Critical Iron Level Associated with Biological Control of Fusarium Wilt

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ABSTRACT

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Chlamydospore germination in vitro of *Fusarium oxysporum* f. sp. *cucumerinum* was significantly decreased over the control in the presence of siderophore-producing *Pseudomonas putida* A12 at Fe^{3+} activities between 10^{-22} M and 10^{-28} M. A nonsiderophore-producing mutant, A1/UV/AB-6, had no effect on fungal spore germination with decreasing Fe activity. Microbial turnover during experimental procedures released 6 μg of Fe per kilogram of solution. When this Fe was available to the fungal chlamydospores, spore germination was 21% higher than when the released Fe was trapped by chelation with ethylenediamine di-o-hydroxyphenylacetic acid (EDDHA). Toxic trace elements in the nutrient solution decreased bacterial survival unless enough EDDHA was present to bind the elements and reduce their effects. These results suggest that the critical level of Fe^{3+} activity in this system, below which chlamydospore germination was suppressed, was between 10^{-19} M and 10^{-22} M and that optimal suppression took place between Fe^{3+} activities of 10^{-22} M and 10^{-27} M.

Additional key words: antagonism, biological control, suppressive soils.

The activity of siderophore-producing pseudomonads leading to Fe competition was suggested as a possible mechanism for the suppression of Fusarium wilt (6). The addition of *Pseudomonas putida* (Trevisan) Migula (11), producing a mixed catechol-hydroxamate siderophore with a high affinity for Fe (16,17), to a conducive soil rendered the soil suppressive to Fusarium wilt pathogens of flax, cucumber, and radish. The same results were achieved with additions of ethylenediamine di-o-hydroxyphenylacetic acid (EDDHA), a synthetic chelate that also has a high Fe stability constant (11). Other studies have demonstrated a direct correlation between the amount of fluorescent siderophore produced by various pseudomonads and their inhibition of chlamydospore germination of *Fusarium* spp. in soil (2,14).

Antagonism due to competition for nutrients is only operable under conditions of low nutrient availability. Iron concentration in the soil solution is related directly to soil pH. As soil pH increases, Fe availability decreases because of the formation of insoluble Fe compounds (8). Scher and Baker (10) converted a suppressive soil to a conducive one by lowering the soil pH from 8.0 to 6.0.

Intense competition for nutrients in the rhizosphere may contribute to the suppressive mechanism. Additions of EDDHA inhibited germination of chlamydospores of *Fusarium oxysporum* Schlect. emend. Snyd. & Hans. in the rhizosphere but not in the surrounding bulk soil (12,14).

This complex system needs careful analysis before management practices can be developed for optimizing the use of pseudomonads as biological control agents of plant pathogens. The objectives of the present study were to describe quantitatively the competition for Fe between the fungus and the bacterial antagonist and to determine the critical Fe level necessary for optimal suppression of chlamydospore germination of *F. oxysporum* by *P. putida*.

MATERIALS AND METHODS

Preparation of organisms. Chlamydospores of *F. o.* f. sp. *cucumerinum* were produced by a method similar to that of Short and Lacy (13). Mycelial plugs were removed from potato-dextrose agar plates, added to 75 ml of potato-dextrose broth (PDB), and agitated on a reciprocal shaker for 3–4 days. Ten milliliters of the resultant microconidial suspension was added to Roux bottles containing 50 ml of PDB and shaken slowly overnight. The germinated microconidia were washed free of excess nutrients and suspended in 75 ml of soil extract prepared by mixing 1 L of distilled water with 1 kg of raw Nunn sandy loam (11). The mixture was allowed to stand for 48 hr, and the supernatant was filtered through a sterilized 2-μm Nuclepore filter (Nuclepore Corporation, Pleasanton, CA).

After 7–9 days, the mixture contained abundant chlamydospores, which were trapped onto a 7-μm Nitex filter (Tetko Inc., Monterey Park, CA), suspended in 0.1 M $MgSO_4$, and stored at 4 C until further use. Chlamydospores in solution were estimated by plating a series of 10-fold dilutions onto Komada's *Fusarium*-selective medium (7).

P. putida A12 (11) and A1/UV/AB-6 strains (obtained from M. Schroth, Department of Plant Pathology, University of California, Berkeley) were maintained on low-iron synthetic medium (SM) containing 20 g of sucrose, 2 g of L-asparagine, 1 g of K_2HPO_4, 0.5 g of $MgSO_4 \cdot 7H_2O$, and 20 g of agar per liter (11). The *P. putida* strain A1/UV/AB-6 was not a mutant of strain A12, however, it was the only nonsiderophore-producing mutant available at the time. One loop of bacteria was transferred to 50 ml of SM liquid medium and cultured at 28 C in a rotary shaker at 60 rpm. After 18 hr, the bacteria were centrifuged and washed three times with sterile water. The absorbance of the bacterial suspension was measured at 780 nm and the suspension was adjusted to the desired population density with the aid of a predetermined absorbance vs. bacterial-population curve.

Nutrient solutions. A complete two-fifths Hoagland nutrient solution (4) with modified Fe levels was used in all experiments. Five levels of Fe^{3+} activity were maintained by the addition of amorphous iron oxide or various combinations of EDDHA and its ferrated form to the solution (Table 1). Solution pH was adjusted to 7.6 with KOH and buffered by additions of 0.4 g of powdered $CaCO_3$ per 200 ml. A modified version of GEOCHEM (15), which included the thermodynamic constants given by Lindsay (8), was used to estimate Fe^{3+} activity in the nutrient solutions. The model predicted a solution of 0.03 ionic strength. The solutions also contained 0.05% glucose as an available carbon source.

Two additional nutrient solutions were used to investigate the system under extreme Fe stress: One contained no added Fe or

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chelate, whereas the other contained 10^{-4} M EDDHA but no added Fe. The nutrient solutions were collected after the experiment, centrifuged, and analyzed for Fe.

Incubation procedures. Desired numbers of bacteria and 3.5×10^4 fungal chlamydospores were added to 50 ml of deionized water, and the suspension was vacuum-filtered onto 0.4-μm Nuclepore filters to give a spore density of 3.4×10^3 cm^{-2}. Each filter was floated on 200 ml of nutrient solution in containers in a dark growth room at 27 C. There were four replicates of each treatment, and the containers were set up in a randomized complete block design. Each experiment was repeated at least twice.

The membrane filters were removed from the nutrient solution after 12 hr and stained by floating on 0.1% Calcofluor (Calcofluor white-ST solution, American Cyanamid Co., Bound Brook, NJ) for 15 min. The filters were placed on slides and viewed with an Olympus BH microscope (Olympus Optical Co., Tokyo, Japan) with both a 530-nm barrier filter and a blue exciter filter, which transmitted 400 nm of light from an epifluorescent illuminator (12). Germination was determined by observing 100 spores from each filter.

Five different population levels of *P. putida* A12, ranging from 2.5×10^5 to 2.5×10^6 bacteria, were added to the system that contained the highest Fe level. This allowed the observation of bacterial population density effects on germination of *F. o.* f. sp. *cucumerinum* when Fe activity was not limiting. There was no significant difference in chlamydospore germination as the pseudomonad population density was increased as compared with the control. An intermediate level of 1×10^6 bacteria was used in all subsequent experiments.

Respiration and siderophore production. Two nutrient solutions were evaluated for their effects on bacterial respiration and siderophore production in the system. One was maintained at the highest Fe^{3+} activity of 10^{-19} M, whereas the other contained no added Fe. Neither solution contained EDDHA, which would have confounded the colorimetric measurement of siderophore production.

Membrane filters supporting the pseudomonads and chlamydospores of *F. o.* f. sp. *cucumerinum* were floated on top of 100 ml of nutrient solution in sealed jars containing vials of dilute NaOH. After 24 hr, the amount of released CO_2 was determined by titration of the base with dilute HCl. Siderophore production was estimated by measuring the absorbance of the centrifuged nutrient solution at 410 nm after the addition of 10^{-4} M Fe (11).

Chelate effects on the population of pseudomonads. Five levels of EDDHA, ranging from 10^{-7} to 10^{-3} M, were added to nutrient solutions containing no added Fe. *P. putida* A12 or A1/UV/AB-6 at 8×10^5 colony-forming units (cfu) was added to the solutions and cultured at 28 C on a rotary shaker at 60 rpm. After 12 hr, the bacterial suspension was centrifuged and bacterial numbers were estimated on King's B agar (5) by use of the method developed by Harris and Sommers (3). The supernatant was analyzed for Fe by inductively coupled plasma spectroscopy.

Three solutions were used to investigate the apparent toxicity of the nutrient solution to *P. putida* A12 at low chelate concentrations: the previously described nutrient solution; a phosphate buffer solution at pH 7.6; and the phosphate buffer plus trace elements, including Cu, Zn, Mn, B, and Mo. The two phosphate buffer solutions also contained C and N additions identical to the original nutrient solution. EDDHA was added to these solutions at 10^{-4} and 10^{-7} M.

Experiments were repeated at least twice with two or four replications. Experimental data were subjected to regression analysis or analysis of variance as appropriate ($P = 0.05$). Fisher's least significant difference tests were used for mean separations.

TABLE 1. Effect of varying the Fe source and excess chelate on Fe^{3+} activity in nutrient solution buffered at pH 7.6

(Fe^{3+})	$[Fe_{TS}]$[a]	Fe source ($mol \cdot L^{-1}$)	Excess EDDHA[b] ($mol \cdot L^{-1}$)
10^{-19}	$10^{-9.3}$	$Fe(OH)_3$ 10^{-4}	0
10^{-22}	$10^{-10.8}$	Fe EDDHA 10^{-4}	10^{-6}
10^{-25}	$10^{-12.2}$	Fe EDDHA 10^{-4}	10^{-5}
10^{-27}	$10^{-13.2}$	Fe EDDHA 10^{-5}	10^{-5}
10^{-28}	$10^{-13.7}$	Fe EDDHA 10^{-5}	10^{-4}

[a] $[Fe_{TS}] = [Fe^{3+}] + [FeOH^{2+}] + [Fe(OH)_2^{+}] + [Fe(OH)_3^{\circ}] + [Fe(OH)_4^{-}]$.
[b] EDDHA = Ethylenediamine di-o-hydroxyphenylacetic acid.

RESULTS

Competition for Fe. The effect of decreasing Fe activity on germination of *F. o.* f. sp. *cucumerinum* was evaluated both in the absence and presence of *P. putida* strains A12 or A1/UV/AB-6. In the absence of the bacteria, lowering Fe^{3+} activity from 10^{-19} to 10^{-28} M resulted in a linear decrease in germination of spores of *F. o.* f. sp. *cucumerinum* (Fig. 1). Inclusion of *P. putida* A12 decreased spore germination in a more complex fashion with decreasing Fe^{3+} activity. In the nonsiderophore-producing mutant, *P. putida* A1/UV/AB-6 had no significant effect on germination of spores of *F. o.* f. sp. *cucumerinum*.

The level of chlamydospore germination was not significantly different in the presence or absence of the pseudomonads at the highest or lowest Fe level. At Fe^{3+} activity levels of 10^{-22}–10^{-27} M, however, spore germination was significantly decreased by the presence of the siderophore-producing bacterial antagonist.

When the nutrient solution contained no added Fe and no added chelate, germination of spores of *F. o.* f. sp. *cucumerinum* in the presence of *P. putida* A12 was 59% (Fig. 2), which is comparable to the spore germination when Fe^{3+} activity was 10^{-19} M (Fig. 1). When 10^{-4} M EDDHA was added, spore germination decreased to 38%. The iron concentration was insignificant in the nutrient solution with no added chelate both before and after the 12-hr incubation. In the nutrient solution containing EDDHA, however, there was an increase of 6 μg of Fe per kilogram of solution before the incubation and an additional 6 μg of Fe per kilogram of solution increase after 12 hr.

Effects of Fe on respiration and siderophore production. Respiration and siderophore production of *P. putida* A12 were affected by the Fe^{3+} activity maintained in the nutrient solution (Fig. 3). There was a significant increase in both CO_2 evolved and siderophore production as Fe^{3+} activity was increased from 0 to 10^{-19} M. A comparison of Figures 1 and 2 shows that these large increases in pseudomonad respiration and siderophore production only led to a slight, nonsignificant decrease in germination of spores of *F. o.* f. sp. *cucumerinum*, from 61.3 to 59%, at these two levels of Fe activity.

Chelate effects on bacteria in nutrient solution. When low amounts of chelate were present in the nutrient solution, bacterial population dropped significantly below the initial level of 7×10^5 cfu/ml after the 12-hr incubation period (Figs. 4 and 5). As chelate concentration increased to 10^{-5} M or greater, the population density of the pseudomonad isolates was maintained at the initial level (Fig. 4). The population density of the nonsiderophore-producing mutant was always slightly below that of *P. putida* A12, but increasing EDDHA in solution had the same relative effect on population size of the two isolates.

Changes in the EDDHA concentration had no effect on the population densities of *P. putida* A12 maintained in the phosphate buffer (Fig. 5). The addition of trace elements to the phosphate buffer solution led to a significant decrease in pseudomonad population density only when EDDHA was present at a low concentration.

DISCUSSION

The concentration of Fe in soil solution is extremely low, in fact below analytical detection limits. The activity of Fe^{3+} is its "effective concentration," which is slightly lower than its concentration due to interactions with other ions (8). In this study, the activity of Fe^{3+} was controlled in the absence of soil by amorphous Fe hydroxide or added chelating agents. The activity of Fe^{3+} could then be calculated through known equilibrium constants.

A

B

FOC ALONE
FOC + A12
FOC + A1/UV/AB⁻6

% GERMINATION

64 60 56 52 48 44

-19 -22 -25 -27 -29

LOG (Fe^{3+})

Fig. 1. Effect of decreasing Fe^{3+} activity on chlamydospore germination of *Fusarium oxysporum* f. sp. *cucumerinum*. **A,** Comparison of fungal spores alone and in the presence of *Pseudomonas putida* A12. **B,** Comparison of fungal spores alone and in the presence of *Pseudomonas putida* A1/UV/AB-6, a nonsiderophore-producing mutant. Ninety-five percent confidence intervals of the regression lines are shown.

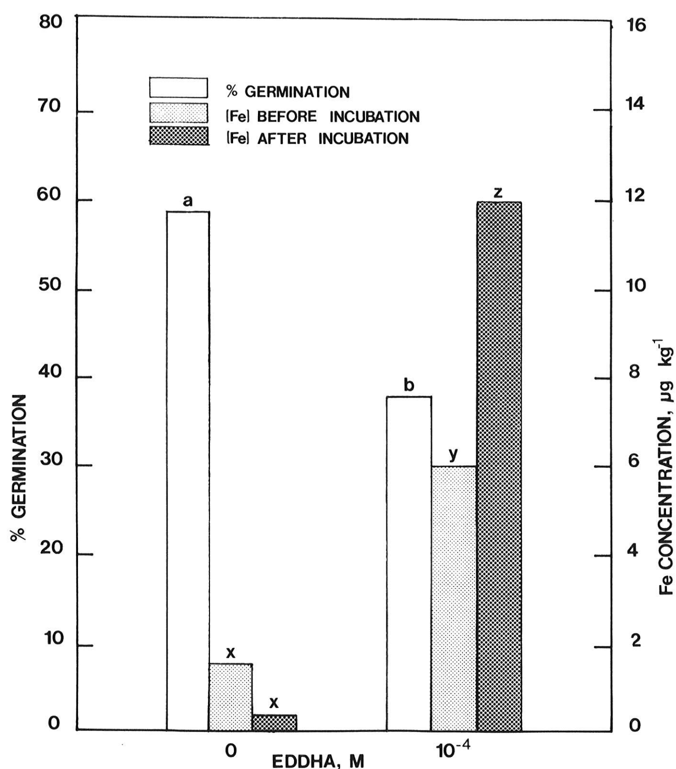

Fig. 2. Effect of added ethylenediamine di-o-hydroxyphenlyacetic acid on chlamydospore germination of *Fusarium oxysporum* f. sp. *cucumerinum* in the presence of *Pseudomonas putida* A12 in solutions containing no added Fe. Changes in nutrient solution Fe concentration are also shown. Columns headed with different letters for a given variable differ statistically at $P = 0.05$.

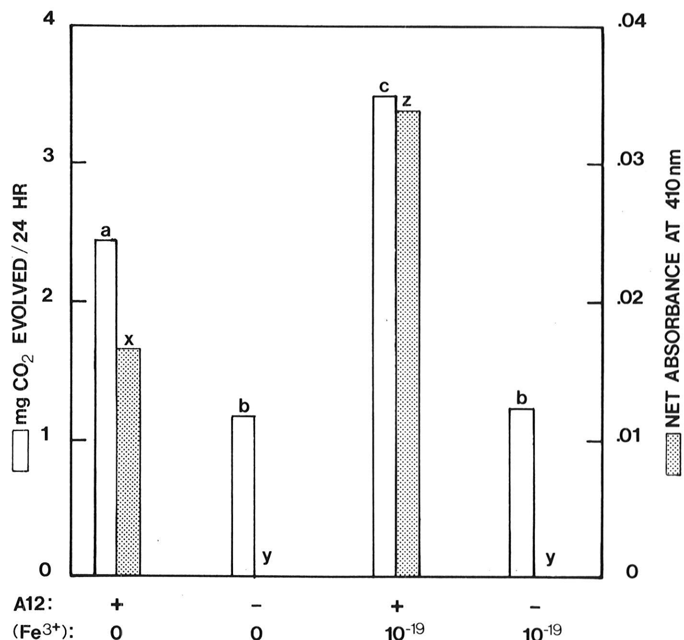

Fig. 3. Influence of different Fe^{3+} activities and the presence of *Pseudomonas putida* A12 on microbial activity (CO_2 evolved) and siderophore production (absorption at 410 nm). Columns headed with different letters for a given variable differ statistically at $P = 0.05$.

Through careful manipulation of Fe^{3+} activity in nutrient solution, the determination of the critical Fe level necessary for suppression of germination of spores of *F. o.* f. sp. *cucumerinum* by *P. putida* A12 in this system was achieved. When Fe^{3+} activity was high, there was enough available Fe for both the fungal pathogen and the bacterial antagonist. As Fe^{3+} activity dropped between 10^{-22} and 10^{-27} M, Fe competition between the two organisms was enhanced and germination of *F. o.* f. sp. *cucumerinum* was significantly decreased (Fig. 1). The critical Fe^{3+} below which chlamydospore germination was suppressed was between 10^{-19} and 10^{-22} M.

As Fe^{3+} activity was decreased below 10^{-27} M, germination suppression of *F. o.* f. sp. *cucumerinum* by the pseudomonad isolate also decreased (Fig. 1). One explanation for this behavior could be that the smaller bacterial cells do not contain as large an internal Fe supply as the larger fungal chlamydospores. Siderophores are secondary metabolites (9), and their production depends on overall metabolic activity. Under extreme Fe stress, *F. o.* f. sp. *cucumerinum*, which contains 0.06 ng of Fe per chlamydospore (2), would have a competitive advantage over the pseudomonad isolates, which face a reduction in metabolic rate and in siderophore production. The significant reduction in both overall respiration and siderophore production by *P. putida* A12 as Fe was decreased to extremely low levels (Fig. 3) supports this explanation. An Fe^{3+} activity between 10^{-22} and 10^{-27} M is, thus, the optimal range for competition between *P. putida* A12 and *F. o.* f. sp. *cucumerinum*.

In a well-aerated soil, the solubility of Fe is generally controlled by a ferric hydroxide, which is referred to as soil-Fe (8). In a soil at pH 7.6 in equilibrium with soil-Fe, the calculated Fe^{3+} activity is 10^{-20} M (8). When all inorganic ferric hydrolysis species are considered, this corresponds to a total solution Fe concentration of 10^{-10} M (8). Thus, the critical Fe^{3+} level, between 10^{-19} and 10^{-22} M, associated with germination suppression of *F. o.* f. sp. *cucumerinum* by *P. putida* A12 could occur in a natural soil solution. The Fe^{3+} activity level for optimal suppression, between 10^{-22} and 10^{-27} M, could occur in the rhizosphere when plant uptake and microbial utilization are considered.

Additional analysis of the system under extreme Fe stress permits quantification of the Fe requirement for chlamydospore germination of *F. o.* f. sp. *cucumerinum* in the presence of *P. putida* A12. During experimental procedures and the 12-hr incubation, death of some organisms is expected. This microbial turnover would lead to a release of Fe to the system. When no Fe or EDDHA was added to the nutrient solution, some of the microbially released Fe became available to *F. o.* f. sp. *cucumerinum*, and chlamydospore germination was comparable to that obtained under the maximum Fe^{3+} activity of 10^{-19} M (Figs. 1 and 2). Iron concentration in this nutrient solution before and after the 12-hr incubation was insignificant (Fig. 2), thus, any Fe released was used by the organisms on the membrane and did not remain in solution.

In contrast, Fe from microbial turnover was not as available to *F. o.* f. sp. *cucumerinum* when EDDHA was in the solution. Released Fe was held in solution by the chelate, which has a much

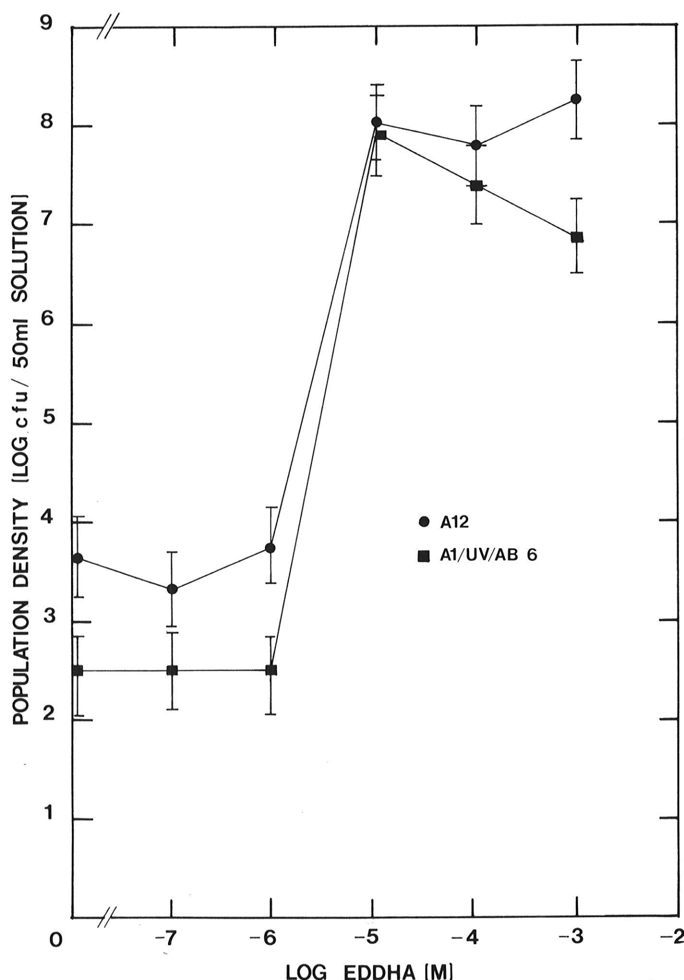

Fig. 4. Effect of increasing amounts of ethylenediamine di-o-hydroxyphenylacetic acid in nutrient solution on the population density of *Pseudomonas putida* strains after a 12-hr incubation period. Initial population density added to 50 ml of solution was $10^{7.6}$. Ninety-five percent confidence intervals of individual means are shown.

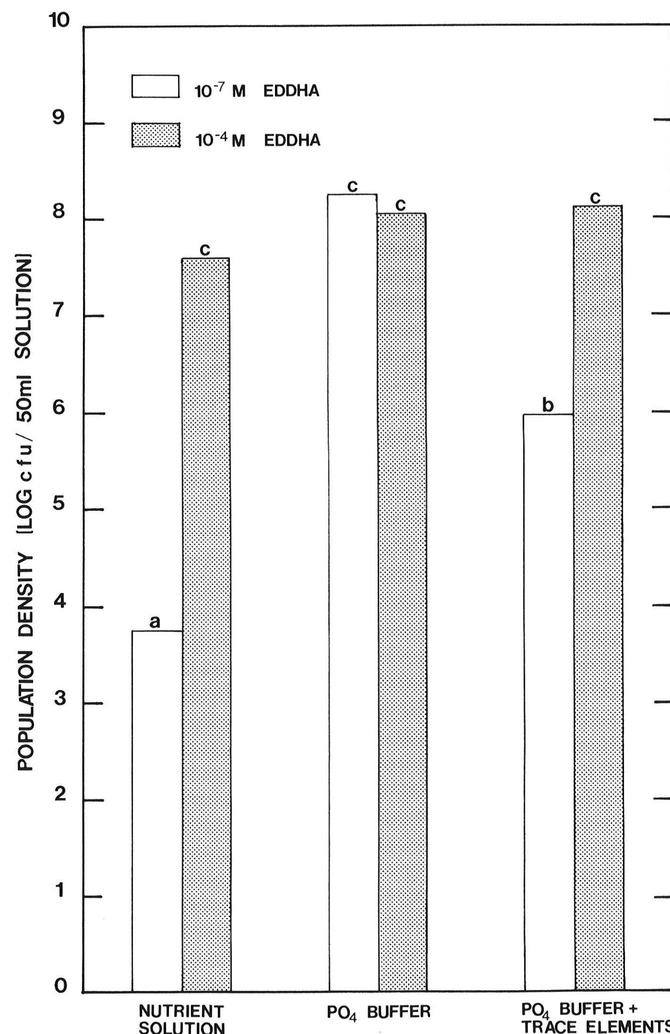

Fig. 5. Effect of low and high amounts of ethylenediamine di-o-hydroxyphenylacetic acid in three different solutions on the population density of *Pseudomonas putida* A12 after 12-hr incubation. Initial population added to 50 ml of solution was $10^{7.6}$. Trace elements include Cu, Zn, Mn, B, and Mo. Columns headed with different letters differ statistically at $P = 0.05$.

stronger affinity for Fe than the Fusarium siderophore (11). The 6 μg of Fe per kilogram increase after incubation (Fig. 2) was a measure of the Fe necessary to increase chlamydospore germination of *F. o.* f. sp. *cucumerinum* by 21%. The 6 μg of Fe per kilogram of nutrient solution that was measured before incubation is probably the result of EDDHA scavenging Fe from the glucose source and keeping this Fe in solution.

Toxicity of the nutrient solution to the pseudomonad isolates when EDDHA is absent or present in low concentrations (Fig. 4) indicated that other elements may have affected the competitive mechanism. This toxicity also helps explain the reduction in respiration and siderophore production of the pseudomonad isolates under extreme Fe stress, as the solutions used did not contain EDDHA. The toxic effects were partly alleviated by the addition of Fe to the solution (Fig. 3), but not enough to affect chlamydospore germination of *F. o.* f. sp. *cucumerinum* significantly.

The detrimental effect of the trace element addition to a simple phosphate buffer on the population density of *P. putida* A12 (Fig. 5) suggests that these were the elements responsible for the observed toxicity. Both Cu^{2+} and Zn^{2+} are strongly bound by EDDHA in the absence of Fe^{3+} (8). Because Cu^{2+} is present in the solution at $10^{-6.5}$ M and Zn^{2+} at $10^{-5.8}$ M, the addition of at least 10^{-5} M EDDHA should be enough to bind these elements, render them unavailable to the bacteria, and relieve their toxic effects.

The Zn/Cu ratio in a soil solution at pH 7 in equilibrium with reported soil-Zn and soil-Cu is around 1,000/1 (8). The Zn/Cu ratio in the two-fifths Hoagland solution, however, is 5/1. This relatively large increase in Cu could account for the toxic effects of the nutrient solution to a bacterium whose natural environment more closely resembles that of soil solution. Ecker et al (1) have noted a decrease in fungal siderophore synthesis brought on by the presence of $10^{-7.1}$ M Cu in a growth medium. Clearly, the interactions of trace elements on the competition between fungal and bacterial siderophores are complex and need further study.

The mechanism of biological control of Fusarium wilt by Fe competition induced by a fluorescent pseudsomonad is a complicated interaction, which is affected by soil minerals, pH and Fe levels, siderophore production, and plant roots. By simplifying the system, quantification of the competitive response is possible. A knowledge of the Fe^{3+} activity necessary for optimal biological suppression could have many applications in the future. Maintenance of the proper Fe level during selection of potential bacterial antagonists is one such application. Another is the use of the value as a predictor for whether biological suppression is possible when other factors affecting the mechanism are known.

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