Influence of Trace Amounts of Cations and Siderophore-Producing Pseudomonads on Chlamydospore Germination of *Fusarium oxysporum*

Yigal Elad and Ralph Baker

Visiting scientist and professor, respectively, Plant Pathology and Weed Science, Colorado State University, Fort Collins 80523. Address of first author: Department of Plant Pathology and Microbiology, Faculty of Agriculture, The Hebrew University of Jerusalem, Rehovot 76100, Israel.

Published with the approval of the director of the Colorado State University Experiment Station as Scientific Journal Series Paper 2964.
Research supported by funds from the Science and Education Administration Project 83-CRCR-1-1259, Western Regional Project W-147, and BARD Research Project US-290-80.
Accepted for publication 10 April 1985.

ABSTRACT

Elad, Y., and Baker, R. 1985. Influence of trace amounts of cations and siderophore-producing pseudomonads on chlamydospore germination of *Fusarium oxysporum*. Phytopathology 75:1047-1052.

A siderophore(s) from several *Pseudomonas* spp. and *P. putida* was concentrated by dialysis and freeze-drying. The concentrated siderophore was active after 3 mo of storage. The cations Mg^{2+}, Mn^{2+}, Ca^{2+}, Co^{2+}, Cu^{2+}, Zn^{2+}, Al^{3+}, K^{1+}, Na^{1+}, NH_4^{1+}, or Ni^{1+} competed with Fe^{3+} on binding sites of the siderophore in vitro; however, affinity of the siderophore to Fe^{3+} was higher than to the other cations. Added cations delayed multiplication of *Pseudomonas* spp. in soil. The concentrated siderophore inhibited germination of chlamydospores of *Fusarium oxysporum* f. sp. *cucumerinum* up to 70.2%. This effect was nullified by an excess of iron in soil. The chelating agent EDDHA (ethylenediaminedi-*O*-hydroxyphenylacetic acid) had similar effects on chlamydospore germination in raw soil as did the siderophore, whereas FeEDDHA was ineffective in raw soil. Both chelators effectively inhibited chlamydospores of *F.o.* f. sp. *cucumerinum* in the rhizospheres of cucumber (*Cucumis sativus*) but did not inhibit germination of *F. solani* f. sp. *phaseoli* in rhizospheres of beans (*Phaseolus vulgaris*).

Competition for iron (Fe) in alkaline soils was proposed as a mechanism for suppression of several *Fusarium oxysporum* formae speciales by *Pseudomonas* spp. (4,12). According to this hypothesis, there is intense competition for binding of Fe^{3+} in rhizosphere soil by pseudomonads and the pathogen; the siderophores bind iron (Fe) so it is not available to the plant pathogen (12). Tientze et al (15) published the structure of a siderophore (pseudobactin) produced by *Pseudomonas putida*.

Sneh et al (14) recently reported that germination of *F. oxysporum* f. sp. *cucumerinum* in soil was inhibited by fluorescent pseudomonads, and available Fe counteracted inhibition of germination. Although Misaghi et al (7) reported that Fe^{3+} and Fe^{2+} were the only microelements modifying siderophore activity in vitro, Sneh et al (14) observed that inhibition also was counteracted to a lesser extent by the addition of trace amounts of Zn^{2+}, Cu^{2+}, and Mn^{2+}; however, the mechanism involved was not clear.

The objective of the present work was to study the effect of siderophores produced by *Pseudomonas* spp. on germination of chlamydospores of *F. oxysporum* in soil and the influence of trace amounts of cations on this process. Another objective was to further elucidate the mechanisms involved in the induction of suppressiveness to *Fusarium* in soil by adding Fe-chelators.

MATERIALS AND METHODS

Bacterial isolates, growth media, and siderophore production. The following strains were used: *Pseudomonas* spp. 346, 2Za, 381, and 61 (14); *P. putida* A12 (12), NIR, and 8c (2). The strains were maintained for short periods on nutrient agar (Becton-Dickinson Corp., Cockysville, MD). Freeze-dried skim milk cultures were used for long-term storage. Siderophore production of these strains was determined by growing the bacteria in low-Fe synthetic medium (SM) containing 20.0 g of sucrose, 2 g of L-asparagine, 1.0 g of K_2HPO_4, and 0.5 g of $MgSO_4 \cdot 7\ H_2O$ (Sigma Chemical Co., St. Louis, MO) in 1 L of distilled water, pH 7 (12). Cultures were grown in 250-ml Erlenmyer flasks each containing 50 ml of SM, at 28 C in a rotary shaker at 60 rpm. Subsequent steps were done in acid-washed glassware. After 24 hr, the liquid cultures were centrifuged at 2,500 *g* for 10 min, supernatants were filtered through 0.4-μm polycarbonate membrane (Nucleopore, Pleasanton, CA), and the pH of each filtrate was adjusted to 5.5 with 0.1 N HCl. Each supernatant was added to two spectrophotometer tubes (3 ml per tube). Ten microliters of fresh 10^{-2} M $FeCl_3$ were added to one tube, and the tube without added Fe served as the control. Absorbance at 410 nm was measured with a Spectronic 20 spectrophotometer (Bausch & Lomb, Rochester, NY).

Concentration of siderophore preparations. Cell-free supernatants of strains A12 or 346 were dialyzed against distilled water for 24 hr and freeze-dried. The freeze-dried preparation was stored at 4 C in the dark until it was dissolved in 0.05 M Na acetate buffer (pH 5.5) at a concentration of 128 μg of protein per milliliter before use. Activity of the concentrates was tested with $FeCl_3$ as mentioned above for crude siderophore preparations.

Assays for measuring the interaction of trace cations with siderophores. The following salts were used for a cation interaction test: $MnCl_2 \cdot 4\ H_2O$, $CuSO_4 \cdot 5\ H_2O$, $ZnCl_2 \cdot 6\ H_2O$, $CoCl_2 \cdot 6\ H_2O$, $Al_2(SO_4)_3 \cdot 18\ H_2O$, $MgCl_2 \cdot 6\ H_2O$, $CaCl_2 \cdot 2\ H_2O$, $FeCl_3 \cdot 6\ H_2O$, $FeSO_4 \cdot 7\ H_2O$, NiCl, NH_4Cl, LiCl, NaCl, and KCl (Fisher Scientific Co., Fair Lawn, NJ). Cation solutions of 10^{-2} M in distilled water were prepared in acid-washed test tubes. Ten microliters of each solution were mixed with 3 ml of siderophore preparations in acetate buffer and incubated for 1 hr at room temperature. Various volumes of a solution of 10^{-2} M $FeCl_3$ solution were added to the siderophore-cation solution. Solutions to which no Fe had been added served as controls. Optical density was recorded after incubation for 1 hr and also for 4 days at room temperature in the dark.

Chlamydospore germination in soil. Infestation of soil with chlamydospores of either *F. oxysporum* Schlecht. emend. Snyd. & Hans. f. sp. *cucumerinum* or *F. solani* (Mart.) Appel. & Wa. f. sp. *phaseoli* (Burk.) Snyd. & Hans. was done according to Sneh et al (14) in Fusarium wilt-conducive soil (Ascalon sandy loam, pH 7.3)

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. § 1734 solely to indicate this fact.

©1985 The American Phytopathological Society

described previously (12). Soil contained an average of 8.9×10^5 or 8.1×10^4 colony-forming units (cfu) per gram for *F. oxysporum* or *F. solani*, respectively.

Bacterial strain isolates were grown on SM agar, collected, washed twice, resuspended in distilled water to obtain the desired concentrations (by adjusting their densities in a spectrophotometer at 780 nm—OD 0.5 equaled 1.9×10^8 cfu/ml) and kept at 4 C until applied to soil. In rhizosphere-simulation studies, 5-g samples of chlamydospore-enriched soil were placed in 16 × 22.5-cm plastic bags. Aliquots (0.1 ml) of bacterial suspensions, glucose and asparagine nutrient solution (14), trace cation solutions, chelators, or H_2O were mixed with soil at the beginning of experiments in which there was only one application of nutrients. When solutions were applied in pulses, 0.05 ml of the desired solutions were added and mixed six times at equal intervals every 2 hr. Water was added in a similar manner to nonpulsed treatments. Bags were not sealed to prevent uneven accumulation of moisture in the soil. Soil was incubated for 20–24 hr and germinating chlamydospores were stained with Calcofluor New M2R (American Cyanamid Co., Bound Brook, NJ) and assayed under a UV-light microscope (Olympus; Tokyo, Japan) by procedures described by Scher and Baker (13) and Sneh et al (14).

To test germination in the rhizosphere, the chlamydospore-enriched soil was treated as mentioned above and placed on a 76 × 25-mm glass microscope slide. Cucumber (*Cucumis sativus* L. 'Straight Eight') or bean (*Phaseolus vulgaris* L. 'Olathe') seeds, surface disinfested in 2% sodium hypochlorite solution for 1 min and washed in sterile distilled water, were germinated at 25 C on autoclaved moist paper towels in a plastic bag. Roots of 5-day-old germinated seedlings were placed in chlamydospore-infested soils, covered with a second glass slide, and tied with two rubber bands. Slides were incubated in a moist paper towel, placed in a plastic bag and maintained at 28 C. Soil adjacent to the roots was collected and assayed for chlamydospore germination 36 hr later (13,14).

Monitoring bacterial population densities. Recovery of *Pseudomonas* spp. added to soil was accomplished by using appropriate dilutions in the drop plate method (10) on solid SM. Densities were expressed as cfu per gram of dry soil. Population densities of strain 346 were monitored by using spontaneous mutants resistant to rifampicin according to the procedures of Dupler and Baker (2). Raw soil, to which pseudomonads were not added, contained an indigenous population density of fluorescent pseudomonads of 1.2×10^6 cfu/g soil.

Change of soil pH. The hydrogen ion concentration of the Fusarium-wilt conducive soil was altered to pH 3 by treating it with 2 N H_2SO_4. This acidified soil was mixed up to 6.5% with the chlamydospore-infested soil to change its pH to 5.0–6.5. These pH-stabilized acidified soils were incubated for at least 3 days before use. Siderophore preparations or strain 346 were applied and chlamydospore germination was monitored.

Production of chlamydospore suspensions. Mycelial disks were introduced into 50 ml of potato-dextrose broth (PDB; Difco Laboratories, Detroit, MI) in 250-ml Erlenmeyer flasks. Cultures were incubated in a rotary shaker for 5 days at 250 rpm and 27 C. Cultures were sieved aseptically through 10 layers of cheesecloth, centrifuged (5,000 *g*), and rinsed three times in autoclaved H_2O. Conidia were introduced into new PDB and incubated at 27 C for 24 hr. Germinated conidia were placed in filter-sterilized soil extract and were incubated for 7 days at 27 C. The chlamydospores that were produced were separated from mycelial fragments and conidia by sieving through 20 and 7 μm Nitex nylon screen (Tetko, Inc., Elmsford, NY) and homogenized to separate them from other portions of the thalli. Chlamydospore suspensions were stored at 4 C until used.

Incorporation of iron chelators into cultures of *Fusarium*. Ethylenediaminedi-*o*-hydroxyphenylacetic acid (EDDHA) was dissolved in 0.1 N KOH and adjusted to pH 6 with 0.05 N HCl. Siderophore or FeEDDHA and EDDHA solutions were added to suspensions of chlamydospores at the desired concentrations at the time of glucose and asparagine application. Cultures were incubated for 16 hr at 28 C. The incidence of germination of chlamydospores and germ-tube length were measured under a light microscope. Half of each replicate of every treatment was added to two sterile test tubes. In one tube, the suspension was centrifuged and rinsed three times in autoclaved H_2O. Both tubes were incubated at 28 C and chlamydospore germination was recorded after another 7 hr.

In other experiments, aliquots (20 μl) of solutions of the chelating agents were added to 5-g samples of chlamydospore-infested soil. Chlamydospore germination was observed as mentioned above (13,14).

Experiments were repeated at least two times with at least four replicates. Appropriate experimental data were subjected to analysis of variance at $P = 0.05$ followed by Fishers least significant difference tests for mean separations.

RESULTS

Production and concentration of siderophore preparations produced by *Pseudomonas* spp. Production of siderophores by 10 selected strains of *Pseudomonas* spp. was evaluated after 24 hr of growth in SM. Optical density (410 nm) of cell-free growth medium containing 0.33×10^{-4} M Fe^{3+} varied from 0.05 to 0.45 for the different strains. Strains A12 and 346 produced the highest concentration of siderophores. The chelating ability and growth in liquid culture of these two strains were compared. Optical densities of the bacterial cultures in the presence of 1.7×10^{-5}, 3.3×10^{-5}, and 5.0×10^{-5} M Fe^{3+} were 0.34, 0.42, and 0.45 for *P. putida* A12 and 0.18, 0.21, and 0.32 for strain 346, respectively. However, when these *Pseudomonas* spp. were grown in Fe-free SM, they produced approximately the same number of cells within the 24-hr incubation period. Siderophore extracts of both isolates were concentrated by dialysis and freeze-drying of their cell-free growth media. Activity of the concentrated siderophores was up to eight times higher than the original activity of the crude extract from the growth medium. Activity remained high for at least 3 mo at room temperature (89% of activity).

Interaction of siderophore with cations. Crude extracts from growth media of *Pseudomonas* spp. and the purified siderophore preparation produced similar results in interactions with the cations tested. Therefore, the data from experiments employing purified extracts are presented here.

The ions Fe^{3+}, Fe^{2+}, Zn^{2+}, Cu^{2+}, Co^{2+}, Mn^{2+}, Mg^{2+}, Ca^{2+}, Al^{3+}, Na^{1+}, Li^{1+}, Ni^{1+}, NH_4^{1+}, or K^{1+} were mixed separately in the siderophore preparation of *P. putida* A12 to obtain a final concentration of 0.33×10^{-1} μm/ml. Chelation with Fe^{3+} at increasing concentrations was tested after addition of the cations (Fig. 1). Chelation of Fe^{3+} by the siderophore was inhibited strongly by incubation with Mg^{2+}, Co^{2+}, Zn^{2+}, or Ni^{1+}. Inhibition was also pronounced with Cu^{2+}, Al^{3+}, or Ca^{2+}. The monovalent cations Na^{1+}, Li^{1+}, NH_4^{1+}, and K^{1+} were less effective in inhibiting chelation of Fe^{3+}.

Chelation of Fe in the siderophore preparation also was tested during a 4-day incubation period after application of the cations mentioned above at the same concentrations (Fig. 2). Except for Al^{3+}, Li^{1+}, and Na^{1+}, all the cations tested significantly reduced the optical density of the Fe-bound siderophore after 2 days of incubation. However, the optical density of the Fe control solution at the end of the fourth day was similar to those of most of the cation treatments except Ca^{2+}, Mn^{2+}, and Zn^{2+}. Similar results were observed with siderophore preparation from strain 346.

The effect of cations on chlamydospore germination and growth of *Pseudomonas* spp in soil. The effect of Fe on chlamydospore germination was tested by adding it to soil at the beginning of the experiment or applying it in six pulses at the same total dose of Fe^{3+} at 1×10^{-3} mol/g of soil. Chlamydospore germination after 24 hr of incubation was 78.5%, whereas the addition of *Pseudomonas* sp. strain 346 to soil reduced germination to 35.2%. Germination in soil treated with this bacterium and one application of Fe^3 at the beginning of the experiment was 56.4%, whereas with pulsed application of Fe^{3+} it was significantly increased to 71.6%.

When Fe was added at the beginning of the experiment, the population density of strain 346 was significantly reduced by 25–30% compared with the untreated control during the first 4–6 hr

(Fig. 3). Pulsed Fe also delayed the log phase of growth of the bacteria in soil by 2 hr; however, the same population level was found in all treatments after 24 hr of incubation. Iron applied at less than 10^{-4} mol/g of soil nullified the effect of the pseudomonads on chlamydospore germination but did not affect the bacterial population density.

Cu^{2+}, Zn^{2+}, and Fe^{3+} were applied to soil at rates of 2×10^{-4} M, 2×10^{-4} M, and 2×10^{-3} mol/g, respectively, with *Pseudomonas* sp. strain 346. Germination of chlamydospores in untreated soil was 77.7%, whereas with the strain 346 alone it was reduced to 38.2%. In the presence of the bacterium, the cations that were tested increased percentages of chlamydospore germination up to 49.8, 65.0, and 71.6, respectively. Growth of the *Pseudomonas* (Fig. 4A) in soil was significantly delayed by 1–4 hr in the presence of cations compared with that in nontreated soil. The log phase of the growth curve of the culture of *Pseudomonas* sp. strain 346 whose application in soil was delayed by 2–3 hr was similar to growth when applications were made at time 0 (Fig. 4B). Germination of chlamydospores was increased up to 45.9, 58.5, and 69.3% when the bacteria were mixed in soil after 1, 2, and 3 hr of incubation, respectively. Germination was 35.6% when bacteria were applied at time 0.

Zn^{2+} and Cu^{2+} at one-tenth of the above concentration did not affect doubling time and rate of bacterial multiplication in soil but did counteract chlamydospore germination.

The effect of *Pseudomonas* strain 346 and its concentrated siderophore preparation and EDDHA on germination of chlamydospores of *F. oxysporum* f. sp. *cucumerinum*. Inhibition of chlamydospore germination by concentrated siderophore preparation in soils of pH 5.0–7.0 was tested along with the effect of *Pseudomonas* strain 346 (Table 1). Significantly more inhibition of

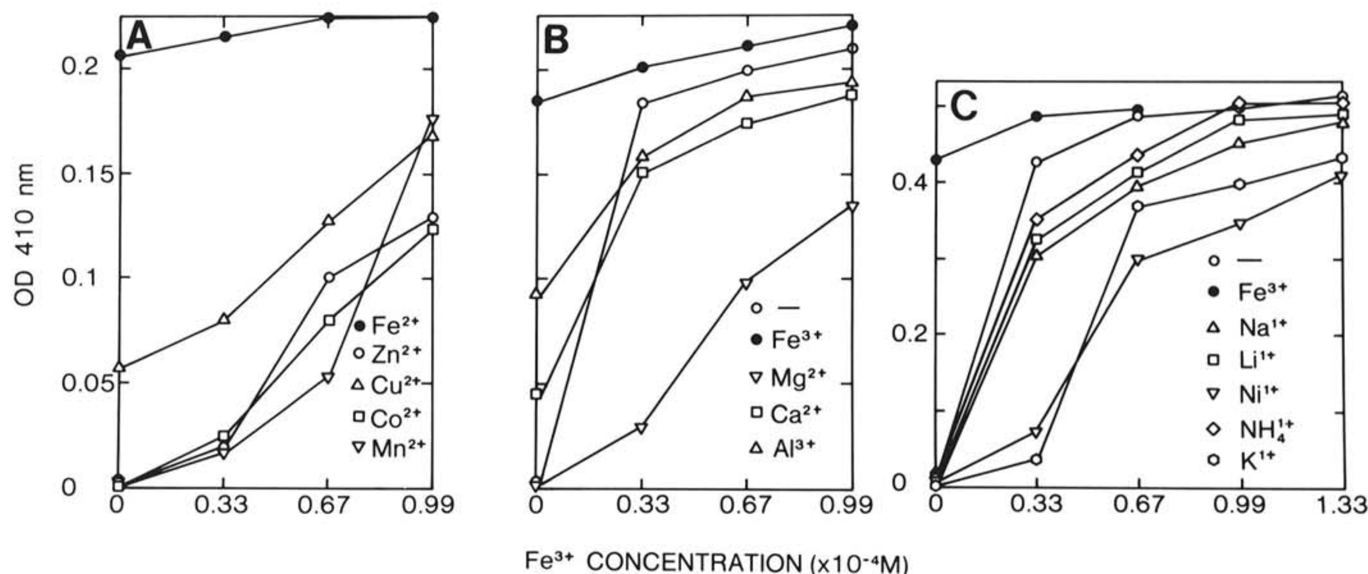


Fig. 1. Absorption at 410 nm of concentrated siderophore preparations of *Pseudomonas putida* strain A12 to which $FeCl_3$ was added at various concentrations after incubation for 1 hr in the solutions with the respective cations added at 0.33×10^{-4} mol/ml.

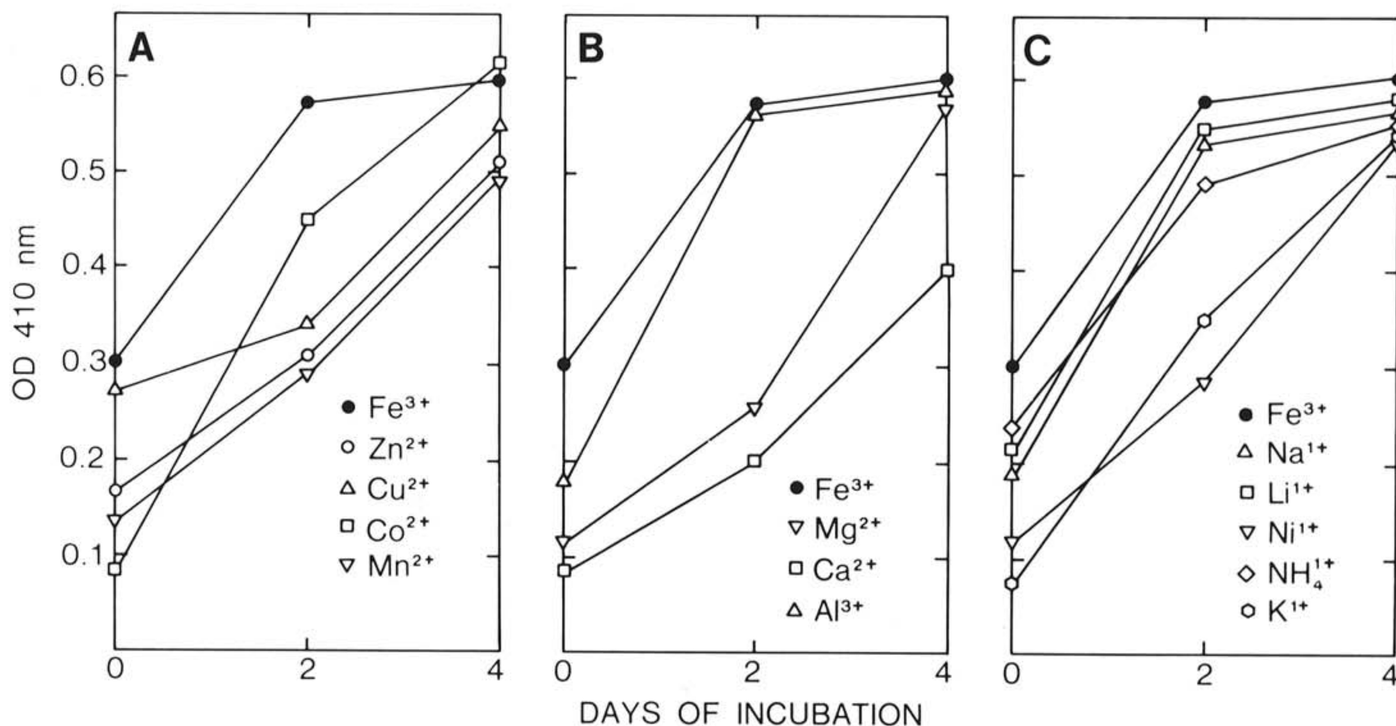


Fig. 2. Absorption curves at 410 nm for concentrated siderophore preparations of *Pseudomonas putida* strain A12 when 0.33×10^{-4} M $FeCl_3$ was added after 1 hr of incubation with 0.33×10^{-4} mol/ml of the respective cations.

chlamydospore germination was obtained in soils at pH 6.0–7.0 compared with inhibition at pH levels of 5.0–5.5 by treatment with either strain 346 or the concentrated siderophore preparation. The applied siderophore preparation reduced chlamydospore germination at pH 5.0–5.5 by 41.2–43.4%, whereas the bacteria reduced germination slightly although strain 346 was present at population levels of 5.7×10^8 cfu/g soil. Significantly higher population densities were detected at pH 6.0 and above. Addition of Fe^{3+} substantially reduced the inhibition of chlamydospore germination induced by the siderophore(s), but this effect was significantly greater at pH 5.0–5.5 than at higher pH levels.

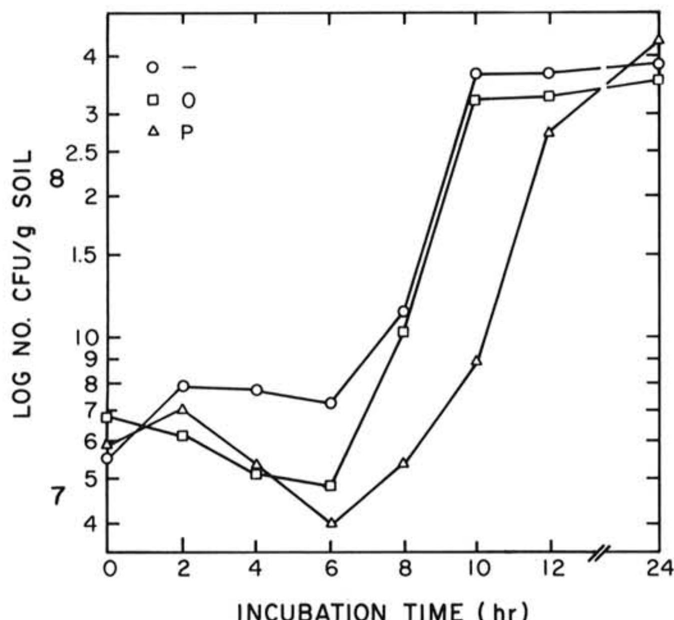


Fig. 3. Growth curves of *Pseudomonas* sp. strain 346 in Fusarium wilt-conducive chlamydospore-infested soil, in the presence of 1×10^{-3} M Fe^{3+}/g of soil. $FeCl_3$ was applied to soil one time at the beginning of the experiment (0) or in six pulses (P), each 2 hr, in same total concentration as at 0. In controls Fe^{3+} was not added (–). Glucose and asparagine were added at the beginning of the experiment at rates of 125:41 μg/g soil.

When the siderophore preparations or EDDHA were dissolved and added to suspensions of chlamydospores, germination and length of germ tubes were inhibited in the presence of the lower concentration of Fe^{3+} compared with the control where Fe was not chelated (Table 2). This effect lasted at least 24 hr. When chlamydospores exposed to treatments for 16 hr were separated from the chelating agents by washing and centrifugation and were resuspended in glucose-asparagine solution, germination was increased as compared with chlamydospores without glucose and asparagine (Table 2) compared with germination of nonwashed chlamydospores of the same age.

EDDHA and FeEDDHA (10^{-4} mol/g) significantly suppressed germination of chlamydospores in the rhizosphere of cucumbers in the presence of Fe^{3+} (7×10^{5} mol/g) or in its absence by 47.7–55.5%, respectively, relative to the control. In nonrhizosphere soil amended with glucose and asparagine, the addition of FeEDDHA or FeEDDHA plus Fe^{3+} did not suppress chlamydospore germination relative to the control (Table 3). However, EDDHA suppressed chlamydospore germination in both rhizosphere and nonrhizosphere soils by 58 and 38%, respectively. A significant reduction in germination was also achieved by adding EDDHA in the presence of added Fe^{3+} in soil. No significant reduction of germination of chlamydospores of *F.s.* f. sp. *phaseoli* or f. sp. *pisi* was obtained with these chelators under the same conditions.

DISCUSSION

The theoretical background has been established for the

Fig. 4. Growth curves of *Pseudomonas* sp. strain 346 in Fusarium wilt-conducive, chlamydospore-enriched soil. **A,** After one application of 2×10^{-4} M Cu^{2+}/g soil, 2×10^{-4} M Zn^{2+}/g, 2×10^{-3} M Fe^{3+}/g, or no cations (–). **B,** Following application of the bacteria 0, 1, 2, or 3 hr after addition of glucose and asparagine (125:41 μg/g soil).

induction of suppressiveness to *Fusarium* in soil through competition for Fe by siderophore-producing *Pseudomonas* spp. and/or soil amended with Fe-chelating compound with stability constants higher than those of the hydroximate siderophores of the pathogens (3,5,6,8,9,12,14,15).

Baker (1) proposed that competition for a particular element can be determined by adding the candidate limiting factor to the system and observing whether biological control was nullified. Thus, Sneh et al (14) added available Fe to soil and found that it counteracted the inhibitory of fluorescent pseudomonads on chlamydospore germination. Misaghi et al (7) reported that Fe counteracted inhibition of fungal growth by a partially purified siderophore in vitro, but other trace elements had no effect; however, partial counteraction of inhibition by siderophore-producing pseudomonads was observed by Sneh et al (14) when soil was amended with Zn^{2+}, Co^{2+}, Mn^{2+}, and MoO_4^{2-}. Two lines of evidence may explain these phenomena. First, added micronutrients delayed multiplication of *Pseudomonas* spp. in soil, thereby extending the lag phase (Figs. 3 and 4A). Indeed, when application of pseudomonads was delayed by 1–3 hr (Fig. 4B), the bacteria did not induce inhibition of chlamydospore germination, again suggesting that this was due to delay in multiplication of added bacteria. Thus, it appears that high population densities (or activity) of the biological control agents are necessary to be effective in the initial stages of chlamydospore germination. This may not explain, however, why partial nullification of germination was observed at lower concentrations of 10^{-4} mM Fe^{3+}, 10^{-5} mM Cu^{2+}, or Zn^{2+}/g of soil.

Second, siderophores produced by pseudomonads apparently lack specificity for binding Fe^{3+}. Unlike chelators such as EDDHA, which specifically bind Fe^{3+} over a wide range of hydrogen ion concentrations (5), siderophores may incorporate or substitute other cations on binding sites (Fig. 1). However, when Fe^{3+} was added to siderophore preparations originally provided with these

TABLE 1. The effect of *Pseudomonas* sp. (strain 346) and its concentrated siderophore preparation on germination of chlamydospores of *Fusarium oxysporum* f. sp. *cucumerinum* at various levels of soil pH

Soil pH	Chlamydospore germination in untreated soil (%)	Reduction in chlamydospore germination (%): *Pseudomonas* sp. (strain 346)	Siderophore[w]	Siderophore + Fe^{3+}[x]	*Pseudomonas* sp.[y] population density after 14 hr of incubation (cfu/g soil)
5.0	71.1 b[z]	15.2 a	43.4 a	1.0 a	5.7×10^8
5.5	70.2 b	11.0 a	41.2 a	0.7 a	5.7×10^8
6.0	79.7 c	39.5 b	67.8 b	4.5 b	1.9×10^9
6.5	62.3 a	35.4 b	70.2 b	6.3 bc	2.1×10^9
7.0	63.6 a	54.9 c	64.6 b	11.5 c	4.7×10^9

[w] Culture medium of strain 346 was centrifuged, dialyzed, and lyophilized.
[x] 2 mM/g soil of Fe^{3+}.
[y] Initial population level of *Pseudomonas* sp. (strain 346) was 2.4×10^7 cfu/g soil.
[z] Numbers in each column followed by a common letter are not significantly different ($P = 0.05$).

TABLE 2. The influence of siderophore, EDDHA, and iron on percentage of germination of chlamydospores of *Fusarium oxysporum* f. sp. *cucumerinum*, in vitro[u]

Treatments		Germ tube length after 16 hr (μm)	Germination after 16 hr (%)	Germination 8 hr after addition of glucose and asparagine (g/a): −g/a[v] (%)	+g/a[v] (%)
None	$-Fe^{3+}$	54.0 c[z]	>100	56.8 b	75.8 bc
None	$+Fe^{3+}$	61.6 b	>100	76.7 bc	81.7 c
Siderophore[w]	$-Fe^{3+}$	30.5 b	10–18	35.5 a	54.3 b
Siderophore	$+Fe^{3+}$	30.7 b	10–95	34.8 a	57.3 b
EDDHA[x]	$-Fe^{3+}$	25.4 a	5–15	31.3 a	54.3 b
EDDHA	$+Fe^{3+}$	27.5 a	5–15	33.3 a	53.4 b

[u] Chlamydospores 5×10^3/milliliter were stimulated to germinate by exposure to 250 μg glucose + 62 μg asparagine per milliliter.
[v] All chlamydospore suspensions were divided into three equal volumes after 16 hr and centrifuged and washed in distilled water. Two volumes of glucose and asparagine were added 125:41 μg/ml to one-third of each treatment.
[w] Lyophilyzed and freeze-dried siderophores of strain 346 was mixed to a final concentration of 128 μg of protein per milliliter.
[x] At 5×10^{-4} mol/ml.
[y] At 1×10^{-4} mol/ml.
[z] Numbers in each column followed by the same letter are not significantly different ($P = 0.05$).

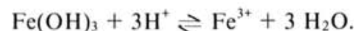
TABLE 3. The influence of EDDHA or FeEDDHA[v] on germination of chlamydospores of *Fusarium oxysporum* f. sp. *cucumerinum* in rhizospheres of cucumbers[w] and in soil amended with glucose and asparagine[x]

Environment associated with chlamydospores	Chlamydospore germination after 24 hr: Control (%)	Control + Fe^{3+} (%)	EDDHA (%)	EDDHA + Fe^{3+} (%)	FeEDDHA (%)	FeEDDHA + Fe^{3+} (%)
Nonrhizosphere soil[x]	29.4 a[z]	31.3 a	18.3 c	23.1 bc	26.4 ab	34.1 a
Rhizosphere soil[y]	26.3 a	28.1 a	11.2 b	14.7 b	12.3 b	13.1 b

[v] EDDHA or FeEDDHA were added to soil at 1×10^{-4} mol/g soil.
[w] Cucumber seedlings were placed on 2 g of soil between two glass slides and incubated for 36 hr.
[x] Incubation with 125:41 μg/g soil glucose and asparagine, respectively.
[y] $FeCl_3$ was added to soil at 7×10^{-5} mol/g.
[z] Numbers across each line followed by a common letter are not significantly different ($P = 0.05$).

cations, Fe gradually replaced the microelements as indicated by a significant increase in optical density of the solution at 410 nm (Fig. 2). This suggests that the affinity of Fe^{3+} to the siderophore-binding site is higher than that of the other cations tested.

Fe^{3+} applied in pulses was more effective than when applied (in total) at the beginning of the experiment in counteracting inhibition of germination of chlamydospores by pseudomonads (Fig. 3). Fe resupplied over time maintains an available pool of Fe^{3+} by the equilibrium (5,6):



$$Fe(OH)_3 + 3H^+ \rightleftharpoons Fe^{3+} + 3\ H_2O.$$

Thus, a relatively large amount of Fe added at the beginning of the experiments may shift the equilibrium to the left and immobilize Fe. Fe supplied in pulses renews the pool of Fe^{3+} each time it is applied.

Obvious inhibition of germination of chlamydospores by *Pseudomonas* sp. strain 346 added to soil was observed at high pH levels but inhibition was at low levels at pH values lower than 6.0 (Table 1). In acid conditions, population levels of pseudomonads were lower than in alkaline conditions (Table 1). According to theory, available Fe was in good supply (5,6); therefore, siderophores were not produced (8). This provides evidence for the hypothesis outlined above. Further support was obtained when concentrated siderophore preparations were added to soil. These induced suppressiveness to chlamydospore germination in soil even at relatively low pH values.

The catechol hydroximate siderophores of fluorescent pseudomonads have a higher stability constant than the hydroximate siderophores of *F. oxysporum* and, thus, are able to compete successfully for available Fe (12). The ligand EDDHA also has a higher stability constant than hydroximate siderophores and induced suppressiveness when added to soil. However, low quantities of Fe limiting germination of chlamydospores would only occur on the host rhizoplane where there is intense competition for this element by the root and rhizosphere bacteria capable of utilizing Fe in FeEDDHA—once stripped of its Fe the ligand binds more Fe from the available Fe^{3+} pool. In contrast, soil distant from root surfaces should maintain a level of Fe^{3+} determined by soil pH regardless of the addition of FeEDDHA since there are no root or active rhizosphere bacteria to compete for the trace element. Thus, FeEDDHA was only effective in reducing chlamydospore germination in the rhizosphere (14, and Table 3) but not in soil (13). The effect of the ligand (EDDHA) alone (Table 2), however, was similar to the effect of purified siderophores (Table 1) in that germination of chlamydospores in soil was inhibited in the presence of Fe in a lower molarity than that of the chelator.

These results expand and confirm developing theory regarding the mechanisms involved in the induction of suppressiveness to *Fusarium* in soil. Also, it is apparent that the presence of excessive amounts of some essential cations results in competition for binding sites on siderophores and inhibits multiplication of pseudomonad biocontrol agents. This diminishes competition for Fe and reduces the efficacy of biological control.

LITERATURE CITED

1. Baker, R. 1968. Mechanisms of biological control of soilborne pathogens. Annu. Rev. Phytopathol. 6:263-294.
2. Dupler, M., and Baker, R. 1984. Survival of *Pseudomonas putida*, a biological control agent, in soil. Phytopathology 74:195-200.
3. Emery, T. 1974. Biosynthesis and mechanism of action of hydroxamate-type siderophores. Pages 107-123 in: Microbial Iron Metabolism. J. B. Neilands, ed. Academic Press, New York. 507 pp.
4. Kloepper, J. W., Leong, J., Tientze, M., and Schroth, M. N. 1980. *Pseudomonas* siderophores: A mechanism explaining disease-suppressive soils. Curr. Microbiol. 4:317-320.
5. Lindsay, W. L. 1974. Role of chelation in micronutrient availability. Pages 507-524 in: The Plant Root and its Environment. E. W. Carson, ed. University Press of Virginia, Charlottesville. 691 pp.
6. Lindsay, W. L. (ed.). 1979. Chemical Equilibria in Soils. John Wiley & Sons, New York. 449 pp.
7. Misaghi, I. J., Stowell, L. J., Grogan, R. G., and Spearman, L. C. 1982. Fungistatic activity of water-soluble fluorescent pigments of fluorescent pseudomonads. Phytopathology 72:33-36.
8. Neilands, J. B. 1973. Microbial iron transport compounds (siderochromes). Pages 167-202 in: Inorganic Biochemistry. Vol. 1. G. L. Eichhorn, ed. Elsevier, Amsterdam. 602 pp.
9. Neilands, J. B. (ed.). 1974. Microbial Iron Metabolism. Academic Press, New York. 507 pp.
10. Reed, R. W., and Reed, G. B. 1948. Drop plate method of counting viable bacteria. Can. J. Res., Sect. E. Med. Sci. 26:317-326.
11. Scher, F. M., and Baker, R. 1980. Mechanism of biological control in a Fusarium-suppressive soil. Phytopathology 70:412-417.
12. 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.
13. Scher, F. M., and Baker, R. 1983. A fluorescent microscopic technique for viewing fungi in soil and its application to studies of a Fusarium-suppressive soil. Soil Biol. Biochem. 15:715-718.
14. Sneh, B., Dupler, M., Elad, Y., and Baker, R. 1984. Chlamydospore germination of *Fusarium oxysporum* f. sp. *cucumerinum* as affected by fluorescent and lytic bacteria from a *Fusarium*-suppressive soil. Phytopathology 74:1115-1124.
15. Tientze, M., Hussain, M. B., Barnes, C. L., Leong, J., and Van der Helm, D. 1981. Structure of ferric pseudobactin, a siderophore from a plant growth-promoting *Pseudomonas*. Biochemistry 20:6446-6457.