

Effect of *Pseudomonas putida* and a Synthetic Iron Chelator on Induction of Soil Suppressiveness to Fusarium Wilt Pathogens

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ABSTRACT

Scher, F. M., and Baker, R. 1982. Effect of *Pseudomonas putida* and a synthetic iron chelator on induction of soil suppressiveness to Fusarium wilt pathogens. *Phytopathology* 72:1567-1573.

When *Pseudomonas putida* isolated from a Fusarium-suppressive soil, ethylenediaminedi-*O*-hydroxyphenylacetic acid (EDDHA), or its ferrated form (FeEDDHA), was added to conducive soil, the soil became suppressive to Fusarium wilt pathogens of flax, cucumber, and radish. Experimental evidence suggested that competition for iron (Fe) was responsible for the suppressiveness in these systems since Fe appeared to be necessary for germ-tube elongation of *Fusarium oxysporum* f. sp. *lini* microconidia. FeEDDHA and siderophores produced by *P. putida* were considered to have higher Fe stability constants than siderophores shown to be produced by various pathogenic fusaria. Suppressiveness was not

Additional key words: biological control, *Fusarium roseum*.

induced by DTPA or FeEDTA, which have lower Fe stability constants than FeEDDHA. Suppressiveness from adding both *P. putida* and FeEDDHA to conducive soil was additive, but not interactive, in short-term experiments. *P. putida* did not produce fluorescent siderophores on agar medium containing 10^{-4} M Fe unless EDDHA or DTPA (2 mg/ml) was added. This indicated that these ligands created an iron deficiency in the medium. Presence of EDTA in the agar medium inhibited production of fluorescent siderophores. It is suggested that the management of Fe availability in the infection court, through Fe competition, can induce suppressiveness to Fusarium wilt pathogens.

Soil in the Salinas Valley of California is suppressive to Fusarium wilt (2,9,16,17,21). A fluorescent *Pseudomonas* sp. isolated from this soil induced suppressiveness to *Fusarium oxysporum* f. sp. *lini*, the flax wilt pathogen, when added to conducive soil (16).

Fluorescent pseudomonads produce siderophores, which are high-affinity Fe³⁺ chelators, that enhance the microbial acquisition of iron (Fe) in iron-deficient environments (11). Kloepper et al (5) suggested that soils suppressive to Fusarium wilt pathogens contain siderophore-producing microorganisms that complex Fe and, thus, make it less available to microflora not capable of producing comparable Fe-transport agents.

The objectives of the present investigation were to study the interrelationships between a fluorescent pseudomonad biocontrol agent comparable to that isolated in the previous study (16), and

the status of Fe when suppressiveness is induced, and to test the hypothesis that competition for Fe³⁺ is a mechanism explaining the biological control observed in Fusarium-suppressive soils.

MATERIALS AND METHODS

Fusarium wilt-conductive soil used in these investigations was a sandy loam with the following characteristics: pH 7.3; lime, 2%; conductivity, 0.4 mmhos/cm; organic matter, 1.1%; NO₃-N, 1 μg/g; P, 9 μg/g; K, 196 μg/g; Zn, 0.5 μg/g; and Fe, 3.2 μg/g. These properties were essentially similar to the physical characteristics of a Fusarium-suppressive Metz fine sandy loam taken from California (16). The conducive soil was stored outside in an exposed bin. Prior to use, it was air-dried and passed through a 4-mm screen. Storage of the suppressive soil was described previously (16).

Substrate for growth of Fusarium inoculum was prepared in 1-L flasks by soaking whole oats in distilled water and allowing imbibition for 1–2 hr. Excess water was decanted and the oats were autoclaved for 1 hr on two consecutive days. Fusarium mycelium was aseptically introduced into the flasks and allowed to colonize the oats for 3–4 wk. Fusaria used were *F. oxysporum* Schlecht.

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emend Snyder & Hans. f. sp. *lini* for flax, f. sp. *conglutinans* (Wr.) Snyder & Hans. for radish, and f. sp. *cucumerinum* for cucumber. The fungus-oat mixture was air-dried, triturated to a fine powder in a Waring Blendor model #5011 (Waring Products, New Hartford, CN 06057), and passed through a 1-mm screen. The powder obtained from the contents of one flask was added to 3 kg of moistened soil that had been autoclaved for 1 hr on two consecutive days. The infested soil was covered and incubated at 25 C for 2 wk to allow the fungus to permeate the soil. The inoculum mixture was air-dried, passed through a 1-mm sieve, and stored in loosely covered plastic containers. The number of mixed propagules of *Fusarium* in the inoculum was determined to be approximately 10^5 colony-forming units (cfu) per gram by a series of 10-fold dilutions onto Komada's medium (6).

The inoculum was added to air-dry conducive soil at the rate of 1,000 cfu of *F. oxysporum* f. sp. *lini*, 200 cfu of *F. oxysporum* f. sp. *cucumerinum*, or 50 cfu of *F. oxysporum* f. sp. *conglutinans* per gram of soil and evenly mixed in a twin-shell blender. These concentrations of inoculum were chosen since they resulted in approximately 50% disease incidence of flax, cucumber, or radish, respectively, by the end of most experiments. After adjusting the soil moisture to -2 bars matric potential by addition of water or the appropriate treatment solution, 250-g portions of infested soil were placed in each of four plastic pots (11-cm-diameter top, 9-cm-diameter bottom, and 7 cm high). Soil in each pot was planted with 15 flax seeds (*Linum usitatissimum* L. 'Taichung #1'), six cucumber seeds (*Cucumis sativus* L. 'Straight Eight'), or 15 radish seeds (*Raphanus sativus* L. 'Scarlet Globe'). After emergence, the seedlings were thinned to 10 per pot for flax and radish, and to five for cucumbers. Pots were placed in a completely random design on benches under continuous illumination supplied by fluorescent lamps (500 lux) at 27 ± 2 C. Plants were watered every day with a nutrient solution described previously (16).

The number of wilted plants was counted every few days; the frequency with which data were recorded depended on the rate of wilting. Seedlings with disease symptoms were pulled and discarded or surface disinfested and plated on Komada's medium (6) to ensure correct diagnosis. Data were subjected to analysis of variance; a Duncan's multiple range test was performed for separation of treatment means. All experiments were performed at least twice.

Isolation and identification of fluorescent pseudomonads. Five nylon screens (1 cm²) with 0.5-mm² holes were placed on potato-dextrose agar culture plates. *F. oxysporum* f. sp. *lini* was introduced into the center of each plate. After 1 wk, the screens, which were covered with hyphae and spores of the fungus, were buried around the roots of flax seedlings growing in suppressive Metz fine sandy loam. The screens were retrieved after 24 hr, rinsed with water, and placed on King's Medium B (KB) agar (4). *Pseudomonas* spp. fluorescing on the agar were transferred to KB. Pure cultures were incubated for 24 hr, then washed off with 10% skim milk, and lyophilized for storage.

Introduction of pseudomonads into soil. Pseudomonads were grown in KB broth on a wrist-action shaker at 28 C for 24 hr. Cells were collected by centrifugation (2,500 g) and were rinsed with, and resuspended in, 0.1 M MgSO₄ (18). Standardized suspensions were prepared based on optical density and a standard absorbance curve at 780 nm.

To add the *Pseudomonas* to soil, the soil moisture was brought up to approximately -2 bars matric potential with the bacterial suspension and the soil was thoroughly mixed by hand. The same volume of 0.1 N MgSO₄ was added to all plants that received no *Pseudomonas*.

Germination of microconidia of *Fusarium* in vitro. Microconidia were produced by growing *F. oxysporum* f. sp. *lini* in potato-dextrose broth on a wrist-action shaker at 28 C for 1 wk. Spores and hyphae were centrifuged (2,500 g) and rinsed five times with sterile water. Hyphae were separated from spores by filtration three times through four layers of sterile cheesecloth.

Filter-sterilized iron chelator ligands were added (1 mg/ml) to molten 2% Noble water agar. Ethylenediaminetetraacetic acid (EDTA) and diethylenediaminetetraacetic acid (DTPA) were

dissolved in water and adjusted to pH 5.5 by the addition of 1 N NaOH. Ethylenediaminedi-*O*-hydroxyphenylacetic acid (EDDHA) was dissolved in 0.1 N NaOH and adjusted to pH 9 with 1 N HCl (14). No chelators were added to the controls. The agar of all treatments was adjusted to pH 6 with 1 N HCl or NaOH and allowed to solidify. It was remelted after 24 hr and poured into sterile plastic petri plates (20 ml per plate). Plates were incubated at 25 C for 3 days to allow metal chelation before microconidia (5,000 per plate) were placed on the agar surface.

Germination percentages and germ tube lengths were recorded after 16 and 30 hr. In some cases, 1-ml volumes of 10^{-2} M FeCl₃ or 10^{-2} M MnCl₂, or water were added to the plates after 16 hr. There were three plates per treatment.

Addition of iron chelators to soil. FeEDTA, FeEDDHA, or EDDHA were added to conducive soil infested with *Fusarium* and with or without *Pseudomonas*. EDDHA was dissolved as before, whereas FeEDTA and FeEDDHA were dissolved in water before addition to soil. When *Pseudomonas* was added to soil, the chelators were mixed into the cellular suspension before addition. In one case, chelators were added to soil that had been autoclaved for 1 hr on two consecutive days (steamed soil) and plants were watered with filter-sterilized nutrient solution.

Siderophore production. Siderophore production by *Pseudomonas* and various fusaria was determined by growing them in low-Fe medium containing 20 g sucrose, 2 g L-asparagine, 1 g K₂HPO₄, 0.5 g MgSO₄·7 H₂O to 1 L of water (pH 7.0). Fe was added (0.1 g of FeCl₃ per liter) to the medium in half the treatments. The test organisms were introduced into 50-ml portions of the medium and incubated on a wrist-action shaker at 27 ± 2 C. After 24 hr for *Pseudomonas* or 1 wk for *Fusarium*, the suspensions were centrifuged (2,500 g). Supernatants were passed through a 0.4- μ m polycarbonate membrane filter (Nuclepore, Pleasanton, CA 94566) to remove residual cells, and the pH of each filtrate was adjusted to 5.5. Each supernatant was added to two spectrophotometer tubes (3 ml per tube). Fifteen microliters of 10^{-2} M FeCl₃ was added to one tube, whereas the other tube, to which no iron was added, served as the blank. Absorbance at various wavelengths was read against the blank with a Bausch & Lomb Spectronic 20 spectrophotometer (Bausch & Lomb, Rochester, NY 14625).

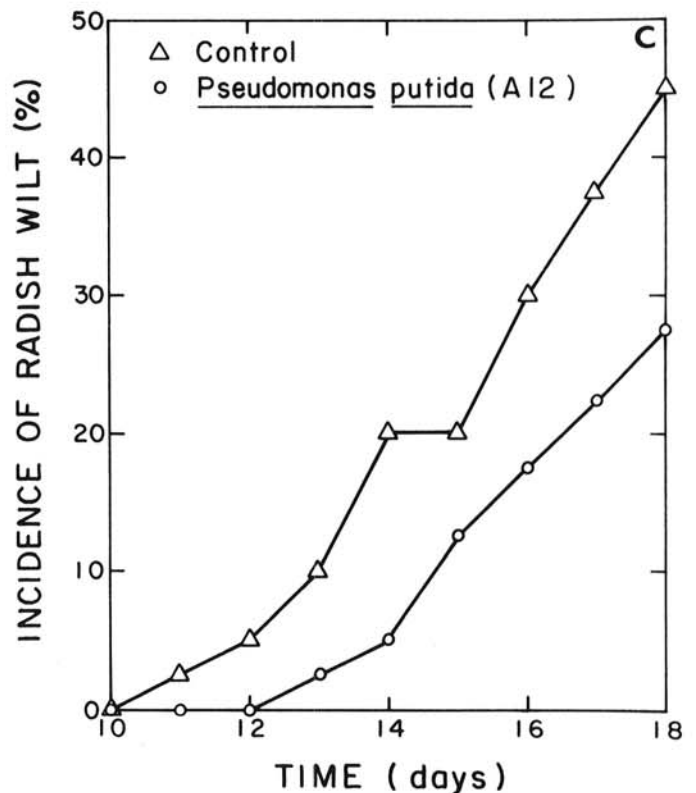
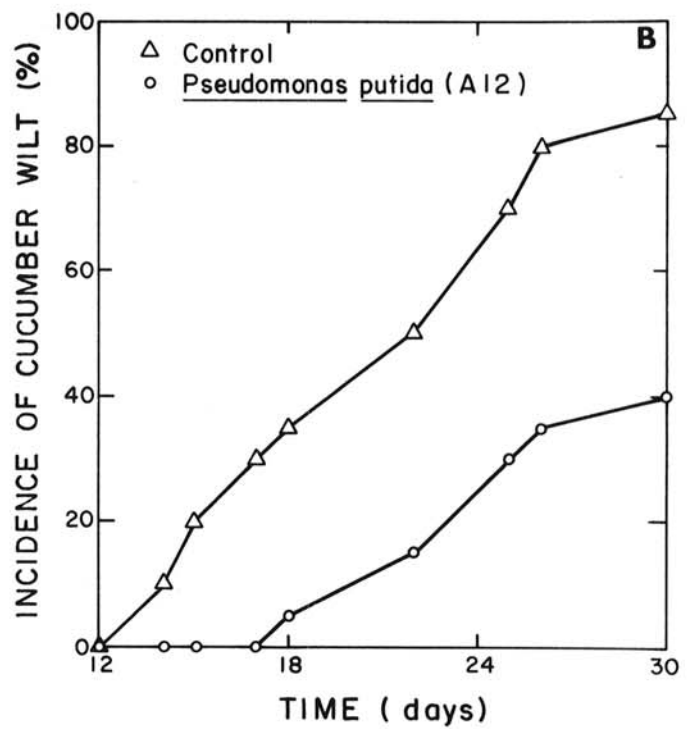
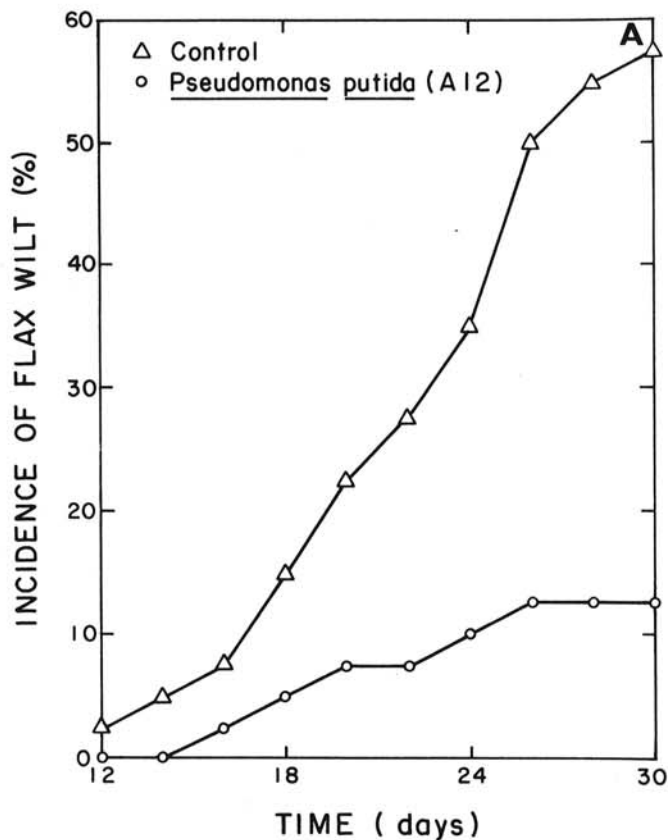
To assess siderophore production by *Pseudomonas* in vitro as affected by iron chelators, filter-sterilized chelators were added to molten KB agar (2 mg/ml agar). FeCl₃ was added to half the treatments to a final concentration of 10^{-4} M Fe. After 24 hr, *Pseudomonas* was streaked on the solid agar. Plates were incubated at 25 ± 1 C for 48 hr, at which time the fluorescence of the bacterial colonies and surrounding agar was evaluated by using an ultraviolet lamp (Blak-Ray Lamp, 366 nm, UV Products, San Gabriel, CA 91778). Fluorescence was considered an indication of siderophore production (19).

RESULTS

Numerous colonies of fluorescent bacteria were observed on KB around nylon screens taken from the rhizospheres of flax roots growing in the Metz fine sandy loam 24 hr after their placement on the agar. Twelve distinct colonies were selected and coded A1, A2, ... A12.

Introduction of *Pseudomonas* into soil as a control for *Fusarium* wilt disease. Isolates A1 through A12 were added at 10^7 cells per gram to soil infested with *F. oxysporum* f. sp. *lini*. Flax wilt incidence after 30 days was 42.5% in the control and 10.0% in the presence of isolate A12. Disease incidence with the addition of other isolates ranged from 12.5 to 35.0% (unpublished). Isolate A12 was selected for use in further experiments due to the low incidence of disease in the A12 treatment. By standard bacteriological testing, it was identified as *Pseudomonas putida* (Trevisan) Migula.

Isolate A12 was added to *F. oxysporum* f. sp. *lini*-, *F. oxysporum* f. sp. *cucumerinum*-, and *F. oxysporum* f. sp. *conglutinans*-infested raw, conducive soil. Incidence of wilt over time is given in Fig. 1. There was a lag period of 2-5 days before disease symptoms appeared in the A12 treatment as compared with the control in each



host system. Addition of *P. putida* (A12) resulted in significant ($P=0.05$) disease control at the end of experiments with all three hosts. Disease incidence was lowered to 22% of that in the control for flax, 40% for cucumbers, and 61% for radish by treatment with *P. putida*.

Effect of iron chelators on the germination of *Fusarium oxysporum* f. sp. *lini* microconidia. After 16 hr of incubation at 25 C, microconidial germination on the Noble water agar plus chelators was evaluated. Although approximately 80% of the conidia germinated in all treatments, there was a striking difference in germ tube lengths among the treatments. Germ tube lengths were significantly ($P=0.05$) less in plates that had received the EDDHA, EDTA, and DTPA treatments than in the controls. Lengths averaged 166 μm in the control of one experiment, but only 66 μm with EDDHA and 56 μm with EDTA. The average length was 164 μm in the control of another experiment, but only 114 μm when DTPA was added.

FeCl_3 , MnCl_2 , or H_2O was added 16 hr after conidia were placed on the agar surface and germ tube lengths were recorded at 30 hr. Increase in germ tube lengths during the ensuing 14 hr are given in Fig. 2. Mean germ tube length in the control plus H_2O (Fig. 2A) increased 395 μm . Addition of FeCl_3 or MnCl_2 did not result in a significant ($P=0.05$) change. Mean length in the EDTA- H_2O treatment increased only 16 μm but the addition of FeCl_3 or MnCl_2 significantly increased this. Mean length in the EDDHA- H_2O treatment was 83 μm . Addition of MnCl_2 had no effect; however, FeCl_3 significantly increased germ tube length. This was not significantly different ($P=0.05$) from the chelator-free controls. Thus, germ tube inhibition by EDDHA was completely reversed by the addition of FeCl_3 .

In a similar experiment, addition of DTPA to the medium significantly inhibited germ tube growth for between 16 and 30 hr (Fig. 2B) compared with controls. Addition of FeCl_3 partially reversed the inhibition, whereas MnCl_2 completely reversed it.

Effect of iron chelators on *Fusarium* wilt incidence. FeEDTA, FeEDDHA, or EDDHA at 100 $\mu\text{g/g}$ was introduced into conducive soil infested with *F. oxysporum* f. sp. *lini*. *P. putida* (A12) was added to half the treatments. Mean incidence of flax wilt after 30 days is given in Fig. 3A. Addition of *P. putida* to soil

Fig. 1. Effect of addition of *Pseudomonas putida* (A12) at 10^7 cells per gram of soil on *Fusarium* wilt incidence in A, *Fusarium oxysporum* f. sp. *lini*-, B, f. sp. *cucumerinum*-, and C, f. sp. *conglutinans*-infested conducive soil. No *P. putida* was added to the control. Wilt incidence in the *P. putida* treatments at the end of each experiment was significantly different ($P=0.05$) from the control for all three host systems. Data represent mean disease incidence of 40 (A and C) or 20 (B) plants.

reduced disease incidence from 60 to 10%. EDDHA and FeEDDHA also reduced disease incidence significantly ($P=0.05$) as compared with the respective control. Greatest reduction in disease incidence consistently was observed when EDDHA or FeEDDHA and *P. putida* were added, although not significantly so when compared with the *P. putida* treatment alone. Addition of

FeEDTA nullified biological control by *P. putida*, confirming a previous report (5), and increased disease incidence to a level significantly higher than in the control.

A similar experiment was performed with radish. *F. oxysporum* f. sp. *conglutinans*-infested soil disease incidence after 18 days is given in Fig. 3B. Addition of FeEDTA nullified biological control by *P. putida*. Wilt incidence was reduced from 45% in the control to 20% when FeEDDHA was added, and to 10% with both FeEDDHA and *P. putida*. Addition of DTPA (100 $\mu\text{g/g}$) to *F. oxysporum* f. sp. *lini*-, *F. oxysporum* f. sp. *conglutinans*-, or *F.*

oxysporum f. sp. *cucumerinum*-infested soil had no effect on biological control of *Fusarium* wilts by *P. putida* (unpublished).

FeEDDHA alone was added to *F. oxysporum* f. sp. *lini*- and *F. oxysporum* f. sp. *conglutinans*-infested soil at 5, 10, 50, and 100 $\mu\text{g/g}$ to determine the level of chelator necessary to reduce disease incidence. In both host systems, 50 $\mu\text{g/g}$ was the minimum level of FeEDDHA tested that significantly ($P = 0.05$) reduced disease incidence (Fig. 4). Disease reduction observed by addition of 50 $\mu\text{g/g}$ was not significantly different from that of 100 $\mu\text{g/g}$ in either system.

Siderophore production. Siderophores produced by *P. putida* in the low-Fe medium were recognizable by their distinct yellow-green pigment and fluorescence. Absorbance of the culture filtrates

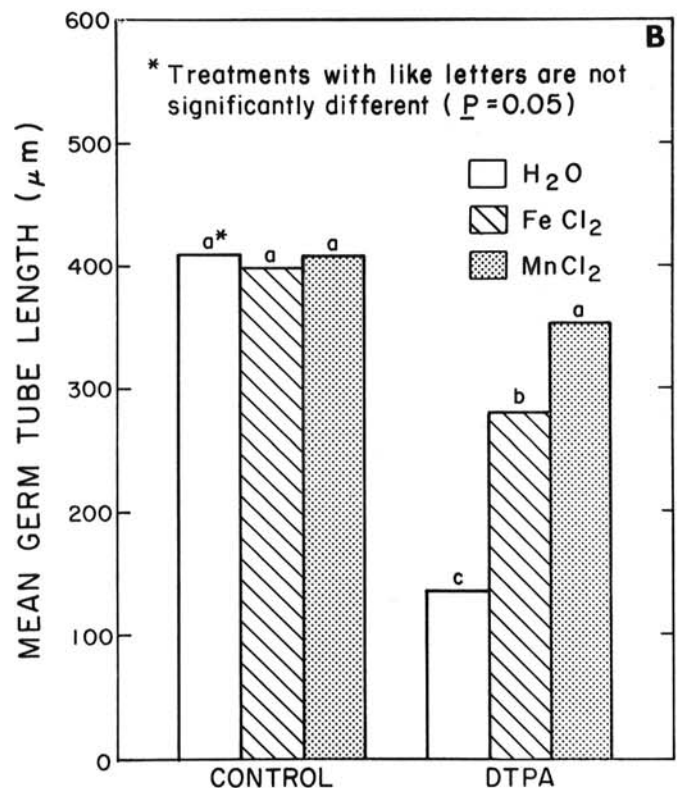
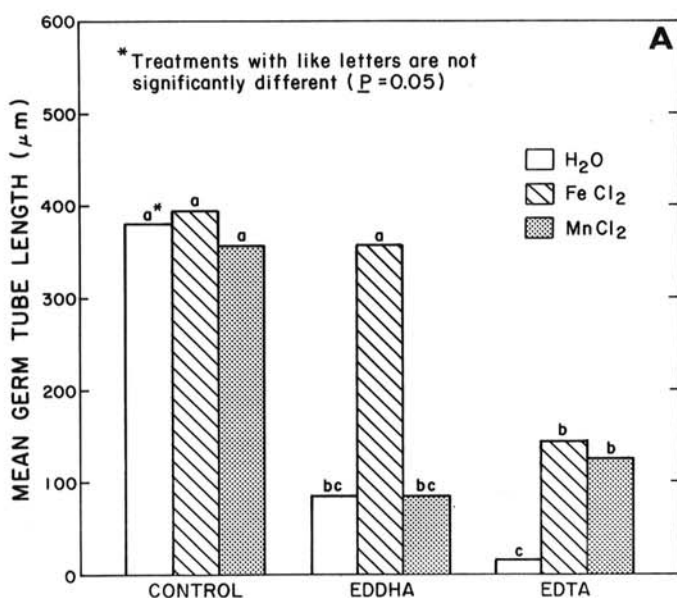


Fig. 2. Germ tube growth between 16 and 30 hr after placement of microconidia of *Fusarium oxysporum* f. sp. *lini* on 2% Noble water agar containing A, EDTA, EDDHA, or B, DTPA. The controls did not contain chelators. FeCl₃, MnCl₂, or H₂O was added 16 hr after conidia were placed on the agar surface. Data are means of six replicates, three from each of two trials.

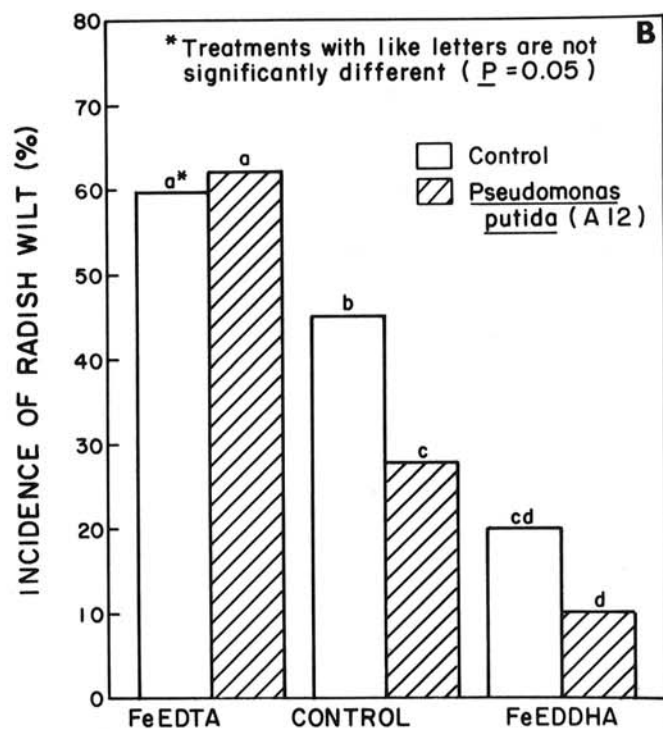
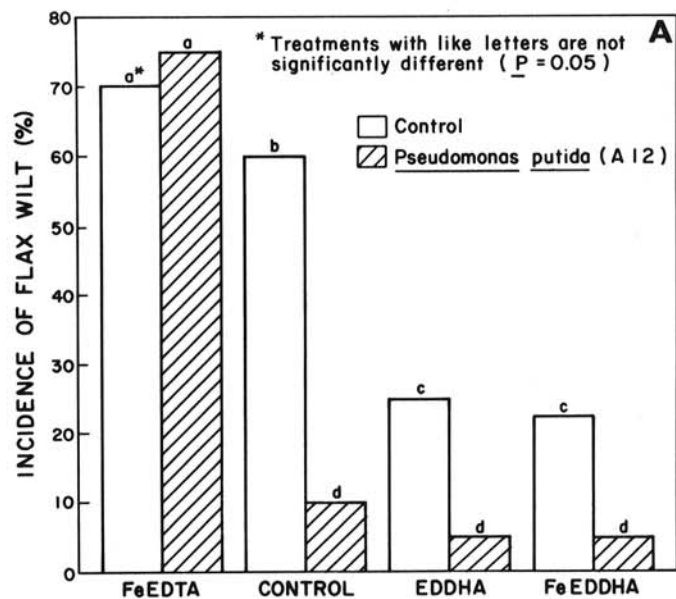


Fig. 3. Mean *Fusarium* wilt incidence when iron chelators (100 $\mu\text{g/g}$ soil) were introduced into conducive soil infested with A, *Fusarium oxysporum* f. sp. *lini* and B, f. sp. *conglutinans* with or without *Pseudomonas putida* at 10^7 cells per gram of soil. Data represent mean disease incidence of 40 plants.

was read from 350 to 500 nm after addition of FeCl_3 . Peak absorbance for the siderophore was at 410 nm (Fig. 5). No peak at 410 nm occurred when Fe was added to the sterile filtrate if Fe previously had been added to the culture medium.

Absorbance at 350–500 nm for culture filtrates of *F. oxysporum* f. sp. *lini*, *F. oxysporum* f. sp. *cucumerinum*, *F. oxysporum* f. sp. *conglutinans*, *F. oxysporum* f. sp. *dianthi* (Prill et Del.) Snyder & Hans., and *F. roseum* emend Snyder & Hans. cultivar *culmorum* was recorded. Each showed a broad absorption peak at 440 nm when Fe was added to the filtrate. No peak was observed for culture filtrates from treatments to which iron was added in the culture medium. Absorbance versus wavelength for the culture filtrate plus Fe of *F. oxysporum* f. sp. *lini* is given in Fig. 6.

Effect of iron chelators on fluorescence of *P. putida* in vitro. *P. putida* growing on KB agar produced fluorescent pigment, but not when iron was added to the medium (Table 1). Presence of EDTA in the agar, regardless of whether Fe was added or not, prevented fluorescence. Fluorescence of *P. putida* occurred if EDDHA or DTPA was added to KB plus Fe.

Effect of iron chelator on incidence of Fusarium wilt in plants growing in a steamed soil. Disease control by EDDHA and FeEDDHA in steamed soil was evaluated to determine if control occurred in steamed soil. Both raw and steamed soil were infested with *F. oxysporum* f. sp. *cucumerinum*, and EDDHA or FeEDDHA was added at 100 $\mu\text{g/g}$ to each. The EDDHA was dissolved as described and no chelator was added to the control. Disease incidence at 30 days is given in Table 2. There was 15% greater disease incidence in the steamed soil control as compared to

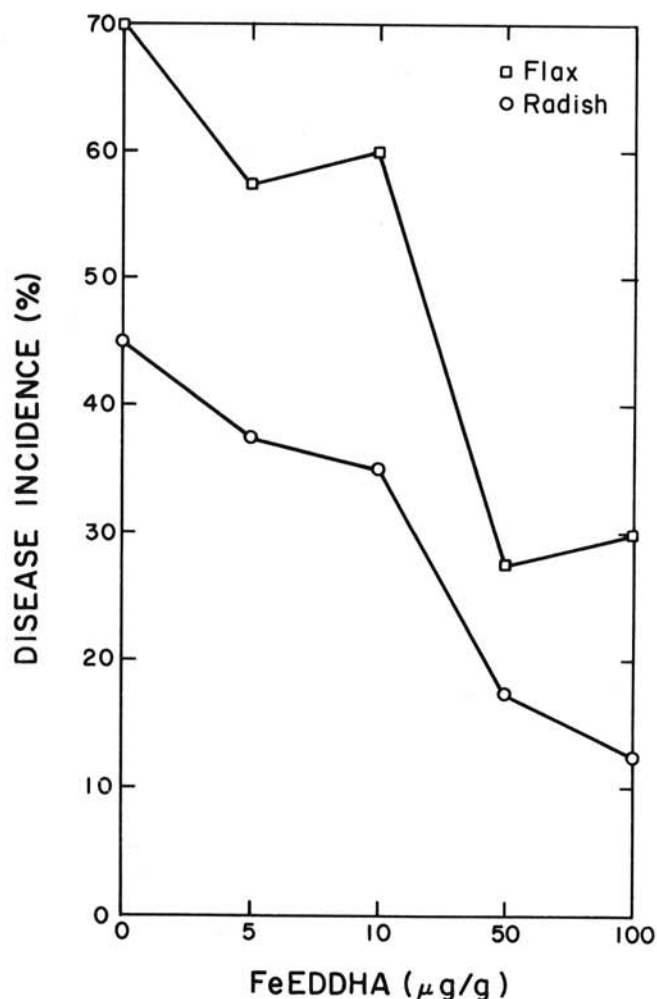


Fig. 4. Mean Fusarium wilt incidence when FeEDDHA was introduced into *Fusarium oxysporum* f. sp. *lini*- or f. sp. *conglutinans*-infested conducive soil at 0, 5, 10, 50, and 100 $\mu\text{g/g}$ soil. Data represent mean disease incidence of 40 plants.

raw soil. EDDHA and FeEDDHA significantly reduced disease incidence in both soils ($P=0.05$). Data, expressed as percentage of the control, were transformed to degrees for the analysis of variance. Results of the analysis indicated that EDDHA or FeEDDHA controlled disease equally as well in raw or steamed soil.

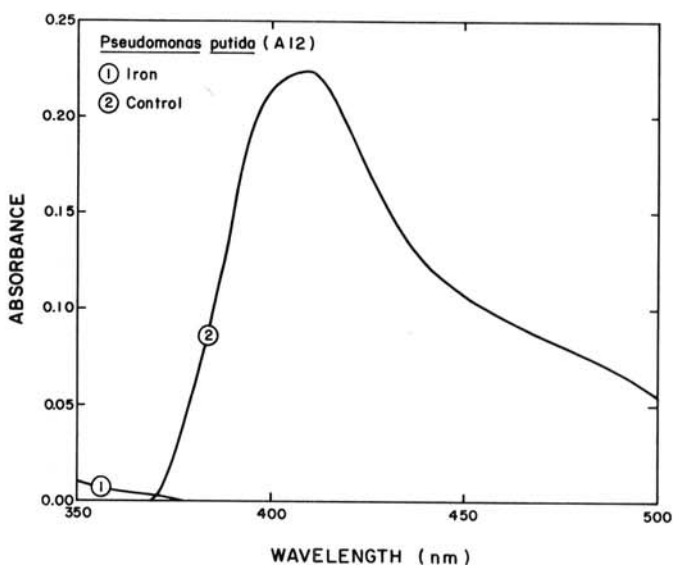


Fig. 5. Absorption spectra for the culture filtrate of *Pseudomonas putida* (A12) when iron (0.5 g FeCl_3/L) was (curve 1) or was not (curve 2) present in the culture medium.

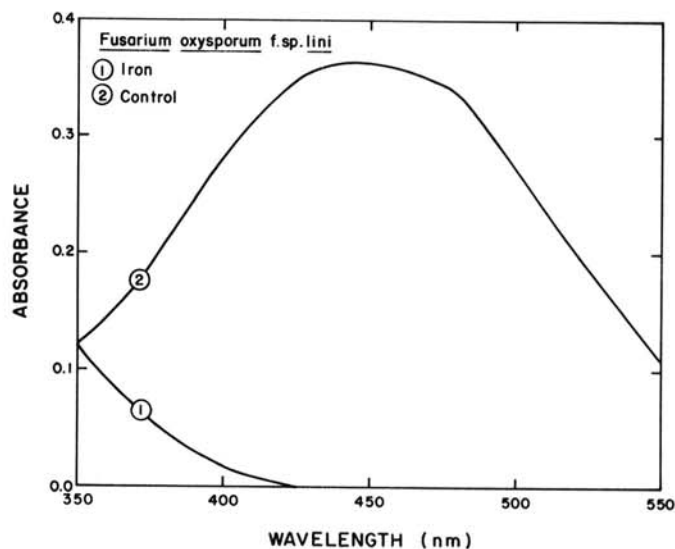


Fig. 6. Absorption spectra for the culture filtrate of *Fusarium oxysporum* f. sp. *lini* when iron (0.5 g FeCl_3/L) was (curve 1) or was not (curve 2) present in the culture medium.

TABLE 1. Presence of UV-fluorescent pigments produced by *Pseudomonas putida* on King's Medium B containing iron chelators^a

| Chelator added | Fluorescence observed | |
|----------------|-----------------------|-----------|
| | Without iron | With iron |
| None (control) | + | - |
| EDTA | - | - |
| EDDHA | + | + |
| DTPA | + | + |

^a Iron (10^{-4}M) as FeCl_3 was added to half the treatments. (+) Signifies fluorescence.

***Pseudomonas* × FeEDDHA interaction in soil.** *P. putida* (A12) was added to *F. oxysporum* f. sp. *cucumerinum*-infested conducive soil at 0, 10³, 15⁵, and 10⁷ cells per gram of soil. FeEDDHA was included in these treatments at 0, 12.5, 25, 50, or 100 µg/g, resulting in a factorial treatment combination. Mean cucumber wilt incidence after 30 days is given in Table 3. Disease incidence was reduced 20% with the addition of *P. putida* at 10⁷ cells per gram, or by FeEDDHA at 100 µg/g. Addition of both *P. putida* and EDDHA at the highest concentration resulted in only 5% disease incidence. An analysis of variance was performed on the data and the treatment sum of squares was partitioned into that for *Pseudomonas*, FeEDDHA, and their interaction. Significant *F* ratios for *P* = 0.05 were obtained for the *P. putida* and FeEDDHA treatments, but not for the FeEDDHA × *P. putida* interaction.

DISCUSSION

To demonstrate mechanisms of competition in biological control, evidence must be established experimentally to confirm the operation of limiting factors affecting pathogenesis. For example, it should be established that the pathogen requires the limiting nutritional factor for germination and subsequent establishment of infection of the host. Only carbon and nitrogen have been established as elements essential in the infection process of soilborne plant pathogens (1). The present study suggests that Fe also may be essential in induction of disease by *Fusarium* wilt pathogens. The percentage germination of microconidia of *F. oxysporum* f. sp. *lini* on Noble water agar was not decreased when EDTA, DTPA, or EDDHA was added; however, these chelators significantly inhibited germ tube elongation. Inhibition by EDDHA was not changed by addition of MnCl₂, but was completely reversed by FeCl₃ (Fig. 2), suggesting that Fe³⁺ was the limiting factor for germ tube elongation. FeCl₃ partially reversed inhibition by EDTA and DTPA. At the substrate pH, EDTA- or DTPA-bound Fe was released from the chelators upon addition of FeCl₃ or MnCl₂; however, EDDHA-bound Fe was not released upon addition of MnCl₂. Thus, only excess addition of Fe nullified inhibition by EDDHA. Misaghi et al (10) reported that a partially purified siderophore of *Pseudomonas* inhibited the growth of various fungi in vitro and that the effect was counteracted by addition of excess iron.

Induction of suppressiveness in soil by addition of EDDHA or *P.*

TABLE 2. Mean incidence of cucumber wilt when chelators EDDHA or FeEDDHA were introduced into raw or steamed conducive soil infested with *Fusarium oxysporum* f. sp. *cucumerinum*^a

| | Cucumber wilt incidence | | | |
|---------|-------------------------|----------------|--------------|----------------|
| | Raw soil | | Steamed soil | |
| | (%) | (% of control) | (%) | (% of control) |
| Control | 70 | ... | 85 | ... |
| EDDHA | 30 | 43 | 40 | 47 |
| FeEDDHA | 25 | 36 | 30 | 35 |

^aFigures are based on disease incidence of five plants in each of four replications per treatment.

TABLE 3. Mean percent incidence of cucumber wilt when FeEDDHA and *Pseudomonas putida* (A12) were added at various levels to *Fusarium oxysporum* f. sp. *cucumerinum*-infested conducive soil^a

| FeEDDHA (µg/g) | <i>Pseudomonas putida</i> (cells per gram of soil) | | | |
|-------------------|--|-----------------|-----------------|-----------------|
| | 0 | 10 ³ | 10 ⁵ | 10 ⁷ |
| 0 | 35 | 35 | 25 | 15 |
| 12.5 | 30 | 30 | 20 | 15 |
| 25.0 | 25 | 25 | 20 | 10 |
| 50.0 | 15 | 20 | 15 | 10 |
| 100.0 | 15 | 15 | 10 | 5 |

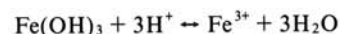
^aFigures are based on disease incidence of five plants in each of four replications per treatment.

putida suggests that competition for Fe could be involved.

The three ligands used in these investigations bind Fe³⁺ at various stability constants: EDDHA, log₁₀ *K* = 33.9 > DTPA, log₁₀ *K* = 27.3 > EDTA, log₁₀ *K* = 25 (8). Also, Fe-binding siderophores have been detected in most of the microorganisms examined for their presence (12). Indeed, the fusaria used in this investigation produced siderophores under Fe-deficient conditions. The broad absorption peak at 440 nm (Fig. 6) suggested that these siderophores are of the hydroxamate class (3), and, thus, may have stability constants in the proximity of log₁₀ *K* = 29 (13). This stability constant is many powers of 10 less than those of most bacterial catechol siderophores (13), and may indicate the relative iron-binding ability of *Fusarium* and bacterial siderophores. *P. putida* produced a siderophore with an absorption peak at 410 nm similar to the 400-nm peak described for the mixed hydroxamate-catechol siderophore pseudobactin by Teintze et al (19).

From a study of these stability constants, therefore, there emerges a hierarchy of Fe-binding compounds, which, hypothetically, can be used to exploit Fe competition in the induction of soil suppressiveness. In the infection court, the siderophores produced by the *Fusarium* wilt pathogens should be able to bind Fe from the soil or FeEDTA, but not from FeEDDHA or a bacterial catechol siderophore. This is not the only factor to consider in the induction of iron competition, however.

The Fe available in soil for growth of plants or microorganisms is defined by the equilibrium reaction (8):



The principal factor mediating the equilibrium of this equation is the pH of the soil; the more alkaline the soil, the less Fe³⁺ is available. The influence of soil pH, therefore, may be considerable in determining whether Fe³⁺ is limiting for the pathogen, and, ultimately, whether a soil is suppressive or conducive. Perhaps an example of this phenomenon was provided by Scher and Baker (16). At the native soil pH of 8.1, there was 4.3 µg/g of DTPA-extractable Fe in the Metz fine sandy loam. This soil was suppressive to *F. oxysporum* f. sp. *lini*. Much of the suppressiveness was lost if the pH was reduced to 7.0. At pH 6.0, the soil was conducive.

We hypothesize that the following mechanisms participate in the phenomenon of Fe competition and contribute to the level of suppressiveness to *Fusarium* wilt pathogens. In a conducive soil, adequate Fe³⁺ is available for germination and penetration through the root tips of the host by the pathogen. Since many *Pseudomonas* spp. have high rhizosphere competence (18), there should be intense competition for Fe³⁺ at the rhizoplane (the infection court) when the *Pseudomonas* produces siderophores. Therefore, Fe³⁺ is bound in such a way that it is unavailable to the *Fusarium*. Thus, application of *P. putida* to soils induced suppressiveness (16; Figs. 1 and 3; Table 3).

The ligand EDDHA apparently has a higher stability constant than the siderophore produced by the pathogen. Fe³⁺ complexed with this ligand in the rhizosphere would be available to the pathogen. EDDHA-bound Fe can be utilized by the host, however. This frees the ligand (EDDHA) to bind more Fe³⁺ at the root surface (7,20). FeEDDHA added to conducive soil induced suppressiveness (Figs. 3 and 4; Table 3). It is interesting that FeEDDHA added to a growth medium improved growth of soybeans beyond the effect of supplying iron (22).

Obviously, this is a dynamic system in which the siderophores produced by pseudomonads, the EDDHA, and the root combine to render Fe³⁺ limiting for pathogenesis by the *Fusarium* wilt microorganisms. How may such competition be demonstrated experimentally? A suggested method is to impose an excess of the limiting factor upon the biocontrol system and notice whether such control is nullified (1). In this case, adding Fe to an alkaline soil does not fulfill the experimental requirements as demonstrated by the equilibrium reaction (above); the amount of available Fe³⁺ is determined by the soil pH. Therefore Fe bound to EDTA was introduced into the system to achieve this effect. At the soil pH of 7.3, Fe³⁺ is released from EDTA, but is temporarily available before

becoming immobilized (7). Thus, soil was conducive when FeEDTA was applied to soil with or without addition of cells of *P. putida* (Fig. 3).

From the Fe equilibrium reaction, it is apparent that more Fe³⁺ is released in soil once Fe is bound to either a siderophore, EDDHA, or is taken up by the plant root. Therefore, the hypothesis outlined above would only allow for limiting quantities of Fe on the rhizoplane where there is intense competition for this element. This suggests that the soil distant from root surfaces should maintain a level of Fe³⁺ determined by soil pH, regardless of addition of ligands. Fe-competition is not expected in the bulk soil and germination of inoculum units of such pathogens should not be inhibited here. Preliminary experiments in which chlamydo spores of *F. oxysporum* f. sp. *lini* were induced to germinate in soil (by the method of Smith and Snyder [17]), with or without the addition of EDDHA, confirmed this extrapolation (15). Chlamydo spores germinated whether conducive soil contained EDDHA or not. Yet, we confirmed the observation of Smith and Snyder (17) that chlamydo spore germination and germ tube development was severely inhibited in the Metz fine sandy loam suppressive soil.

Addition of the ligand EDDHA, but not EDTA, induced the formation of siderophores of *P. putida* in vitro. Also, when EDDHA or FeEDDHA is added to soil, the only microorganisms capable of utilizing the Fe bound to this ligand should be those producing siderophores with a stability constant for Fe higher than that of EDDHA. These factors suggest that such siderophore-producing entities should have a competitive advantage over the rest of the soil microflora in obtaining Fe from EDDHA in soils where Fe³⁺ is in short supply. Thus, besides the Fe competition described above, there could be an added increment of biocontrol induced by EDDHA attributed to increased activity of certain components of the soil microflora. Two methods were used to test this hypothesis. The first approach was to determine whether suppressiveness by FeEDDHA could be induced whether siderophore-producing microorganisms were present or not. Addition of EDDHA or FeEDDHA to steamed soil reduced disease incidence significantly, and control was comparable to that occurring in raw soil (Table 2). In addition, a possible interaction between *P. putida* and FeEDDHA in soil was tested statistically by means of a factorial design (Table 3). The analysis of variance indicated a nonsignificant *P. putida* × FeEDDHA interaction. This demonstrated that disease control by *P. putida* and FeEDDHA was not interactive but was additive. These two lines of evidence suggest that the addition of EDDHA to soil did not selectively stimulate the activity of siderophore-producing biocontrol agents over that occurring in unamended soil in the short-term experiments described in this report.

The isolation of fluorescent pseudomonads responsible for suppressiveness of soils to the Fusarium wilt pathogens and the addition of such biocontrol agents to raw conducive soils for control of disease (16) could represent a significant step in the strategy for application of plant disease control technology. Significant control of flax, radish, and cucumber Fusarium wilt was obtained in these investigations through the application of *Pseudomonas putida* and/or FeEDDHA to soil. By employing management systems that selectively reduce the amount of Fe available to pathogens for initiation of infection, soil suppressiveness may be induced.

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