. This wavelength gave maximum OD readings of the cells in the broth. Data are reported as percentage of growth in air measured at 36 hr, when the cultures were near the end of the logarithmic growth phase.

Fruits. Healthy Castlemart and Royal Flush tomatoes were handpicked at the breaker stage from plants grown at Davis and Tracy, CA, respectively. Breakers were green tomatoes with light pink stripes at the blossom end. All fruits in each experiment were of one cultivar. Fruits were washed, surface-sterilized in a 0.02% NaOCl solution, rinsed in water, dried, and stored at 12.5 C. Fruits were sorted and matched in lots of 10, each with mean weight of 1,500–1,600 g. Four replicates of 10 fruits were used in each treatment. Fruits were wiped with sterile cotton saturated with 95% ethanol, air-dried, and rinsed with sterile glass-distilled water. Each fruit was wounded on two opposite sites with a flame-sterilized scalpel. The wounds were about 8 mm long and 2 mm deep. A 0.01-ml portion of inoculum was added to each wound with a sterile 1-ml syringe with a needle 12 mm long and 0.05 mm in diameter. Sterile water was added to the wounds in the control fruits. Ten fruits were placed in a 19.4-L glass container, which was sealed and connected either to humidified air or the appropriate line containing an O_2:CO_2 mixture.

Fruits were examined after 14 days at 12.5 C for decay incidence and severity of lesions was rated using a decay index (DI). Lesions were placed into one of four categories: 0 = dry spot (no infection), 1 = wet spot (slight decay), 2 = growth and some white/yellow slime (more decay), and 3 = growth with abundant white/yellow slime (most decay) as shown in Figure 1. The DI for each lot was calculated as the number of sites per category times the score per total number of inoculated sites. Fruits were left in air at 12.5 C for an additional 4 days before discarding.

Preparation of gas compositions. CAs were obtained by mixing water-saturated gases (CO_2 and N_2 from pressurized cylinders and O_2 as air) into lines with capillaries of known resistance. The gases passed through flow boards with

capillaries that gave a gas flow rate of 2.5 L/hr into both the culture flasks and the glass jars. Gas compositions were checked throughout the experiment with a Carle 8000 thermal-conductivity gas chromatograph (6). A 10-ml gas sample was withdrawn from the flow board and at the inlet and outlet of the flask and glass container to determine O_2 and CO_2 concentrations.

RESULTS

Effect of O_2 and CO_2 on in vitro growth of *P. marginalis* and *fluorescens*. Growth curves of *P. marginalis* and *P. fluorescens* on buffered asparagine-yeast extract broth under various CA conditions at 12.5 C are shown in Figure 2. Growth was reduced by all CA treatments, and the inhibition pattern was similar for both organisms except at 4% O_2. The initial growth rate of the two bacteria at 4% O_2 was greater in the first 18 hr than in the other treatments. After 18 hr, however, the growth rate of *P. marginalis* declined, leading to the major difference in the growth of the two organisms (Fig. 2).

The percentage of growth (relative to the control) after 36 hr under the different CAs is given in Table 1. Growth of *P. marginalis* was best (47% of that in air) at 4:2. Growth was reduced to 36, 33, and 16% of that in air at 4:0, 4:10, and 1:2, respectively. Growth of *P. fluorescens* was faster than that of *P. marginalis* and was reduced at 4:0, 4:2, 4:10, and 1:2 to 55, 54, 30, and 17% of the control, respectively. The pH of the medium was changed to different extents under the different CAs but the range observed (6.5–8.3) was suitable for growth of the two bacteria. Gas mixtures remained constant throughout the experiments except at 4:0, where the CO_2 level equilibrated to that of air, 0.03%.

Effect of O_2:CO_2 on decay incidence and severity. Ripe tomato fruits inoculated with 10^7 cells of *P. marginalis* and stored for 14 days at 12.5 C under CA 4:10 and 1:2 showed less decay incidence and severity than those under air (Table 2). Ripe fruits, however, were softened after storage under these two conditions. Decay incidence and severity at 4:0 and 4:2 were the same and were not significantly different from those of the air control. When fruits were left for an additional 4 days in air at 12.5 C, symptomless sites did not develop infections. Furthermore, wound sites in control fruits treated with sterile distilled water were free of decay.

DISCUSSION

Decay of tomato fruits inoculated at the breaker stage with *P. marginalis* and stored at 12.5 C for 14 days was reduced both in incidence and severity by CAs of 4:10 and 1:2. The fruits were soft at the end of the storage period, however, which supports the finding of Parsons et al (15) that tomatoes held either at very low O_2 concentrations or high CO_2 concentrations develop injury. Exposure of inoculated fruits to 4:0 and 4:2 did not reduce decay significantly at the end of 14 days. This result closely approximates that of Parsons and Spalding (16), who observed that decay incidence in tomatoes inoculated with *E. carotovora* was not reduced in a CA of 3:5 after 6 days at 12.5 C but the size of lesions was reduced. The difference in the observations may be explained by the fact that a CA of 3:5 has both a lower O_2 concentration and a higher CO_2 concentration than 4:0 or 4:2 and hence would be more effective in reducing lesion size without necessarily affecting initiation of soft rot by the pathogen. The storage period could also be a factor affecting lesion size (1).

It was possible to show by the measurement of changes in OD with time in artificial medium that cell division of *P. marginalis* and *P. fluorescens* was inhibited by all CAs used. The pattern of inhibition in all CAs was identical except at 4:0, which supports other evidence that the two species are closely related (18). *P. fluorescens* had a faster growth rate at 12.5 C and inhibition of growth at 4:0 and 4:2 was the same, 55 and 54% of air control, respectively, whereas growth of *P. marginalis* at 4:0 was reduced to 36%, equal to that at 4:10 and less than at 4:2. This observation indicates that under 4% O_2, *P. marginalis* was sensitive both to a very low CO_2 concentration at 4:0 and a high CO_2 concentration at 4:10, whereas *P. fluorescens* was affected by only the high CO_2 concentration at 4:10. This observation supports that of Wells (20), who reported a decrease of growth rates of *P. fluorescens* by concentrations of CO_2 in excess of 10% but not by the absence of CO_2. *P. marginalis*, on the other hand, was affected in the same way as the two *Erwinia* spp., which were inhibited both by the absence of CO_2 and by CO_2 concentrations higher than 10%.

Because the causation of disease, even one as supposedly primitive as bacterial soft rot, involves more than growth of the pathogen, it may be improper to compare the results from the in vitro with in vivo tests. Nevertheless, because in atmospheres of low O_2 and high CO_2 (1:2 and 4:10), cell division was inhibited in broth, the increased generation time could possibly account for reduced growth in tomato fruits leading to lower production of pectinolytic enzymes and hence reduced decay despite physiological induced softness of the fruit. At 4:0 and 4:2, growth was significantly lower than in the air control, but decay in fruit was not different from that in the air control. This indicates that even if these CAs inhibited growth in the fruit, there were other factors such as host reactions of the type reported by Bartz (2) inhibiting soft rot even in the air control. These observations support the notion that caution should be exercised in the application of in vitro results to the inhibition of bacterial decay on stored agricultural commodities as suggested by Wells (20). Additional study should be carried out to determine the growth rates of the pathogens within the fruit at these CAs. The effect of an alternative CA, such as 4:0 and 4:2 combined with carbon monoxide (7,14), on decay of tomato fruits by *P. marginalis* could be examined. Finally, we suggest that because growth of *P. marginalis* is inhibited by a very low CO_2 concentration, the best atmosphere to check soft rot by *P. marginalis* of tomatoes stored at 12.5 C is 4% O_2, 0% CO_2, and 96% N_2.

Table 1. Effect of O_2 and CO_2 concentrations on growth of *Pseudomonas marginalis* and *P. fluorescens* on a buffered asparagine-yeast extract broth at 12.5 C

Treatment (O_2:CO_2 ratios)	*P. marginalis*		*P. fluorescens*	
	$OD_{550\ nm}$[x]	Growth (%)[y]	$OD_{550\ nm}$[x]	Growth (%)[y]
Control (air)	0.90	100 a[z]	1.00	100 a[z]
4:0	0.32	36 b	0.55	55 b
4:2	0.42	47 c	0.54	54 b
4:10	0.30	33 b	0.30	30 c
1:2	0.14	16 d	0.17	17 d

[x] Optical density (OD) measured at 550 nm on a Bausch & Lomb Spectronic 20 spectrophotometer after 36 hr.
[y] Expressed as a percentge of the growth in control after 36 hr.
[z] Mean separation of four replicates not labeled by the same letter differ significantly at the 5% level by Duncan's multiple range test.

Table 2. Incidence and severity of decay of tomato fruits inoculated with *Pseudomonas marginalis* and held for 14 days at 12.5 C in air and controlled atmospheres

Atmosphere (O_2:CO_2 ratios)	Percent decay	Decay index[y]
Control (air)	73.8 a[z]	1.63 a
4:0	71.3 a	1.50 a
4:2	66.3 a	1.60 a
4:10	37.5 b	0.83 b
1:2	27.5 c	0.13 c

[y] Estimated on a scale of 0 (no infection) to 3 (severe decay). Data are the means of four replicates, each composed of 10 fruits.
[z] Mean separation of four replicates in columns by Duncan's multiple range test; 5% level is indicated by letters.

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