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**Siderophore production of fluorescent pseudomonads is sensitive
to fluctuations in the levels of oxygen and carbon dioxide**

Kim, Do Hoon, M.S.

The University of Arizona, 1989

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**SIDEROPHORE PRODUCTION OF FLUORESCENT PSEUDOMONADS
IS SENSITIVE TO FLUCTUATIONS IN THE LEVELS OF OXYGEN AND
CARBON DIOXIDE**

by

DO HOON KIM

A Thesis Submitted to the Faculty of the
DEPARTMENT OF PLANT PATHOLOGY
In Partial Fulfillment of the Requirements
For the Degree of
MASTERS OF SCIENCE
In the Graduate College
THE UNIVERSITY OF ARIZONA

1989

STATEMENT BY AUTHOR

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ABSTRACT

Four strains of the fluorescent pseudomonads were studied to determine the effect of controlled atmospheres on the growth and fluorescent siderophore production at pH 6.0 and 7.8. Bacterial strains were grown in Liquid King's Medium B for 48 hr in the presence of O₂/CO₂ combination percentages of 21.0/0.03, 18.3/3.0, 15.0/6.0, 12.0/9.0, and 9.0/12.0. The bacterial biomass was determined after centrifugation and the siderophores were isolated, partially purified, and quantified spectrophotometrically. Results showed a steady decline in growth and in siderophore production per unit biomass with decreases in the O₂/CO₂ ratio at pH 7.8 and to a lesser extent at pH 6.0. The average percentage changes in siderophore production levels, relative to control were +0.8, -1.2, -18.2, and -40.6 at pH 6.0 ; -33.0, -50.4, -66.8, and -64.1 at pH 7.8 in the presence of O₂/CO₂ levels of 18.0/3.0, 15.0/6.0, 12.0/9.0, and 9.0/12.0, respectively.

CHAPTER 1

INTRODUCTION

Fluorescent pseudomonads have received increasing attention in the past few years because of their potential to increase plant growth and to suppress diseases caused by certain soil-borne plant pathogens. While the mechanisms of disease suppression and of plant growth promotion are not clearly elucidated, antibiosis (Howell and Stipanovic, 1979, 1980) and competition for food and minerals (Stolp, and Gadkari, 1981) seem to be important parameters. Almost all of the fluorescent pseudomonads produce fluorescent siderophores with a high chelating affinity for Fe^{3+} ion (Bossier et al., 1988). These siderophores chelate available Fe^{3+} from the environment and thus make it unavailable for the competing microorganisms. Fluorescent siderophore-mediated iron competition seems to be an effective weapon used by the fluorescent pseudomonads to remove iron from the environment and to deprive competing microorganisms from this vital element. Unfortunately, however, fluorescent siderophore-mediated iron competition has thus far remained an in vitro phenomenon and unequivocal evidence for fluorescent

siderophores production in the soil has not yet been provided. The production of fluorescent siderophores is a common feature of almost all the fluorescent pseudomonads and it is, therefore, highly unlikely that such a common trait would have no specific function in nature.

The early reports of in vitro siderophore-mediated iron competition led to the assumption that the phenomenon is an important biological control mechanism which operates non-contingently under natural conditions. The assumption is not necessarily correct because siderophore production in vitro is known to be sensitive to fluctuations in the levels of mineral nutrition (Lenhoff, 1963), pH (Misaghi et al., 1988), temperature (Garibaldi, 1971 ; Loper, 1986), and the cations such as Mg^{2+} (Georgia and Poe, 1931), Zn^{2+} (Baghdiantz, 1952), and Fe^{3+} (Becker and Cook, 1988). Siderophore production is expected to fluctuate in the soil because of the variability of the above parameters. The contingency of fluorescent siderophore-mediated iron competition as a biological control mechanism in natural soil has been pointed out by Misaghi et al. (1988) who provided evidence that not only production but the activity of the fluorescent siderophores was modulated by pH which is highly variable from soil to soil.

Another parameter which may influence siderophore production by the fluorescent pseudomonads is O_2 and CO_2

levels in the soil which are highly variable with respect to their levels. The O₂ and CO₂ contents of agricultural soils vary depending on temperature (Yamaguchi at el., 1967), moisture (Miller, and Johnson, 1964), organic matter content (Epstein, and Kohnke, 1957), and the type of crops (Abrosimova, and Revult, 1964). The O₂ levels may drop to below 10% from the ambient level of about 21% and CO₂ levels may increase to more than 10% from the ambient level of 0.03% in heavy, moist and/or deep soils (Buyanovsky, and Wagoner, 1983 ; Ioannou at el., 1977 ; Lyda, and Burnet, 1975 ; Scott, and Evans, 1955 ; Stolzy at el., 1975). Any drop in O₂ level in the soil is associated with an increase in CO₂ concentration so that the sum of the two gases approximate 21% (Griffin, 1972). The levels of O₂ and CO₂ also fluctuate as a function of the respiratory rate of roots and microorganisms due to steady accumulation of CO₂ and drop in O₂ levels. Such fluctuations are more pronounced in compact and wet soils due to slow diffusion of these gases and also in deep soils due to restricted permeability of gases. Fluctuations in the levels of O₂ and CO₂ in the soil are also more pronounced in soil with high organic content which is conducive to accelerated growth of roots and microorganisms.

Bacteria (Harrison, and Pirt, 1967 ; King, 1966 ; Smith, and Johnson, 1954 ; Wells, 1974), plants (Tackett, and

Pearson, 1964 ; Whitney, and Gardner, 1943), and fungi (Burgess, and Fenton, 1953 ; Chet, 1986 ; Durbin, 1959 ; Griffin, 1963 ; Imolehin, and Grogan, 1980 ; Rishbeth, 1978 ; Stolzy, and Gundy, 1968 ; Zilberstein, Chet, and Henis, 1983) as well as interactions of microorganisms with plant roots (Bergman, 1959 ; Louvet, and Bulit, 1964 ; Zentmyer, 1965) are sensitive to changes in the levels of O₂ and CO₂. Vertical distribution of certain fungi (Durbin, 1959 ; Morrison, 1976) also are influenced by CO₂ concentration. The sensitivity of soil microorganisms to the soil gaseous environment, to soil moisture, and to other variable soil parameters may explain why many of them possess specific ecological niches. Sensitivity of microorganisms to changes in the level of O₂ and CO₂ differs. The following microorganisms are listed in order of their increased sensitivity : Sclerotium rolfsii < Erwinia spp. and Pseudomonas fluorescens < P. fragi < P. aeruginosa < Bacillus cereus, Saccharomyces cerevisiae < Streptococcus cremoris (Enfors and Molin, 1980).

The effect of CO₂ on plants, microorganisms, and on their interactions may be indirect through a CO₂-induced drop in pH. The drop in pH is brought about by dissociation of carbonic acid (formed from CO₂ and water) to H⁺ and bicarbonate (Umbreit, 1957). The magnitude of the drop in soil

carbonate. The ratios are in a constant state of flux due to changes in the levels of CO_2 , bases (e.g., CaCO_3 , MgCO_3 , Na_2CO_3), and water. The CO_2 -induced changes in soil pH also depend on soil temperature, because the solubility of CO_2 in water is decreased with increases in temperature (Umbreit, 1957). Soil pH also increases with increases in soil water content.

My preliminary observations showed that the growth and siderophore production by the fluorescent pseudomonads are also sensitive to fluctuations in the level of O_2 and CO_2 . I, therefore, designed a study to examine in details the effect of O_2 and CO_2 on siderophore production and the growth of four selected strains of fluorescent pseudomonads.

CHAPTER 2

MATERIALS and METHODS

Bacterial Isolates

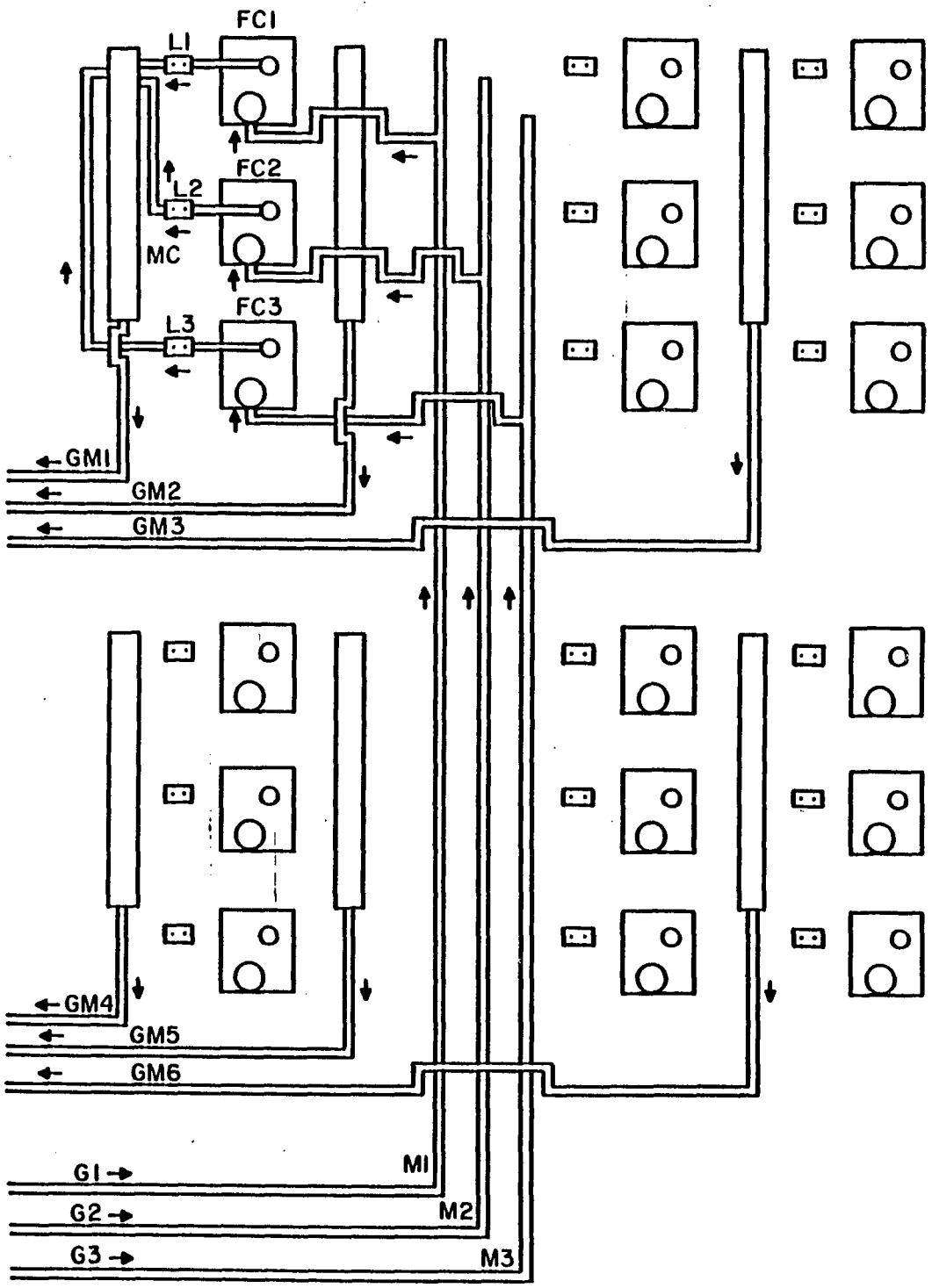
Bacterial strains used in this study were isolated from roots of cotton, tomato, and cucumber plants grown in the field. Roots were shaken gently to remove loose soil particles. About 200 mg of root sections were placed in a 2.5 ml centrifuge tube. The tubes were shaken at high speed on a vortex shaker platform for 20 min and different dilutions of the root washings were plated out on King's Medium B (KB: proteose peptone #3, 2.0% ; glycerol, 1.0% ; MgSO₄, 0.15% ; K₂HPO₄, 0.15% ; Agar, 2.0%) agar. Discrete bacterial colonies were selected after 24 hr of incubation at 27 C and their purity was tested by repeated streaking on KB plates.

Four selected strains were characterized using standard biochemical tests (Misaghi and Grogan, 1969) and API diagnostic tests (API Analytab Products, Plainew, N.Y.).

Gas mixing system

Controlled atmospheres (CA) containing different levels of O₂, CO₂ and N₂ were prepared by using a portable gas mixing system designed and constructed to study the effect of gas

Figure 1: A diagram of a device for preparing and delivering of gas mixtures. It consists of three manifolds (M1-M3), 18 span flow controlers (F1, F2,..., F18), 18 bypass loops (L1, L2,..., L18), and six mixing chambers (MC). Gas flow paths through the device are shown only for one of the six gas mixtures (GM1).



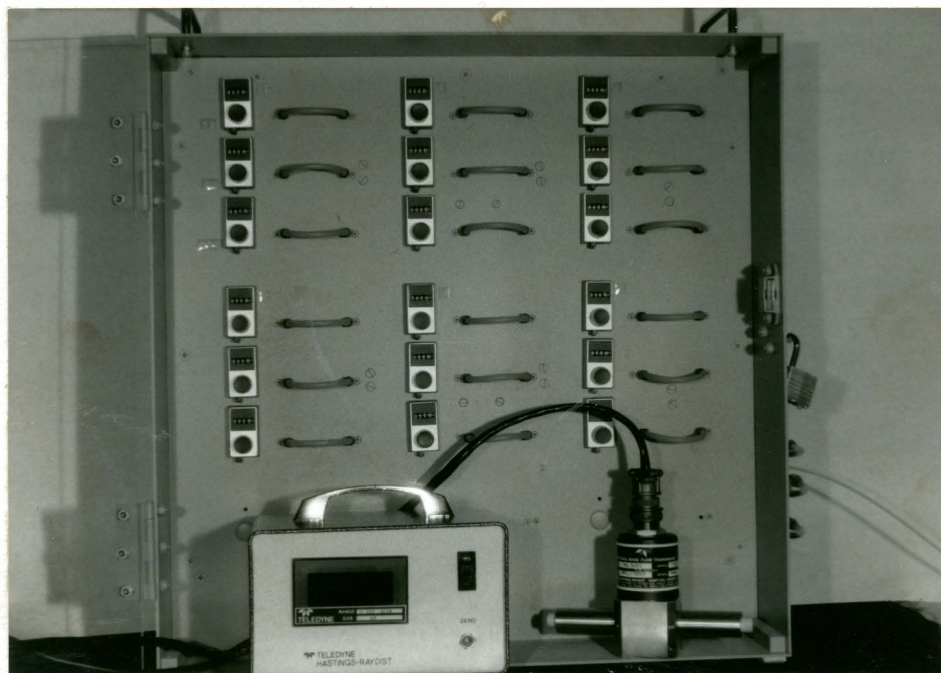


Figure 2: The front side of the device showing span flow controller and bypass loops.

composition on growth and development of fungi and bacteria (Fig. 1). This system mixes and delivers precise, predetermined quantities of any three gases in six different combinations at a constant flow rate up to 15 lb/hr. Flow rates were controlled by 18 adjustable span flow valves and monitored by a linear mass flow meter (Teledyne Hasting-Raydist, Hampton, VA 23661) (Fig. 2.). Flow rates and gas composition were held constant over a 9-day period.

CA and gas analysis

The following percentage combinations of O₂/CO₂ were used ; 20.9/0.03 (ambient atmosphere), 18/3, 15/6, 12/9, and 9/12 with N₂ making the balance. The selected combinations represent O₂/CO₂ concentrations found in cultivated soils (Buyanovsky and Wagoner, 1983). CA were introduced into gas tight 3.8-l glass jars through inlet ports. Gases were analyzed by a gas chromatography.

The effect of CA on growth and siderophore production

The selected strains were tested for the ability to grow and to produce siderophores at pH 6.0 and pH 7.8 in the presence of different levels of O₂ and CO₂, using Liquid King's Medium B (proteose peptone #3, 2.0% ; glycerol, 1.0% ; MgSO₄, 0.15%). The pH of the medium was adjusted with 0.1 M phosphate

buffer.

Three μ l of a 48-hr-old bacterial suspension containing 1×10^8 cfu/ml was added to 3 ml of LKB in one of the three compartments of a 15 x 16 mm plastic petri dish. The petri dishes were then placed in a glass jar. The jars were sealed and each was connected to one of the four CA. To promote exchange of the CA into the medium jars were placed inside a radial shaker incubator at 27 C and shaken gently. Forty eight hours after incubation, 1.5 ml of each culture were transferred into a microcentrifuge tube ; bacterial cells were pelleted by centrifugation at 12,000 rpm for 4 min, dried ,and were weighed. To isolate siderophores, 0.5 ml of cell-free supernatant was centrifuged at 12,000 rpm for 4 min after addition of two volumes of acetone. Siderophores were precipitated from the extract by adding two additional volumes of acetone followed by centrifugation at 12,000 rpm for 4 min. Siderophores were washed three times with reagent-grade acetone and dissolved in 1 ml of deionized water. Siderophores were quantified by measuring their optical density at 403 nm and values were calculated on the basis of milligram of bacterial dry weight. All tests were repeated three times each with three replications. The data were subjected to statistical analysis to determine significance levels using SPSS/PC data analysis package (SPSS

Inc., Chicago, Illinois).

CHAPTER 3

RESULTS

Identification of bacterial strains

Strains, M16, M30, and M31 were identified as Pseudomonas fluorescens and strain M18 was identified as P. putida.

Gas mixing system

The gas mixing system used in this study proved to be capable of preparing gases accurately and reproducibly. After the required initial 15 min warm-up time for mass flow meter, it took about 5 min to prepare one gas mixture containing three different gases. Concentrations of gases in the mixtures were found to be about 91% to 95% of the desired values. The flow rates of the gases during the nine-day test period fluctuated by about 2.0%.

The effect of CA on bacterial growth

The growth response of tested strains to fluctuations in O_2/CO_2 levels was variable. While some strains grew faster with decreases in O_2/CO_2 ratio, others grew slower (Table 1). The general trend, however, was a decreases in growth with

Percent Changes in Growth with Drop in O₂/CO₂ Ratio at pH 6.0

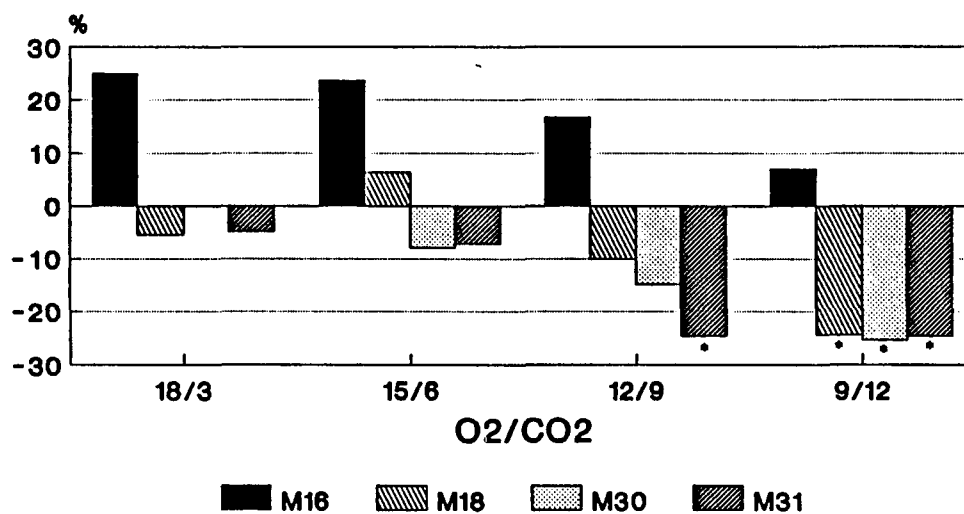


Figure 3: Percent changes in growth of four fluorescent pseudomonads in response to fluctuations in the levels of O₂ and CO₂ relative to control at pH 6.0.

Percent Changes in Growth with Drop in O₂/CO₂ Ratio at pH 7.8

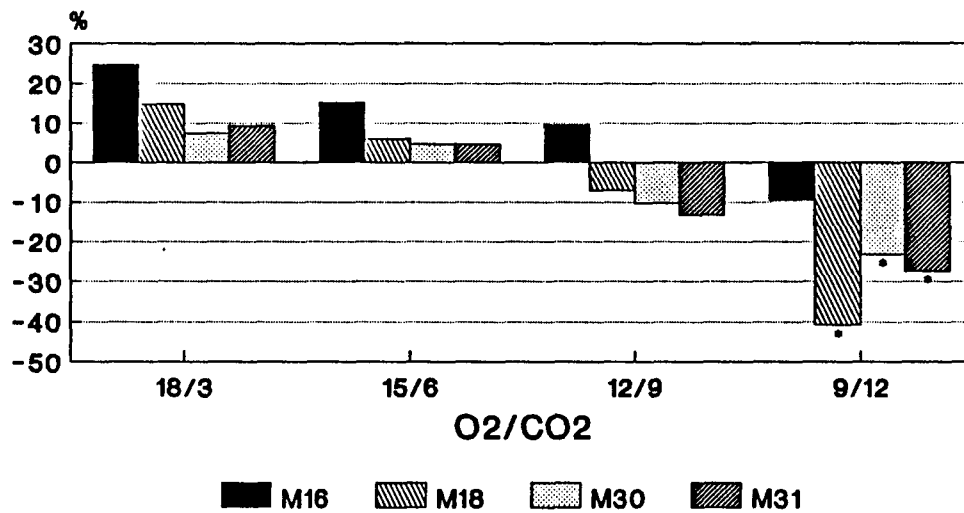


Figure 4: Percent changes in growth of four fluorescent pseudomonads in response to fluctuations in the levels of O₂ and CO₂ relative to control at pH 7.8.

Percent Changes in Siderophore Production with Drop in O₂/CO₂ Ratio at pH 6.0

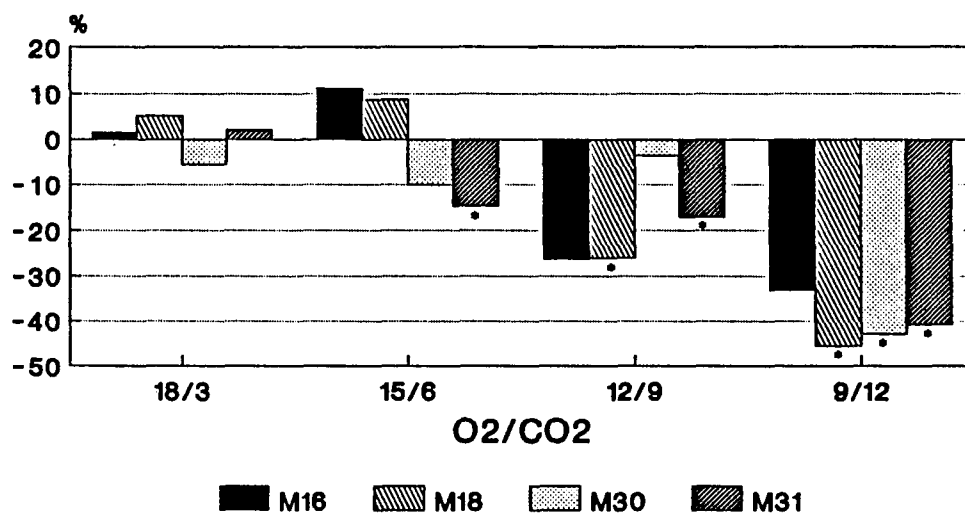


Figure 5: Percent changes in siderophore production by four fluorescent pseudomonads strains in response to fluctuations in the levels of O₂ and CO₂ relative to control.

Percent Changes in Siderophore Production with Drop in O₂/CO₂ Ratio at pH 7.8

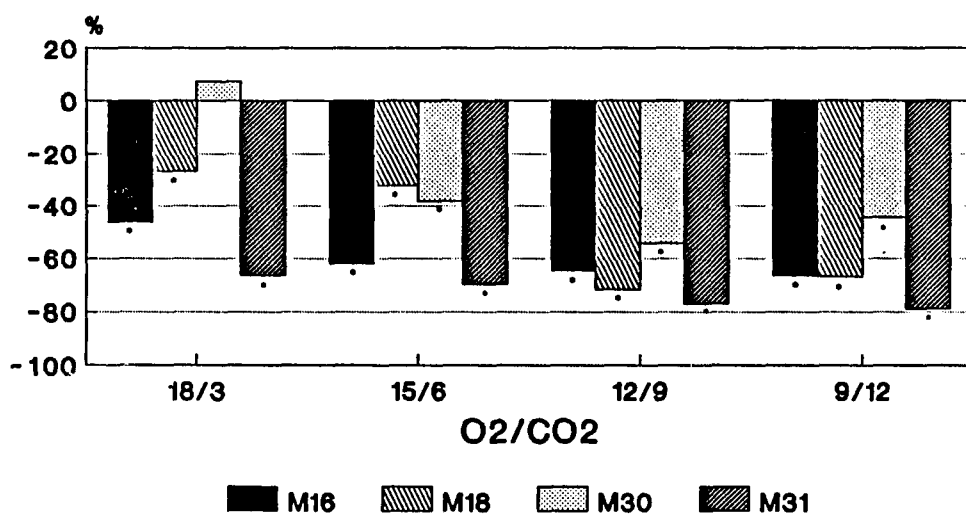


Figure 6: Percent changes in siderophore production by four fluorescent pseudomonads strains in response to fluctuations in the levels of O₂ and CO₂ relative to control.

decreases in O₂/CO₂ ratio at pH 6.0 (Fig. 3). Similar response were observed at pH 7.8. However, strains were much more tolerant of the high concentrations of CO₂ up to 6% at pH 7.8 compared to pH 6.0 (Fig. 4). Changes in growth which are statistically different from control at the 5% level are indicated by asterisks.

The Effect of CA on siderophore production

Siderophore production was also influenced by fluctuations in the levels of O₂ and CO₂. Here again responses of the strains to O₂ and CO₂ were not the same (Table 2). There was a steady decline in siderophore production with decreases in O₂ and increases in CO₂ at pH 7.8 (Fig. 6). However, percent changes in siderophore production with drop in O₂/CO₂ ratio at pH 6.0 were less pronounced than those at pH 7.8 (Fig. 5). Siderophore levels were expressed on the basis of unit biomass to take into account changes in bacterial growth rate in response to changes in O₂ and CO₂ level. Fluctuations in siderophore production which are statistically different from the control at the 5% level are indicated by asterisks.

Table 1. Effect of changes in the levels of O₂ and CO₂ on the growth of four strains of fluorescent pseudomonads at pH 6.0 and pH 7.8

Bacterial isolate	Percent O ₂ /CO ₂	<u>Bacterial dry weight (mg)**</u>	
		pH 6.0	pH 7.8
M16	20.9/0.03 *	0.0072 a	0.0073 a
	18 / 3	0.0090 a	0.0091 a
	15 / 6	0.0089 a	0.0084 a
	12 / 9	0.0084 a	0.0080 a
	9 / 12	0.0077 a	0.0066 a
M18	20.9/0.03	0.0111 a	0.0101 a
	18 / 3	0.0105 a	0.0116 a
	15 / 6	0.0118 a	0.0107 a
	12 / 9	0.0090 a	0.0094 a
	9 / 12	0.0084 b	0.0060 b
M30	20.9/0.03	0.0115 a	0.0108 a
	18 / 3	0.0115 a	0.0116 a
	15 / 6	0.0106 a	0.0113 a
	12 / 9	0.0098 a	0.0097 a
	9 / 12	0.0086 b	0.0083 b
M31	20.9/0.03	0.0126 a	0.0106 a
	18 / 3	0.0120 a	0.0116 a
	15 / 6	0.0117 a	0.0111 a
	12 / 9	0.0095 b	0.0092 a
	9 / 12	0.0095 b	0.0077 b

* represent the ambient atmosphere level.

** indicate means of three replications. Those values followed by different letters are significantly different relative to control at $P \leq 0.05$, according to Dunnett's procedure ; comparing treatments to a control.

Table 2. Effect of changes in the levels of O₂ and CO₂ on siderophore production (absorbance at 403nm / mg dry weight) by four strains of fluorescent pseudomonads at pH 6.0 and pH 7.8

Bacterial isolate	Percent O ₂ /CO ₂	Siderophore production**	
		pH 6.0	pH 7.8
M16	20.9/0.03 *	2.97 a	13.06 a
	18 / 3	3.02 a	6.96 b
	15 / 6	3.30 a	4.96 b
	12 / 9	2.22 a	4.62 b
	9 / 12	2.00 a	4.36 b
M18	20.9/0.03	5.49 a	25.48 a
	18 / 3	7.71 a	18.73 b
	15 / 6	8.01 a	17.25 b
	12 / 9	4.52 b	7.28 b
	9 / 12	3.76 b	8.49 b
M30	20.9/0.03	14.58 a	21.51 a
	18 / 3	13.78 a	23.11 a
	15 / 6	13.12 a	13.31 b
	12 / 9	14.06 a	9.02 b
	9 / 12	8.34 b	11.98 b
M31	20.9/0.03	33.44 a	100.57 a
	18 / 3	34.12 a	33.99 b
	15 / 6	28.54 b	30.62 b
	12 / 9	27.72 b	23.23 b
	9 / 12	19.81 b	21.44 b

* represent the ambient atmosphere level.

** indicate means value of three replications. Those values followed by different letters are significantly different relative to control at $p \leq 0.05$, according to Dunnett's procedure ; comparing treatments to a control.

CHAPTER 4

DISCUSSION

Siderophore-mediated iron competition is most likely a major mechanism of biological control by the fluorescent pseudomonads under field conditions. However, the phenomenon is contingent due to the sensitivity of siderophore production to certain parameters which may vary from soil to soil and even within one soil. The results of this study show that the production of siderophore is highly sensitive to O_2 and CO_2 levels which fluctuate in the soil depending on certain factors such as moisture, soil depth, and microbial activities.

The sensitivity of siderophore production to changes in the levels of O_2 and CO_2 has important ramifications with respect to the effectiveness of the fluorescent pseudomonads as biological control agents. These bacteria are expected to be active against pathogens around shallow roots where O_2/CO_2 ratios are higher than around deep roots where O_2/CO_2 ratios are low.

The fluorescent pseudomonads may, therefore, be more effective against pathogens such as Pythium spp. which attack

shallow roots rather than those pathogens attacking deeper roots.

Soil texture and compactness may also influence the effectiveness of fluorescent pseudomonads as biological control agents. The O_2/CO_2 ratio are expected to be low in heavy soil because of the slow diffusion of these gases and concomitant reduction in the biological activity of the fluorescent pseudomonads.

The effectiveness of the fluorescent pseudomonads as biological control agents is also influenced by the activity of microorganisms around roots because of the influence on the concentrations of O_2 and CO_2 . The greater the activity of the microorganisms, the lower the ratio of O_2/CO_2 and the lower the concentration of siderophores. Intense microbial activity in the rhizosphere not only makes it more difficult for the fluorescent pseudomonads to compete for the ecological niches, but also reduces the likelihood of effective deployment of siderophore-mediated iron competition as an effective weapon.

The intensity of siderophore-mediated iron competition is also influenced by soil moisture because moisture effects O_2 and CO_2 diffusion in the soil. Increases in soil moisture encourages the activity of competing microorganisms and reduces the diffusion rate of gases, both of which are non-conducive to the effective operation of siderophore-mediated

iron competition.

The sensitivity of siderophore-mediated iron competition to O_2 and CO_2 concentrations shows that this important biological mechanism is a highly contingent phenomenon and, therefore, may not be expected to operate consistently in the soil. The fluorescent pseudomonad may be used most effectively as biological control agents by providing conditions which are more conducive not only for the competitiveness but also for the production of siderophores. This may be achieved by cultural practices which help increase the ratio of O_2/CO_2 including deep plowing to increase the gas exchange rate, soil moisture management, and the type of irrigation practices utilized.

Strains of the fluorescent pseudomonad used in this study were variable with respect to the sensitivity to O_2/CO_2 ratios. The development of the most effective biological control practices may require selection of bacterial strains whose siderophore production is less sensitive to fluctuations in O_2 and CO_2 concentrations. This goal may be also achieved through genetic engineering techniques. It is possible to select or reconstruct a bacterial strain which would perform effectively under variable conditions in terms of siderophore production. The achievement of such a goal requires a clear understanding of the ways in which siderophore production is

influenced by O_2/CO_2 ratios.

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