Biocontrol Performance of Two Isolates of *Pseudomonas fluorescens* in Modified Soil Atmospheres

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ABSTRACT

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The incidence of tomato wilt, caused by *Pseudomonas solanacearum*, and the biocontrol performance of two isolates of *Pseudomonas fluorescens* (M29 and M40) against *P. solanacearum* were measured under four soil atmospheres containing O_2/CO_2 concentrations of 210:0.3 (210 and 0.3 ml of O_2 and CO_2 per liter, respectively, ambient concentrations), 180:30, 150:60, or 120:90. Altering the O_2 and CO_2 concentrations of the soil atmosphere did not cause a statistically significant ($P \leq 0.05$) change in disease incidence. However, suppression of wilt by M29 and M40 was significantly ($P \leq 0.05$) decreased when roots were exposed to atmospheres containing lower than ambient levels of O_2 and higher than ambient levels of CO_2. Isolate M29 significantly ($P \leq 0.05$) reduced incidence of tomato wilt only under the ambient atmosphere and not under any of the three modified atmospheres tested. Isolate M40 reduced disease incidence significantly ($P \leq 0.05$) only under the ambient atmosphere and one of the modified atmospheres containing O_2/CO_2 concentrations of 180:30 ml/liter.

The failure of many biocontrol agents to be effective in the field is most likely due to the complexity and variability of physical, chemical, microbiological, and environmental factors in the field and the sensitivity of biocontrol microorganisms to these factors (2,4,11,19,24). Therefore, achieving efficient and consistent performance of biocontrol agents in the field requires a more thorough knowledge of the environmental forces that regulate the growth and activity of biocontrol microorganisms.

One of the factors that potentially can affect the establishment and activity of biocontrol agents in the rhizosphere is the concentration of O_2 and CO_2 in the soil. This is because both plants (6, 20,23) and microorganisms (5,7,10,12,14,25) are sensitive to changes in O_2 and CO_2 concentrations.

Concentrations of O_2 and CO_2 vary from field to field and site to site within the same field, depending on temperature and moisture (27), organic matter content (1), soil depth (26), and the type of crop (3). The N_2 concentration remains constant regardless of O_2 and CO_2 concentrations. Soil atmospheric composition in the top few centimeters of dry soil is near that of the ambient atmosphere, composed of 780 ml of N_2, 210 ml of O_2, and 0.3 ml of CO_2 per liter. However, the CO_2 concentration may increase to 100 ml/liter, and the O_2 concentration may decrease to below 100 ml/liter in deep, heavy, or wet soils (3). Changes in O_2 and CO_2 concentrations in the soil are mainly due to the respiration of soil fauna, flora, roots, and microorganisms (26), as well as to the slow exchange of these gases between the soil and the atmosphere (22).

The objective of this study was to determine the potential effect of changes in soil O_2 and CO_2 concentrations on the incidence of tomato wilt caused by *Pseudomonas solanacearum* and on the biocontrol performance of two *P. fluorescens* isolates against *P. solanacearum*.

We have chosen to test fluorescent pseudomonads because these bacteria are the most widely studied for their potential to protect plants against disease and to promote plant growth (4).

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MATERIALS AND METHODS

Bacterial isolates. *P. solanacearum* (strain 90-1) was obtained from R. E. Stall, University of Florida, Gainesville, and tested for pathogenicity on tomato. To preserve virulence, the culture was inoculated on tomato seedlings and reisolated periodically.

Two fluorescent pseudomonad isolates, M29 and M40, were recovered from roots of field-grown tomato plants by procedures described previously (17). These isolates are capable of reducing the incidence of *P. solanacearum*-induced wilting in the greenhouse (11).

Preparation of modified atmospheres. A modified atmosphere is defined as an atmosphere containing a lower than ambient level of O_2, a higher than ambient level of CO_2, and an ambient level of N_2. Atmospheres containing defined O_2 and CO_2 concentrations were prepared with a portable gas-mixing device (18). The device uses adjustable flow control valves and a mass flow meter to mix and deliver precise, predetermined quantities of any three gases in different combinations at a constant flow rate of up to 15 liters/h.

The four atmospheres used contained O_2/CO_2 concentrations of 210:0.3 (210 ml of O_2 and 0.3 ml of CO_2 per liter, respectively, ambient concentrations), 180:30, 150:60, or 120:90. Concentrations of O_2 and CO_2 in each modified atmosphere were within the range present in cultivated field soils (3,22). In all modified atmospheres tested, CO_2 concentrations were higher and O_2 concentrations were lower than the ambient concentrations, because a decline in O_2 concentration in field soils is always associated with a proportional increase in CO_2 concentration, so the sum of the two gases approximates 210 ml/liter (3,8,22; D. H. Kim and I. J. Misaghi, *unpublished data*).

Effect of modified soil atmospheres on disease incidence and biocontrol performance. *P. solanacearum* and isolates of *P. fluorescens* were grown in nutrient broth and King's medium B broth, respectively, for 24 h. Aqueous bacterial suspensions were prepared by centrifugation and resuspension twice (17). The *P. solanacearum* suspension was added to a pasteurized planting mix (sandy loam soil/sand/peat moss, 3:1:1, vol/vol) to give ~5×10^7 CFU/g of dry soil. The planting mix was pasteurized to eliminate propogules of *Pythium* sp. The matric potential of the inoculated

planting mix was adjusted to –3 kPa based on a matric-potential moisture-content curve by adding the appropriate amount of water. The water content was high enough to provide adequate moisture for the seedlings during the course of the experiment and low enough to allow adequate gas exchange in the soil. *P. solanacearum*-infested soil was placed in 50-ml plastic containers. The wall of each container was perforated with about 150 holes (~1 mm diameter) to facilitate gas exchange.

One 4-week-old tomato seedling was transplanted into each perforated beaker. For biocontrol treatments, roots were placed in a suspension (1×10^8 CFU/ml) of one of the test biocontrol isolates of *P. fluorescens*, M29 or M40, for 30 min prior to transplanting into planting mix infested with *P. solanacearum*. For the control treatments without biocontrol agents, seedlings were placed in sterile distilled water for 30 min prior to transplanting into planting mix infested with *P. solanacearum*. Five seedlings inoculated with one of the test biocontrol agents and five nonincoculated seedlings were placed randomly in one root chamber for exposure to one of the four test atmospheres. Root chambers (16 × 26 × 32 cm), constructed from stainless steel as described previously (12), allowed roots to be exposed to one of the test atmospheres and shoots to the ambient atmosphere. Leaks around stems protruding through 3-mm-diameter holes in the root chamber's lid were sealed with putty mix (clay dough). The sealed chambers were flushed with 100% nitrogen until the O_2 and CO_2 concentrations in the gas samples taken from the outlet tube were near zero. Root chambers were placed in a controlled environmental chamber with a daily cycle of 12 h at 30 to 32°C in the light (372 E m^{-2} s^{-1}) and 12 h at 25 to 27°C in the dark. Each root chamber was connected to a tube supplying an atmosphere containing O_2/CO_2 concentrations of 210:0.3 (210 ml of O_2 and 0.3 ml of CO_2 per liter, ambient concentrations), 180:30, 150:60, or 120:90, respectively. Gas flow was maintained at 10 liters/h. Gas samples around roots in each chamber were collected with a syringe from the top of a tube inserted next to the roots of one of the 10 seedlings and analyzed daily with a gas chromatograph.

To minimize moisture loss from the soil during the course of the experiment, the modified atmospheres were humidified by allowing them to flow through water before entering the root chambers. Water loss from the planting mix in each container was determined gravimetrically in a separate experiment and was replaced on a daily basis. Water was added to each container by inserting a 20-gauge needle attached to a syringe through the putty mix next to the seedling stems.

For each test atmosphere, the incidence of *P. solanacearum*-induced wilting (percent mortality) was determined after 15 days among five seedlings (in each root chamber) inoculated with *P. solanacearum* alone and among five seedlings inoculated with *P. solanacearum* plus isolate M29 or M40. Biocontrol performance of isolates M29 and M40 under each test atmosphere was determined by calculating the percent reduction in disease incidence in seedlings inoculated with one of the test isolates relative to disease incidence in seedlings not inoculated with the test isolate.

The experiment was performed five times with each test isolate, M29 or M40. That is, the experiment was replicated over time, with each isolate tested at a time. In each experiment and for each test isolate, four root chambers were used, one for each of the four test atmospheres.

Statistical analysis. The disease-incidence value obtained for each of the three modified atmospheres was compared with the value obtained for the ambient atmosphere by analysis of variance protected LSD tests ($P \leq 0.05$) with Minitab (Minitab Inc., University Park, PA). Biocontrol performance values were analyzed similarly. The data also were analyzed by Dunnett's test (21). The linearity of the relationship between biocontrol performance values and O_2/CO_2 concentrations was tested by regression analysis with Minitab.

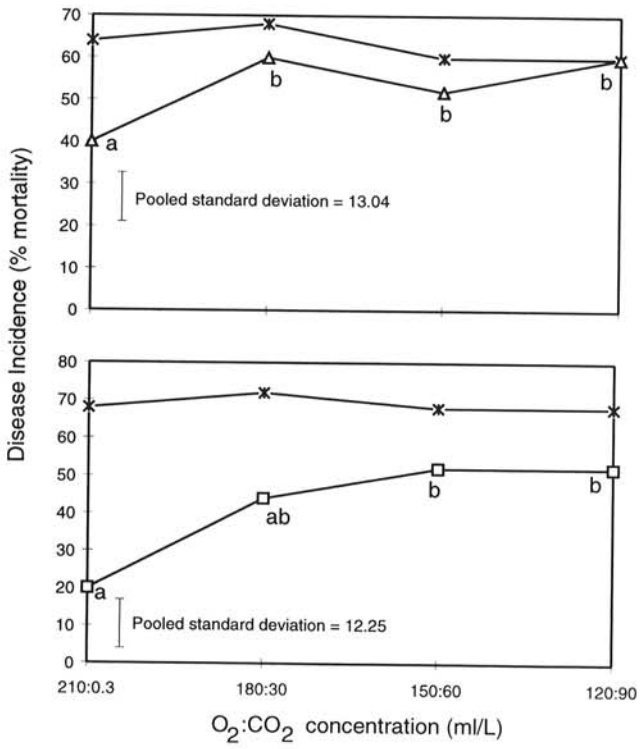

Fig. 1. Disease incidence (percent mortality) of 3- to 4-week-old tomato seedlings inoculated with *Pseudomonas solanacearum* alone (*–*), *P. solanacearum* plus *P. fluorescens* M29 (△–△), or *P. solanacearum* plus *P. fluorescens* M40 (□–□) under different soil atmospheres. In plants inoculated with *P. solanacearum* plus M29 or M40, disease incidence values marked by different letters were significantly ($P \leq 0.05$) different.

RESULTS

The two bacterial isolates used in the study were identified as *P. fluorescens* on the basis of their reactions in API (Analytical Products, Plainview, NY) and Biolog (Biolog, Inc., Hayward, CA) diagnostic tests.

Results of statistical analyses by protected LSD tests and Dunnett's test were identical in all respects. Altering O_2 and CO_2 concentrations in the soil atmosphere did not cause statistically significant ($P \leq 0.05$) changes in disease incidence (percent mortality) in tomato seedlings inoculated with *P. solanacearum* alone (Fig. 1). Biocontrol activity of both M29 and M40 was significantly ($P \leq 0.05$) decreased when roots were exposed to atmospheres containing higher than ambient levels of CO_2 and lower than ambient levels of O_2. Isolate M40 reduced disease incidence significantly ($P \leq 0.05$) only under O_2/CO_2 concentrations of 210:0.3 (ambient levels) and 180:30 ml/liter and not 150:60 or 120:90 ml/liter (Table 1). Isolate M29 reduced disease incidence significantly ($P \leq 0.05$) only under the ambient O_2/CO_2 concentrations and not under

TABLE 1. Biocontrol performance[a] of two isolates of *Pseudomonas fluorescens* (M29 and M40) in 3- to 4-week-old tomato seedlings inoculated with *P. solanacearum* under different soil atmospheres

Isolate	O_2/CO_2 concentration (ml/liter)			
	210:0.3	180:30	150:60	120:90
M29	37.5	6.3*	6.7*	0*
M40	58.8	38.9	23.5*	17.6*

[a] Biocontrol performance values were calculated from the data in Figure 1 as percent reduction in disease incidence in plants inoculated with *P. solanacearum* and with either M29 or M40 relative to disease incidence in plants inoculated with *P. solanacearum* alone. For each isolate, an asterisk denotes that its biocontrol performance under the modified atmosphere was significantly ($P \leq 0.05$) reduced compared to its performance under the ambient atmosphere.

any of the three modified O_2/CO_2 concentrations (Table 1). The biocontrol performance of isolate M40 declined gradually with incremental increases in CO_2 concentration and corresponding decreases in O_2 concentration up to a O_2/CO_2 concentration of 150:60 ml/liter ($r^2 = 0.15$, $P = 0.001$). The correlation was poor for isolate M29 ($r^2 = 0.08$, $P = 0.08$).

Gas chromatographic analysis of gas samples collected from around roots in root chambers showed no appreciable changes in O_2 and CO_2 concentrations during the course of the experiment. The pH values also remained unchanged in soils exposed to the test atmospheres during the course of the experiment.

DISCUSSION

The results of the study clearly showed that the biocontrol performance of the two isolates of *P. fluorescens* against *P. solanacearum* on tomato was sensitive to changes in the levels of O_2 and CO_2 in the soil atmosphere. However, disease incidence was not significantly ($P \leq 0.05$) affected by changes in O_2 and CO_2 concentrations. This is the first report of the effects of soil O_2 and CO_2 concentrations on the incidence of a disease and biocontrol performance of two *P. fluorescens* isolates.

One of the major obstacles in the development of biocontrol measures is the lack of performance or the inconsistent performance of many greenhouse-efficient biocontrol agents in the field. Although the reasons for such failure are unknown, variability in soil factors among different fields, within one field, and between field and greenhouse has been implicated as a contributing factor (4). This study implicates soil atmospheric composition as one possible factor. Concentrations of O_2 and CO_2 in small volumes of soil in pots used in greenhouse experiments are probably close to those present in the top few centimeters of field soils and significantly different from those prevailing at greater soil depths. A biocontrol microorganism that is sensitive to O_2 concentrations lower than the ambient level and to CO_2 concentrations higher than the ambient level may function in pots in the greenhouse but not in the field, particularly in deep soils. Based on these observations, we propose a role for soil atmospheric composition in the success or failure of some biocontrol fluorescent pseudomonads and perhaps other microorganisms in the field.

The mechanism of the observed decrease in biocontrol performance of bacteria mediated by altered soil atmospheric composition is unknown. Because changes in soil atmospheric composition can potentially affect both plants (6,20,23) and microorganisms (5,7,10,12,14,25), the phenomenon may result from direct effects of O_2 and CO_2 on the plant, pathogen, biocontrol bacteria, and interactions among these entities.

The modified atmosphere-mediated decline in biocontrol performance does not seem to be due to the CO_2- and O_2-induced changes in growth and activity of *P. solanacearum.* This is because the incidence of the disease in tomato seedlings inoculated with *P. solanacearum* alone did not significantly ($P \leq 0.05$) change in response to changes in CO_2 and O_2 concentrations. The observed drop in biocontrol performance also may not be due to the CO_2- and O_2-induced decrease in the growth of the test *P. fluorescens* isolates. The results of an earlier study showed that the growth of three isolates of *P. fluorescens* (including M29 used in this study) and one isolate of *P. putida* in the tomato rhizosphere significantly increased when roots were exposed to the same modified atmospheres used in this study (12). However, we do not know with certainty whether the growth of *P. solanacearum* and the test *P. fluorescens* isolates was affected by changes in CO_2 and O_2 concentrations, because populations of these bacteria in the rhizosphere were not monitored in this study.

Changes in O_2 and CO_2 concentrations may induce physiological alterations in roots. Root growth (23) and fine root development (9) increase in the presence of higher than ambient concentrations of CO_2. Root-associated microorganisms also can alter root exudation rates (15,16). These changes potentially may influence microbial competition among rhizosphere-residing microorganisms, including the introduced biocontrol bacteria. In this study, no visible morphological changes were observed in tomato seedlings after exposure to the test atmospheres.

The decline in biocontrol performance may be a consequence of a CO_2- and O_2-induced reduction in the level of fluorescent siderophores produced by M29 and M40. The fluorescent siderophores produced by almost all fluorescent pseudomonads inhibit the growth of microorganisms by chelating available iron in the environment, depriving the microorganisms of this vital element (24). Previously, we showed that in vitro siderophore production by four isolates of fluorescent pseudomonads is reduced in the presence of lower than ambient levels of O_2 and higher than ambient levels of CO_2 (10). Moreover, *P. solanacearum* is inhibited in vitro by fluorescent siderophores produced by M29 and M40, and the inhibition is significantly ($P \leq 0.05$) less pronounced under the modified atmospheres used in this study than under the ambient atmosphere (D. H. Kim and I. J. Misaghi, *unpublished data*). However, whether siderophore-mediated iron deprivation is a mechanism of biocontrol for M29 or M40 against *P. solanacearum* on tomato is unknown. The decline in biocontrol performance also may be due to CO_2- and O_2-induced changes in production of antibacterial products (13). Studies are underway to determine the mechanism of CO2- and O2-induced alteration in the biocontrol performance of the two test isolates in the *P. solanacearum*-tomato pathosystem.

The response pattern of isolates M29 and M40 to changes in O_2 and CO_2 concentrations in the soil atmosphere with respect to biocontrol performance differed. If differential sensitivity of bacterial isolates is confirmed in future studies, it may be possible to select a biocontrol bacterial isolate whose performance is not significantly affected by changes in soil O_2 and CO_2 concentrations. The selected isolate may perform better in the field than isolates chosen randomly. It also may be possible to genetically construct bacteria that are less sensitive to fluctuations in soil atmospheric composition.

The results of the study may have practical applications for formulating disease management strategies. For example, if the biocontrol performance of a bacterium is favored by a specific soil atmospheric composition, it may be possible to boost its performance in the field by implementing measures that help generate an optimal atmospheric composition. Agricultural practices that can alter soil atmospheric composition include the type and frequency of irrigation, addition of organic matter, mulching, etc. It also may be possible to select bacteria that can manifest their useful traits under the conditions prevailing in the rhizosphere of a specific plant in a particular field.

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