Chlamydospore Germination of *Fusarium oxysporum* f. sp. *cucumerinum* as Affected by Fluorescent and Lytic Bacteria from a Fusarium-Suppressive Soil

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ABSTRACT

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Over 700 bacteria and actinomycetes were isolated from a Fusarium-suppressive Metz fine sandy loam collected from the Salinas Valley in California. Isolates were screened for ability to produce the fungal cell-wall degrading enzyme, chitinase, to lyse dead and living germ tubes of chlamydospores, and to produce fluorescent compounds (siderophores) in iron (Fe)-deficient nutrient medium. Representative isolates from groups with or without these attributes were added to conducive soil where they were evaluated for influence on chlamydospore germination of *Fusarium oxysporum* f. sp. *cucumerinum* in rhizosphere and nonrhizosphere soil, and for ability to induce suppressiveness to Fusarium wilt of cucumber. Nonfluorescent bacteria generally induced little or no inhibition of chlamydospore germination except for *Enterobacter cloacae* and isolate 691 which induced lysis in vitro. Those isolates capable of inducing lysis in vitro did not induce significant germ-tube lysis in rhizosphere or nonrhizosphere soil. There was a direct correlation ($r = 0.99$) between siderophore production by various fluorescent pseudomonads and their inhibition of chlamydospore germination in soil. Adding Fe^{2+}, Zn^{2+}, Co^{2+}, Mn^{2+}, and MoO_4^{2-} to soil partially counteracted inhibition induced by fluorescent pseudomonads. The same concentration of micronutrients did not inhibit the growth of pseudomonads in soil. A chelator, ferric ethylenediaminidei-*O*-hydroxyphenylacetic acid (FeEDDHA), previously reported to induce suppression of *Fusarium* in soil, inhibited chlamydospore germination in the rhizosphere but not in nonrhizosphere soil. This supported theory that Fe^{3+} is involved in the mechanism of competition. Disease was suppressed more strongly by the highly fluorescent siderophore-producing pseudomonad isolates than by other isolates; however, some nonfluorescent bacteria also suppressed disease. Combinations of lytic and fluorescent bacteria did not increase suppressiveness.

The Metz fine sandy loam, found in the Salinas Valley, CA, is suppressive to Fusarium wilt diseases (2,16,25,28,33). Soil suppressiveness was transferred to conducive soil by introducing fluorescent pseudomonads from the suppressive soil (6,25,26). Kloepper et al (10) suggested that siderophore production in soil by fluorescing bacterial isolates is responsible in part for soil suppressiveness.

Theory expansion was provided by Scher and Baker (26). Siderophores or ligands of iron (Fe) chelating agents, with a higher stability constant than the Fe-acquiring facilities of the pathogen, may induce soil suppressiveness when Fe^{3+} is in relatively low concentration in the soil. Therefore, the suppressive Metz fine sandy loam soil in California (pH 7.6–8.1), containing low amounts of available Fe, became conducive when acidified (25) and suppressiveness was most easily induced in alkaline soils.

Mitchell and Alexander (18,19) observed biological control of Fusarium wilt diseases and *Pythium* sp. by the use of fungal cell-wall-lytic bacterial isolates. Koths and Gunner (13) and Sneh (29) also demonstrated biological control of diseases caused by *Fusarium* on carnation by using a chitinolytic *Arthrobacter* sp. Therefore, the addition of lytic mechanisms to siderophore production may increase the suppression and control of pathogens in soil, and broaden the types of conducive soils that could be induced to become suppressive.

The objective of the present work was to evaluate the inhibitory effect of a wide range of lytic, fluorescent, and other bacteria isolated from the rhizosphere, on chlamydospore germination of Fusarium wilt pathogens in rhizosphere or in simulated-rhizosphere soil. The potential of selected bacteria to induce soil suppressiveness and provide biological control of Fusarium wilt of cucumber was also evaluated.

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MATERIALS AND METHODS

Soil. Fusarium wilt-conducive soil (Ascalon sandy loam) was collected near Nunn, CO. It was a sandy loam with pH 7.3. Soil moisture (25) and physical and chemical characteristics (26) were described previously. The physical soil characteristics were similar to those of the Fusarium wilt-suppressive soil taken from the Salinas Valley in California (25).

Isolation and characterization of bacteria. Over 700 isolates of bacteria and actinomycetes initially were isolated for the present study from mycelial mats of *Fusarium oxysporum* Schlecht. emend. Snyd. Hans. f. sp. *cucumerinum* incubated for 5–12 days on roots of cucumber seedlings growing in the suppressive soil. Isolations were made on nutrient agar (NA) (Becton-Dickinson Corp., Cockeysville, MD 21030) for general bacteria and King's B agar (KB) (9) for fluorescent pseudomonads. Isolates were characterized for potential lytic ability by the use of colloidal chitin agar (CA) for chitinolytic bacteria and germ-tube agar for germ-tube-lytic bacteria essentially based on Morris's carbon-deficient

medium (20), in which 20 mg of colloidal chitin were added per milliliter of agar for CA or 10^7 germinated conidia of *F. oxysporum* f. sp. *cucumerinum* (24 hr of incubation) were added per milliliter of medium before autoclaving for germ-tube agar. A cleared zone around the bacterial colonies in the CA medium indicated the production of chitinase. Clearing in the vicinity of colonies growing in germ-tube agar suggested that those isolates could lyse nonviable germ tubes of conidia.

The following isolates also were utilized: *Pseudomonas putida* A12 (26), B10 (31), and N1R (6), *Serratia liquifasciens*, *Hafnia* sp. (29), *Arthrobacter* sp. P35 (13), *Enterobacter cloacae*, and *Erwinia herbicola* (G. A. Harman, New York State Agric. Exp. Stn., Cornell University, Geneva 14456). Pure cultures of the isolates were grown on KB plates for 24 hr, washed from the agar surface with 10% skim milk, and freeze-dried.

Enrichment of soil with chlamydospores of *Fusarium*. Mycelial mats of *F. oxysporum* f. sp. *cucumerinum* were grown in 100-ml aliquots of sterile potato-dextrose broth (PDB) supplemented with 0.2% yeast extract, in Roux bottles. After 5 days of incubation at 27 C, mats were washed with running distilled water for 5 min on four layers of cheesecloth (to remove the medium and conidia). Twelve mats were blended in 150 ml of distilled water for 1 min and centrifuged for 10 min at 2,500 *g*. The pellet was resuspended in 150 ml of H_2O and added to 1 kg of air-dried Nunn soil (−0.3 bar). The soil was well mixed, placed in plastic bags and incubated for 4 wk at 27 C. Soil was then air-dried to 10% moisture, sieved through a 2-mm screen, mixed well, and stored moist in plastic bags in the refrigerator until use. The soil contained an average of 4.5×10^5 colony-forming units (cfu) of *F. oxysporum* f. sp. *cucumerinum* per gram as determined on Komada's selective medium (12). Colonies predominantly originated from chlamydospores.

Chlamydospore germination tests in soil. Bacterial isolates were grown on KB plates for 24 hr, collected, washed twice, and resuspended in 0.1 M $MgSO_4$ solution to the desired concentrations. Samples of 5 g of chlamydospore-enriched soil were placed in medium polystyrene weighing boats (Fisher Scientific Co., Denver, CO 80112). Aliquots of 0.1 ml of glucose and asparagine solutions, bacterial suspensions, and micronutrient solutions or H_2O were added to conducive or suppressive soils to give the desired concentrations at 15% (−0.3 bar) water content. The soil was thoroughly mixed with a bent spatula, placed in three wells of a tissue culture multiwell (Falcon 3000; VWR Scientific, Inc., Denver, CO 80239), and incubated at 27 C in plastic bags for 24 hr. Three 0.5-g soil subsamples of each treatment were transferred to test tubes for processing and staining (27). Aliquots of 1 ml of filtered 0.3% Calcofluor White M2R New (American Cyanamid Co., Bound Brook, NJ 08805) solution were added to each tube. After 5 min, the excess solution was removed with a Pasteur pipette and replaced with 1 ml of H_2O. Soil was suspended and a drop of the suspension was placed on a microscope slide for observation at ×400 under a UV light microscope (Olympus, Japan) for fluorescence observation. When samples could not be counted immediately, two drops of chloroform were added per tube to prevent microbial activity. Tubes could be kept in the refrigerator for at least 14 days. One hundred chlamydospores were counted for each of the three replicates per treatment. Lysis of germinated chlamydospores was determined by the reduction in percentage of chlamydospores with germ tubes observed after 8 days of incubation and by observation of lysed germ tubes.

Chlamydospore germination in the rhizosphere. Cucumber (*Cucumis sativus* L. 'Straight Eight') seed (disinfected for 3 min in 2% sodium hypochlorite solution, and washed three times in sterile distilled water) were pregerminated at 27 C for 24 hr on autoclaved moist paper towels in trays and covered with plastic bags. Cells of the bacterial isolates thoroughly mixed in samples of 5 g of soil enriched with chlamydospores were evenly spread on microscope slides in three replicates providing a 2- to 3-mm layer. A germinated seed was placed on the upper portion of each slide. The soil was wrapped with a nylon net, secured with two rubber bands, and the slides were slightly slanted in moist soil in plastic pots (8 cm diameter, 8 cm high). The pots were covered with plastic bags and incubated in a growth room at 27 C for the desired period while the roots grew along the slides. When the slides were recovered from soil, the rubber bands were gently removed with the nylon net. The soil with the root system was separated from the original slide with a razor blade, transferred to another slide, and placed on a piece of millimetric paper. After the soil at a distance >0.5 mm from the rhizoplane was removed, the root region desired with the adhering 0.5 mm of rhizosphere soil was transferred to test tubes and treated for microscopic observation as described above. Three plants were used for each treatment.

Germination-lysis of chlamydospores and subsequent survival. Various concentrations of glucose or ethanol were added to a chlamydospore-enriched soil. Germination percentage was determined after 24 hr, as described above, and survival was determined on dilution plates with Komada's selective medium (12).

Recovery of *Pseudomonas* spp. isolates added to soil. Bacterial isolates were selected for tolerance to chloramphenicol (150 μg/ml), streptomycin (150 μg/ml), and nalidixic acid (170 μg/ml). Cell suspensions of the desired concentrations were added to the chlamydospore-enriched soil together with glucose and asparagine, with or without micronutrients as described. Soil dilutions were made after 0 and 24 hr of incubation at 27 C and plated on KB agar with or without the antibiotics by use of the drop plate method (24).

Quantitative analysis of siderophore production by fluorescent pseudomonad isolates. Fluorescent, siderophore-producing isolates were grown at 27 C in wrist-action shaking cultures for 24 hr. Ingredients of the broth medium were: 1.0 g of glutamine, 10.0 g of glycerol, 1.32 g of $(NH_4)_2SO_4$, 0.25 g of $MgSO_4 \cdot 7H_2O$, 5.9 g of HEPES buffer, 5 ml of 0.1 M phosphate buffer (pH 7.5), 2 ml of 4×10^{-4} M $MnCl_3$, and 1 ml of 4×10^{-2} M $ZnSO_4$. The pH was adjusted to 7.5 with 0.1 N NaOH.

Culture broth was centrifuged (5,000 *g*) for 10 min. The supernatant was adjusted to pH 6.0 (with 0.1 N HCl), $FeCl_3$ was added to give 0.2% (w/v), and the broth was stirred until the color changed to red-brown. The pH was adjusted to 7.5 (with 0.1 N NaOH) and the broth was diluted 1:2 with 0.1 M HEPES buffer (pH 7.5), stirred well, and centrifuged (5,000 *g* for 10 min). Relative concentration of siderophore in the supernatant was read in the spectrophotometer at 420 nm.

Induction of soil suppressiveness to Fusarium wilt by the addition of bacteria. Bacterial isolates were grown in KB broth for 24 hr at 27 C in a wrist-action shaker. Cells were harvested by centrifugation (2,500 *g* for 15 min), washed, and resuspended in 0.1 M $MgSO_4$. Optical density was determined at 780 nm and dilutions in the same solution were made accordingly. Preparation of inoculum of *F. oxysporum* f. sp. *cucumerinum* and plant growth conditions were previously described (26).

Propagule density of the pathogen in soil was ~200 cfu/g soil. Bacterial isolates were mixed in the soil at 10^8 cfu/g of soil. Surface-disinfected cucumber seeds were pregerminated for 48 hr as described above. They were dipped in the bacterial suspension before being planted. Five plants were planted in each pot containing 500 g of soil, in randomized complete blocks with three replicates. Experiments were repeated three times. Disease incidence was recorded daily for 22 days. Diseased plants were removed each day.

Experimental data were analyzed statistically with a one-way analysis of variance.

RESULTS

All bacteria and actinomycetes were screened for fluorescence, chitinase production, and for ability to lyse dead germ tubes of chlamydospores of *F. oxysporum* f. sp. *cucumerinum*. None of the isolates demonstrated both fluorescence production and chitinase- or germ-tube-lytic ability. Most of the isolates (93%) that lysed germ tubes also produced chitinase, some isolates produced chitinase but could not lyse germ tubes.

Chlamydospore germination in soil amended with glucose and asparagine. In conducive Nunn sandy loam soil, increasing concentrations of glucose and asparagine (4:1) from 0 to 0.8 mg of glucose + 0.2 mg of asparagine per gram of soil resulted in increased

chlamydospore germination of *F. oxysporum* f. sp. *cucumerinum* from 0 to 89%. A further increase in nutrient concentrations did not significantly increase chlamydospore germination (Fig. 1A). Germ-tube length increased linearly with increasing concentrations of glucose and asparagine (Fig. 1B). The response was evident also at high concentrations (25 mg of glucose and 0.625 mg of asparagine per gram of soil) (Fig. 1B). No germination was observed in soil if nutrients were not added.

Addition of a fluorescent *Pseudomonas* sp. (isolate 712, at 10^8 cfu/g of soil) strongly inhibited chlamydospore germination; however, increasing concentrations of glucose and asparagine reduced the magnitude of inhibition (Fig. 1A). In addition, the germinated chlamydospores formed short germ tubes (Figs. 1B and 2B) as compared with those in soil to which the bacterium had not been added (Fig. 2A). In suppressive soil, chlamydospore germination was inhibited in a way similar to the effect observed when the *Pseudomonas* sp. was added to conducive soil. However, at higher glucose and asparagine concentrations, inhibition in suppressive soil was weaker than that induced when the *Pseudomonas* sp. was added to soil at 10^8 cfu/g (Fig. 1A).

The results in Fig. 1 indicate that a concentration of 0.8 and 0.2 mg of glucose and asparagine, respectively, per gram of soil may provide the optimum required sensitivity for systematic testing of introduced, potential inhibitors of chlamydospore germination. When those concentrations were introduced in the conducive soil, increasing concentrations of the fluorescent *Pseudomonas* sp. (712) resulted in decreasing the percent germination of chlamydospores (Fig. 3A). Also, a corresponding decrease was observed in germ-tube length (Fig. 3B). However, *S. liquifasciens*, which is a strong chitinase-producing isolate, induced no significant inhibition (Fig. 3A). When both isolates were added, the inhibitory effect was not as great as when *Pseudomonas* sp. 712 was added alone.

Chlamydospore germination in the rhizosphere of cucumber. To determine the suitable root region and incubation time required for assessing chlamydospore germination in the rhizosphere, samples of rhizosphere soil containing chlamydospores were taken at various distances from the tip of the radical after various incubation periods. In an actively growing root, there were almost no germinated chlamydospores 0–10 mm from the root tip (Table 1), and those that had germinated had short germ tubes (3 μm). At 10–20 mm from the tip, 6–7% of the chlamydospores germinated; germ tubes were still short (5 μm). From 20 mm from the tip to the crown, chlamydospore germination was above 25% and, in some instances, to 46%. Germ-tube lengths ranged from 13 to 100 μm. Occasionally long germ tubes (230 μm) were observed. Less chlamydospore germination (10–15%) occurred in the rhizosphere soil layer 0.5–1.0 mm from the rhizoplane. There were no significant increases in germination over 2- to 5-day incubation periods. Lengths of germ tubes of chlamydospores germinated in the rhizosphere were similar to those induced by the addition of glucose at 0.2–0.4 mg/g of soil and asparagine at 0.05–0.1 mg/g of soil in nonrhizosphere soil. In subsequent experiments for analyses of chlamydospore germination in the rhizosphere, the samples were taken 30–60 mm from the root tip after 2 or 3 days of incubation.

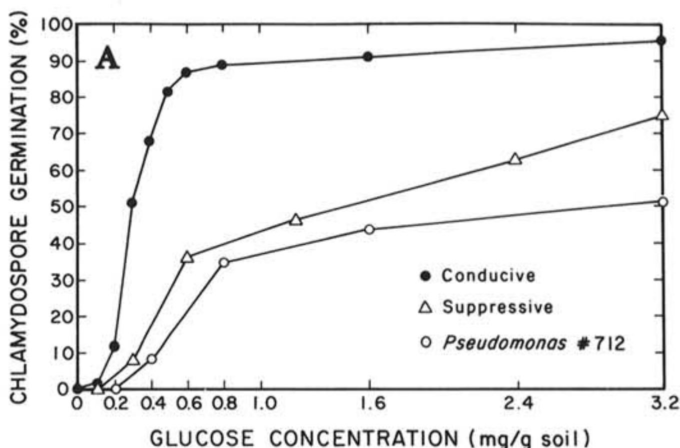
TABLE 1. Chlamydospore germination of *Fusarium oxysporum* f. sp. *cucumerinum* in rhizosphere soil[a] of cucumber seedlings

Distance from root tips (mm)	Chlamydospore germination (%) after incubation for: 43 hr	70 hr	120 hr
0–10	0	1	—[b]
10–20	7	6	—[b]
20–30	25	25	30
30–40	27	30	31
40–50	30	32	30
50–60	30	33	28
60–70	—[b]	33	28
Crown	46	37	28
Lateral roots	—	26	15

[a] A 0.5-mm layer of soil was collected from around the taproot.
[b] Root parts were missing.

Chlamydospore germination in amended soil or rhizosphere as affected by addition of bacterial isolates. Representative bacterial isolates having various antagonistic properties in vitro, inhibited chlamydospore germination in the amended soil (Table 2) and in the rhizosphere (Table 3). In the rhizosphere soil, germination was lower (35%) in the control compared to the soil amended with 0.6 mg of glucose and 0.15 mg of asparagine (82%) per gram of soil. Since inhibition of chlamydospore germination by the bacteria in the rhizosphere was relatively lower in the rhizosphere soil (Tables 2 and 3) and the amended soil provided a more sensitive and simple method for evaluating factors affecting chlamydospore germination, the latter method was used for subsequent assays.

The fluorescent pseudomonad isolates were the strongest inhibitors of chlamydospore germination (Table 2). Within that group of isolates, some were more effective than others. Most isolates of the other groups induced a relatively weak inhibitory effect; however, *E. cloacae* demonstrated a relatively strong inhibition (germination 20% that of the control) whereas *Erwinia herbicola* inhibited only moderately (germination 72% that of the



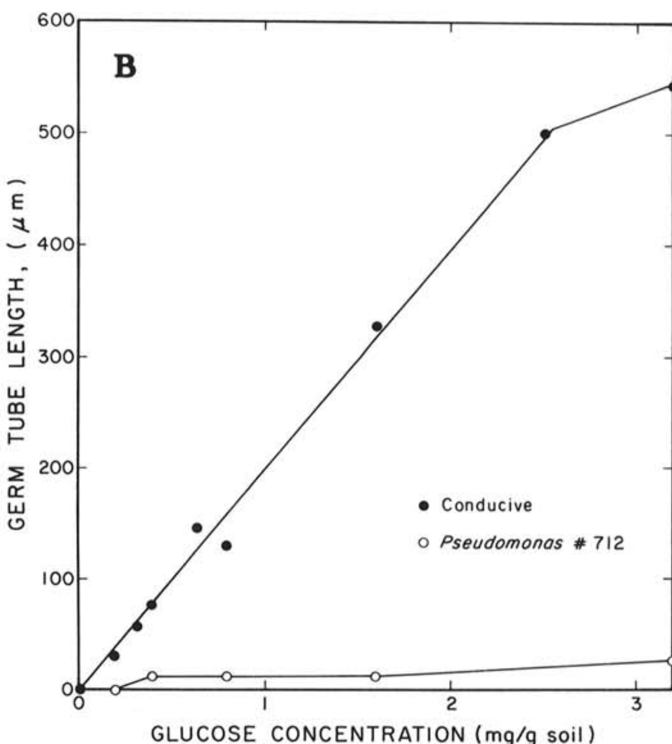


Fig. 1. Effect of glucose and asparagine (4:1) on chlamydospore germination of *Fusarium oxysporum* f. sp. *cucumerinum* in conducive soil, suppressive soil, and in conducive soil supplemented with a fluorescent *Pseudomonas* sp. 712 (10^8 cfu/g soil). **A,** Chlamydospore germination. **B,** Germ-tube elongation.

control). Among the germ-tube-lytic isolates, 691 (72% of control) and 710 (76% of control) induced some inhibition. Isolates 346, 381, 712, and N1R demonstrated the strongest inhibition in the amended soil (germination 16–18% that of the control). However, in the rhizosphere, they induced a much weaker inhibition (germination 46–65% that of the control, Table 3).

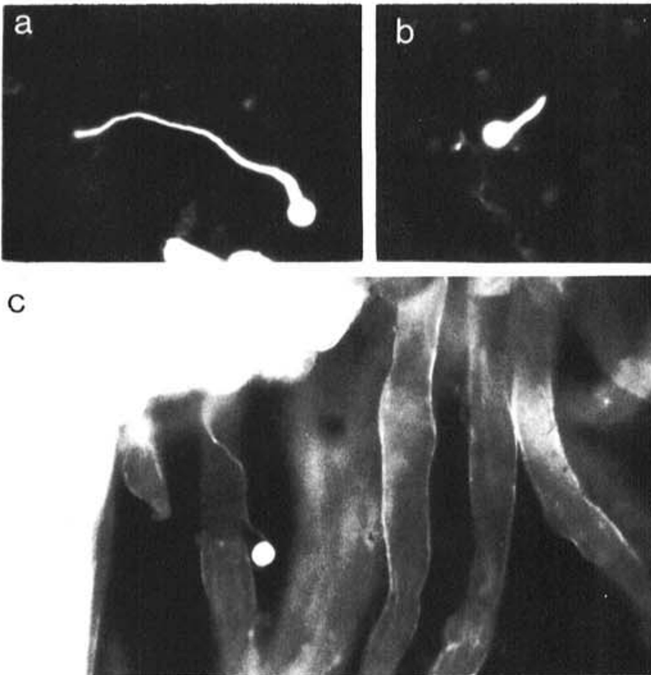


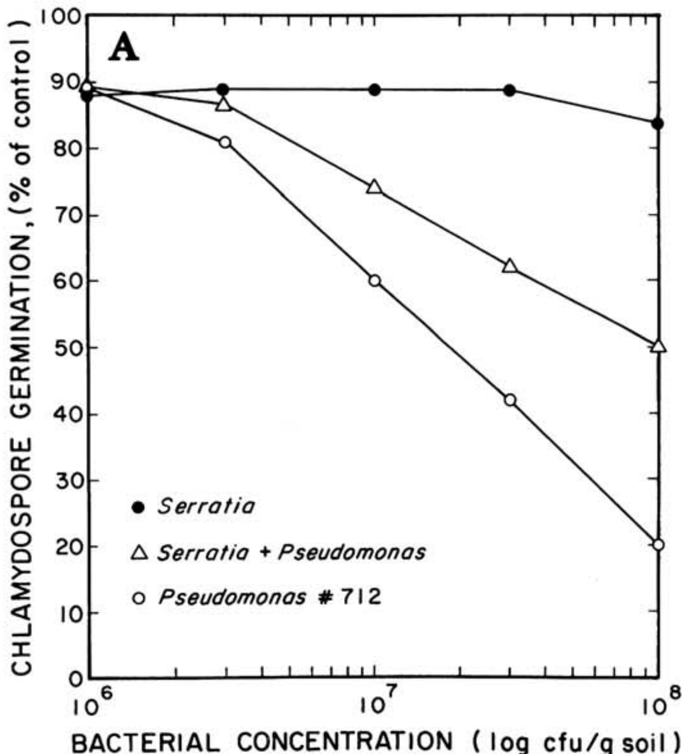
Fig. 2. Germinating chlamydospores of *Fusarium oxysporum* f. sp. *cucumerinum* in soil smears stained with 0.3% Calcofluor and observed under a fluorescence microscope (×400). **A,** In soil amended with glucose (0.6 mg/g soil), and asparagine (0.15 mg/g soil) and incubated for 24 hr. **B,** In soil amended with glucose (0.6 mg/g soil) and asparagine (0.15 mg/g soil) and incubated for 24 hr with a fluorescent *Pseudomonas* sp. 712 (10^8 cfu/g soil). **C,** In rhizosphere of a 3-day-old cucumber seedling.

Lysis of germ tubes of germinating chlamydospores in soil containing added bacterial isolates. To determine the possible role of lysis induced by soil bacteria in biological control of Fusarium wilt diseases, chlamydospores were induced to germinate by incubating soil with glucose and asparagine for 24 hr. Fluorescent, germ-tube-lytic, and other isolates were then added at 10^8 cfu/g of soil. There were no significant differences among the treatments in the numbers of chlamydospores that still had germ tubes after 8 days and these were not different from the control (Table 4). About one-half of the germ tubes appeared to be undergoing lysis. Similarly, after 8 days in the rhizosphere, no significant effect on lysis could be observed in soil treated with bacteria; again, about 50% of the germ tubes observed apparently were lysing (Table 5). However, isolates N1R, 346, 61, 691, and *E. cloacae* inhibited germination in the rhizosphere, confirming the results in Table 3.

Germination-lysis of chlamydospores and survival of *F. oxysporum* f. sp. *cucumerinum* in soil amended with glucose or ethanol. At 0.1 mg/g of soil, ethanol induced 60% chlamydospore germination, whereas almost no germination was induced at the same concentration by glucose; however, about 90% germination occurred when each of those materials was added at 1.0 mg/g of soil (Fig. 4). The survival of *F. oxysporum* f. sp. *cucumerinum* after 3 wk was lowest when each amendment was added at the highest concentration, 1.0 mg/g of soil.

Chlamydospore germination in soil treated with a chelating agent. The addition of fluorescent *Pseudomonas* spp. to soil reduced chlamydospore germination both in the rhizosphere (from 36 to 21%) and in soil amended with glucose and asparagine (from 57 to 8%) (Table 6). The addition of the iron chelator, ferric ethylenediaminidei-*O*-hydroxyphenylacetic acid (FeEDDHA), at 0.1 mg/g of soil, had no effect on chlamydospore germination in soil amended with glucose and asparagine; however, in rhizosphere soil the chelator reduced germination from 36 to 20%. An additional increment of inhibition was observed in the rhizosphere above that induced with *Pseudomonas* sp. 346, from 21 to 13%, by the addition of FeEDDHA.

Chlamydospore germination in soil amended with micronutrients. Previous studies (10,25,26) indicated that siderophore production by fluorescent pseudomonads in soil inhibited development of Fusarium wilt diseases because of competition for Fe^{3+} (26).



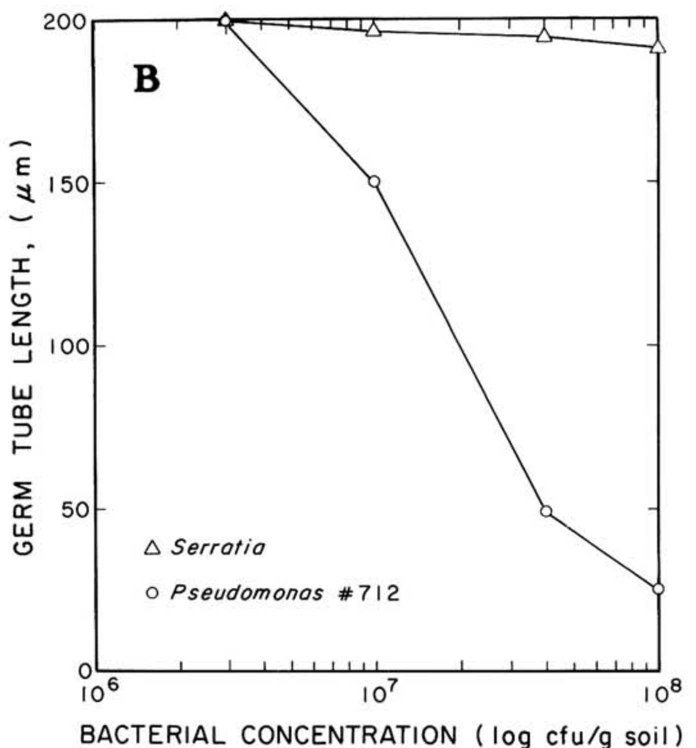


Fig. 3. Effect of a fluorescent *Pseudomonas* sp. 712, and *Serratia liquifasciens* on chlamydospore germination of *Fusarium oxysporum* f. sp. *cucumerinum* in soil amended with glucose (0.8 mg/g soil) and asparagine (0.2 mg/g soil). **A,** Chlamydospore germination. **B,** Germ-tube length of chlamydospores.

Therefore, the addition in excess of Fe to the system should nullify competition and reduce the inhibitory effect on the pathogen. Isolates 346 and N1R of fluorescent *Pseudomonas* sp. and *E. cloacae* significantly inhibited chlamydospore germination in soil amended with glucose and asparagine from 83% in the control to 10, 15, and 12%, respectively (Table 7). Addition of Fe^{2+} at 0.2 mg/g of soil significantly reduced the inhibitory effect of the bacteria to 46, 50, and 40% germination, respectively. In the control soil, the addition of Fe also increased chlamydospore germination significantly from 83 to 94%.

Addition of Cu^{2+}, Zn^{2+}, Co^{2+}, Mn^{2+}, and MoO_4^{2-} also induced a significant reduction of the inhibitory effect caused by *Pseudomonas* isolate 346, but there was no effect upon the addition of H_3BO_3 or Mg^{2+} (Table 8). All increases in germination percentage were accompanied by increased germ-tube elongation.

To determine the stimulatory effect of the micronutrients in raw soil with its natural microflora, the soil was amended with a smaller amount of glucose and asparagine (0.3 and 0.075 mg/g of soil, respectively). This resulted in lower germination (51%) of chlamydospores in the control soil without addition of micronutrients (Table 8); Fe^{2+}, Cu^{2+}, Zn^{2+}, Co^{2+}, Mn^{2+}, and MoO_4^{2-} significantly increased chlamydospore germination in soil, whereas H_3BO_3 and Mg^{2+} had no significant effect.

To determine whether the stimulatory effect on chlamydospore germination was due to a toxic effect of micronutrients on the bacterial populations which were responsible for the inhibition of chlamydospore germination in soil, Fe^{2+}, Zn^{2+}, and Cu^{2+} as well as *Pseudomonas* sp. 346 were added to soil amended with glucose and asparagine. Soil dilution counts were made at 0 and 24 hr incubation on King's B agar plates. Indigenous soil-bacterial

TABLE 2. Effect of bacterial isolates[a] on germination of chlamydospores of *Fusarium oxysporum* f. sp. *cucumerinum* in soil amended with glucose and asparagine[b]

Bacterium	Isolate no.	Characteristics	Chlamydospore germination	
			Percent	Percent of control
Control			82	100
Pseudomonas sp.	A12	Fluorescent	31	38
Pseudomonas sp.	B10	Fluorescent	43	52
Pseudomonas sp.	61	Fluorescent	64	78
Pseudomonas sp.	346	Fluorescent	13	16
Pseudomonas sp.	381	Fluorescent	14	17
Pseudomonas sp.	712	Fluorescent	13	16
Pseudomonas sp.	N1R	Fluorescent	15	18
Unidentified	3c	Chitinolytic	71	87
Unidentified	12c	Chitinolytic	69	84
Serratia liquifasciens		Chitinolytic	74	90
Arthrobacter sp.	P35	Germ-tube lytic	72	88
Unidentified	163	Germ-tube lytic	68	83
Actinomycete	227	Germ-tube lytic	82	100
Unidentified	496	Germ-tube lytic	68	83
Unidentified	691	Germ-tube lytic	59	72
Unidentified	710	Germ-tube lytic	62	76
Unidentified	711	Germ-tube lytic	70	86
Enterobacter cloacae		None[c]	16	20
Erwinia herbicola		None	61	75
Bacillus sp.	443	None	78	95
Unidentified	700	None	75	92
LSD ($P = 0.05$)			15	

[a] Bacteria were added to soil at 10^8 cfu/g.
[b] Glucose and asparagine were added to soil at 0.6 and 0.15 mg/g, respectively.
[c] Isolates which were not fluorescent, chitinolytic, or germ-tube lytic.

TABLE 3. Effect of bacterial isolates[a] on the germination of chlamydospores of *Fusarium oxysporum* f. sp. *cucumerinum* in the rhizosphere of cucumber seedlings[b]

Bacterium	Isolate no.	Characteristic	Chlamydospore germination	
			Percent	Percent of control
Control			35	100
Pseudomonas sp.	61	Fluorescent	27	77
Pseudomonas sp.	346	Fluorescent	17	49
Pseudomonas sp.	712	Fluorescent	16	46
Pseudomonas sp.	N1R	Fluorescent	23	65
Serratia liquifasciens		Chitinolytic	35	100
Arthrobacter sp.	P35	Germ-tube lytic	35	100
Actinomycete	227	Germ-tube lytic	35	100
Unidentified	691	Germ-tube lytic	25	71
Unidentified	710	Germ-tube lytic	27	77
Unidentified	711	Germ-tube lytic	27	77
Enterobacter cloacae		None[c]	17	49
Bacillus sp.	443	None	35	100
LSD ($P = 0.05$)			9	

[a] Bacteria were added to soil at 10^8 cfu/g.
[b] A 0.5-mm layer of soil was collected from around the taproots of 3-day-old seedlings.
[c] Isolates that were not fluorescent, chitinolytic, or germ-tube lytic.

populations were increased by ~4.5-fold during 24 hr whereas the *Pseudomonas* sp. 346 added to soil was increased by ~9.4-fold (Table 9). Addition of Fe^{2+}, Zn^{2+}, and Cu^{2+} had no significant effect on bacterial counts in soil.

Correlation of chlamydospore germination inhibition and siderophore production by various isolates of *Pseudomonas* spp. *Pseudomonas* spp. isolates 381b, 458, 61, 8c, 346r, 712, and N1R produced different amounts of fluorescent siderophores in liquid medium (Fig. 5). The isolates also demonstrated differences in inhibitory effects on chlamydospore germination in soil. There was a direct correlation between the amount of siderophore produced by each isolate and inhibition of chlamydospore germination induced by the addition of these isolates to soil amended with glucose and asparagine (r = 0.99). After 24 hr of incubation, populations of all isolates in soil were not significantly different ($1–2 \times 10^9$ cfu/g soil).

Effects of bacterial isolates on Fusarium wilt of cucumber seedlings. Various isolates reduced disease incidence to different levels, but no single isolate provided complete protection (Table 10). Among the fluorescent isolates, the best control was provided by isolates N1R, B10, 381, 346, 8c, and 609 (33–43% of disease incidence of the control). The combination of three fluorescent isolates (B10, 346, and 712) provided the best protection (19% of the control). Among the chitinolytic isolates, *S. liquifasciens* provided moderate protection (56% of the control). Among the germ-tube-lytic isolates, 691, 406, 411, and 632 provided significant protection (37–43% of the control). Among the others, *Bacillus* sp. 443, *E. herbicola*, and *E. cloacae* also were effective (41–55% of control). Combining a fluorescent isolate with a germ-tube-lytic one did not increase the effectiveness in reducing disease incidence above that provided by one of the isolates.

DISCUSSION

A major objective of the present study was to evaluate lysis as a mechanism of biological control. Such a mechanism was suggested for the control of *Fusarium* spp. (13,18,28,29) and *Pythium* sp. (19) when cell-wall-lytic bacteria were added to soil. Such heterolytic activity was not confirmed in vivo in these studies, however. On the contrary, Ko and Lockwood (11) supplied evidence that autolysis

TABLE 4. Effect of bacterial isolates on the lysis of pregerminated chlamydospores of *Fusarium oxysporum* f. sp. *cucumerinum* in soil[a]

Isolates	Isolate no.	Characteristic	Incubation (8 days): Chlamydospores with germ tubes (%)	Incubation (8 days): Germ tubes with lysis (%)
Control			24[b]	10[b]
Pseudomonas sp.	61	Fluorescent	22	11
Pseudomonas sp.	346	Fluorescent	22	11
Unidentified	691	Germ-tube lytic	21	13
Unidentified	710	Germ-tube lytic	23	13
Unidentified	711	Germ-tube lytic	24	14
Enterobacter cloacae		None[c]	21	10

[a] Bacteria were added to soil at 10^8 cfu/g after 24 hr of incubation in soil amended with glucose (0.6 mg/g) and asparagine (0.15 mg/g). During the 24-hr incubation, 78% of the chlamydospores were induced to germinate.
[b] Results of all treatments in each column are not signifcantly different (P = 0.05). The results are average of three independent experiments, each with three replications.
[c] An isolate that was not fluorescent, chitinolytic, or germ-tube lytic.

TABLE 5. Effect of bacterial isolates[a] on germination and subsequent lysis of chlamydospores of *Fusarium oxysporum* f. sp. *cucumerinum* in the rhizosphere[b] of cucumber seedlings[c]

Bacterium	Isolate no.	Characteristic	Incubation period: 2 days: Germination (%)	8 days: Chlamydospores with germ tubes (%)	8 days: Germ tubes with lysis (%)
Control			43	23	12
Pseudomonas sp.	N1R	Fluorescent	28	23	11
Pseudomonas sp.	61	Fluorescent	34	25	12
Pseudomonas sp.	346	Fluorescent	26	23	11
Serratia liquifasciens		Chitinolytic	47	24	14
Unidentified	691	Germ-tube lytic	28	19	8
Unidentified	710	Germ-tube lytic	40	23	12
Unidentified	711	Germ-tube lytic	38	20	10
Enterobacter cloacae		None[d]	19	16	7
Pseudomonas sp. and *Serratia liquifasciens*	61	Fluorescent Chitinolytic	29	25	12
Pseudomonas sp. and unidentified	N1R 691	Fluorescent Germ-tube lytic	33	26	18
Pseudomonas sp. and unidentified	N1R 711	Fluorescent Germ-tube lytic	34	16	7
LSD (P = 0.05%)				11	7

[a] Bacteria were added to soil at 10^8 cfu/g.
[b] A 0.5-mm layer of soil was collected from around the taproots of 3-day-old cucumber seedlings.
[c] Results are an average of three independent experiments, each with three replications.
[d] An isolate that was not fluorescent, chitinolytic, or germ-tube lytic.

due to competition for carbon substrate was involved in the lytic phenomenon. Autolysis is frequently observed in pure cultures (34). Lysis was also suggested as a mechanism when chitin was added to soil for biological control of *Rhizoctonia solani* (19). While such an amendment should increase the activity of chitinolytic microorganisms, the mechanism implicated was the production of inhibitory polar lipids produced by the soil microflora during the decomposition of chitin (30). In our experiments, no evidence was obtained, indicating that lysis of germ tubes of chlamydospores was induced in soil by any of the microorganisms exhibiting this attribute in vitro (Tables 4 and 5). Even so, Campbell and Ephgrave (3,4) demonstrated that hyphal lysis induced by *Bacillus* sp. in soil was involved in the mechanism of biological control of *Gaeumannomyces graminis*.

Further evidence was accumulated to suggest that competition for available Fe^{3+} is involved in induction of Fusarium wilt-

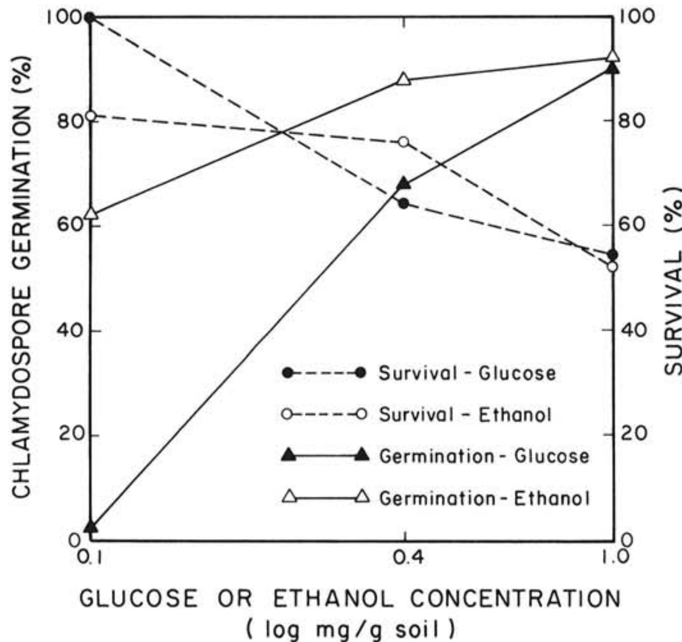


Fig. 4. Effect of glucose or ethanol on chlamydospore germination (after 24 hr) and survival (after 3 wk) of *Fusarium oxysporum* f. sp. *cucumerinum* in soil.

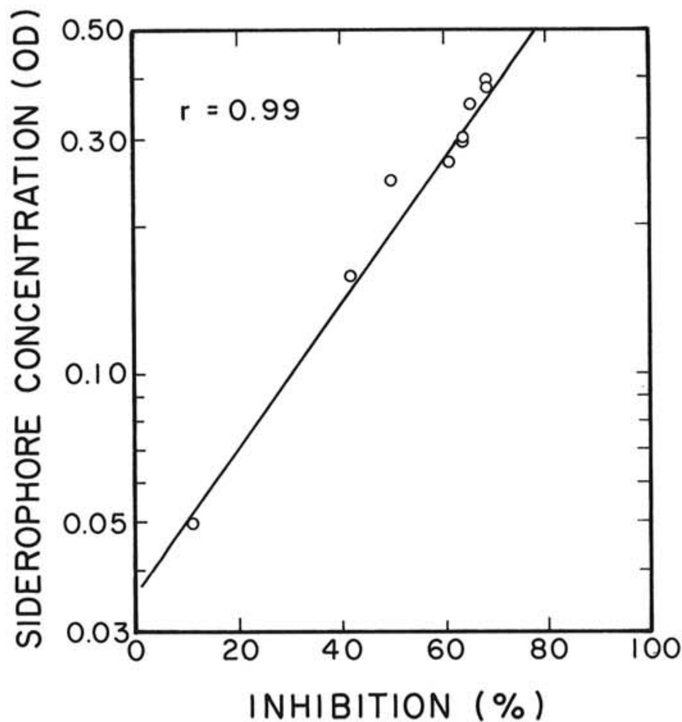


Fig. 5. A correlation between siderophore production by eight isolates of *Pseudomonas* sp. (as measured by optical density of a siderophore extract at 420 nm) and inhibition of chlamydospore germination of *Fusarium oxysporum* f. sp. *cucumerinum* in soil amended with glucose (0.6 mg/g soil) and asparagine (0.15 mg/g soil).

TABLE 6. Effect of an iron-chelating agent and a fluorescent *Pseudomonas* isolate 346[a] on germination of chlamydospores of *Fusarium oxysporum* f. sp. *cucumerinum* in soil amended with glucose and asparagine[b] or in the rhizosphere of cucumer seedlings

Isolate	FeEDDHA	Germination (%) Amended soil	Rhizosphere[d] soil
Control	−	57	36
	+	55	20
Pseudomonas sp. 346	−	8	21
	+	10	13
LSD ($P = 0.05$)		7	6

[a] Initial level in soil was 10^8 cfu/g.
[b] Glucose and asparagine concentrations in soil were 0.4 and 0.1 mg/g.
[c] FeEDDHA was added to soil at 0.1 mg/g.
[d] A 0.5-mm soil layer was collected from around taproots of 3-day-old cucumber seedlings.

TABLE 7. Effect of iron and bacterial isolates[w] on germination of chlamydospore of *Fusarium oxysporum* f. sp. *cucumerinum* in soil amended with glucose and asparagine[x]

Bacterium	Isolate no.	Germination (%) Without Fe^{2+}	With Fe^{2+}[y]
Control		83 b[z]	94 a
Pseudomonas sp.	346	10 d	46 c
Pseudomonas sp.	N1R	15 d	50 c
Enterobacter cloacae		12 d	40 c

[w] Initial levels of bacterial cells were 10^8 cfu/g of soil.
[x] Soil was amended with 0.6 mg of glucose and 0.15 mg of asparagine per gram.
[y] Fe^{2+} added at concentrations of 0.2 mg/g of soil as $Fe_3(SO_4)_2$.
[z] Numbers followed by the same letter are not significantly different ($P = 0.05$).

TABLE 8. Effect of micronutrients and a *Pseudomonas* sp. on the inhibition of chlamydospore germination in *Fusarium oxysporum* f. sp. *cucumerinum*

Micronutrient and concentration (μg/g soil)	Germination (%) of chlamydospores[a]: Without *Pseudomonas* sp. 346 glucose 0.3 mg/g, asparagine 0.075 mg/g soil	With *Pseudomonas* sp. 346[b] glucose 0.6 mg/g, asparagine 0.15 mg/g soil
Control without *Pseudomonas* sp. 346	51	85
Control with *Pseudomonas* sp. 346	−	17
Fe^{2+}, 200	70	60
Cu^{2+}, 10	68	67
Zn^{2+}, 10	81	53
Co^{2+}, 10	59	46
Mn^{2+}, 10	56	40
MoO_4^{2-}, 30	70	35
H_3BO_3, 10	53	25
Mg^{2+}, 100	55	24
LSD ($P = 0.05$)	7	11

[a] Glucose and asparagine were added to induce germination of chlamydospores.
[b] *Pseudomonas* sp. isolate 346 added at 10^8 cfu/g of soil.

suppressive soil. Previous theory expansion (26) provided evidence that manipulation of a previously conducive system by Fe management induced the soil to become suppressive. Fe-acquiring compounds (siderophores) produced by soil microflora or chelates (such as FeEDDHA) with a higher stability constant than those of the pathogen's siderophores induced suppressiveness. Such a system in the presence of roots, which also take up Fe^{3+}, promotes intense competition for this element which is necessary for complete germination and infection by formae speciales of *F. oxysporum* in environments low in Fe-availability due to high pH.

Inhibition of germination of chlamydospores was observed in the rhizosphere but not in soil amended with nutrients, to simulate root exudations, when FeEDDHA was added (Table 6). These results were predicted (26) since the Fe equilibrium reaction, $Fe(OH)_3 + 3H^+ \leftrightarrow Fe^{3+} + 3H_2O$, releases more Fe^{3+} in the soil mass

TABLE 9. Effect of micronutrients on the development of indigenous bacteria and fluorescent *Pseudomonas* sp. isolate 346 in soil amended with glucose and asparagine[x]

Soil treatment	Population (cfu/g soil)[y] after incubation for: 0 hr	24 hr
Control, without *Pseudomonas* sp. 346	2.9×10^6 a[z]	1.3×10^7 c
Control, with *Pseudomonas* sp. 346	1.6×10^7 b	1.5×10^8 c
Fe^{2+} 200 μg/g soil	2.4×10^7 b	2.5×10^8 c
Zn^{2+} 10 μg/g soil	1.1×10^7 b	1.6×10^8 c
Cu^{2+} 10 μg/g soil	1.7×10^7 b	1.8×10^8 c

[x]Soil was amended with 0.6 mg glucose and 0.15 mg asparagine per gram.
[y]Tenfold serial dilutions of soils were carried out and aliquots were inoculated on King's B in five replicates per each treatment; colonies were counted after incubation for 24 hr.
[z]Numbers followed by the same letter are not significantly different (P = 0.05%).

where it is bound to a siderophore or to the ligand EDDHA (14,15). On the rhizoplane, however, there is more intense competition for Fe^{3+} by the root and siderophores produced by rhizosphere bacteria (both of which can utilize Fe from FeEDDHA). Once Fe is removed from the ligand it binds more Fe^{3+} from the pool available from the equilibrium reaction. The combination of a fluorescent pseudomonad and FeEDDHA added to soil significantly reduced chlamydospore germination in the rhizosphere compared with either treatment alone (Table 6). This provides an explanation for the additive effects of these amendments in inducing soil to become suppressive in short-term experiments (26).

Other experiments provided further evidence that competition for Fe^{3+} is associated with the induction of suppressiveness. Fluorescent pseudomonads, producing siderophores with a higher stability constant than those produced by Fusarium wilt pathogens, inhibited chlamydospore germination in soil (Tables 2, 6–8; Figs. 2, 3, and 5) or in the rhizosphere (Tables 3 and 5). Other bacteria also inhibited germination (for example, *E. cloacae, E. herbicola*, and isolate 691); however, this does not deny the hypothesis that siderophores participate in the inhibition of chlamydospore germination. Most of the microorganisms so far tested produce Fe-chelating compounds; bacteria produce siderophores of the catechol group which bind Fe much more strongly (stability constant = 10^{40}) than the hydroxymate siderophores produced by the fungi (stability constant of 10^{28}) (7,21–23,32). Evidence that Fe-competition could be implicated in inhibition of chlamydospore germination by *E. cloacae* is presented in Table 7 in that Fe^{2+} counteracted the inhibition.

The ability of the limiting factor of an added candidate to nullify or ameliorate inhibition or suppressiveness provides evidence for the operation of the factor in competition (1). Thus, Scher and Baker (26) demonstrated that conidium germination of *Fusarium* was inhibited by EDDHA in vitro and excess of Fe counteracted inhibition. Misaghi et al (17) reported inhibition of fungal growth

TABLE 10. Effect of addition of bacterial isolates[a] on disease induced by *Fusarium oxysporum* f. sp. *cucumerinum*

Bacterium	Isolate no.	Characteristic	Disease incidence (% of control)[c]
Pseudomonas sp.	B10	Fluorescent	37
Pseudomonas sp.	A12	Fluorescent	51
Pseudomonas sp.	N1R	Fluorescent	33
Pseudomonas sp.	8c	Fluorescent	43
Pseudomonas sp.	61	Fluorescent	76
Pseudomonas sp.	346	Fluorescent	40
Pseudomonas sp.	381	Fluorescent	38
Pseudomonas sp.	544	Fluorescent	70
Pseudomonas sp.	712	Fluorescent	67
Pseudomonas sp.	452	Fluorescent	100
Pseudomonas sp.	609	Fluorescent	43
Unidentified	3c	Chitinolytic	70
Unidentified	12a	Chitinolytic	70
Unidentified	409	Chitinolytic	62
Hafnia sp.		Chitinolytic	71
Serratia liquifasciens		Chitinolytic	56
Arthrobacter sp.	P35	Germ-tube lytic	62
Unidentified	163	Germ-tube lytic	71
Unidentified	496	Germ-tube lytic	79
Unidentified	710	Germ-tube lytic	66
Actinomycete	227	Germ-tube lytic	81
Unidentified	406	Germ-tube lytic	43
Unidentified	411	Germ-tube lytic	45
Unidentified	632	Germ-tube lytic	43
Unidentified	641	Germ-tube lytic	73
Unidentified	682	Germ-tube lytic	53
Unidentified	691	Germ-tube lytic	37
Unidentified	711	Germ-tube lytic	52
Bacillus sp.	443	None[b]	41
Unidentified	509	None	82
Unidentified	700	None	79
Erwinia herbicola		None	42
Enterobacter cloacae		None	55

(continued)

TABLE 10 (continued). Effect of addition of bacterial isolates[a] on disease induced by *Fusarium oxysporum* f. sp. *cucumerinum*

Pseudomonas sp.	B10	Fluorescent	
and *Pseudomonas* sp.	346	Fluorescent	
and *Pseudomonas* sp.	712	Fluorescent	19
Pseudomonas sp.	61	Fluorescent	
and *Serratia liquifasciens*		Chitinolytic	40
Pseudomonas sp.	N1R	Fluorescent	
and unidentified	163	Germ-tube lytic	40
Pseudomonas sp.	B10	Fluorescent	
and *Arthrobacter* sp.	P35	Germ-tube lytic	49
Pseudomonas sp.	712	Fluorescent	
and unidentified	691	Germ-tube lytic	49
Pseudomonas sp.	B10	Fluorescent	
and *Serratia liquifasciens*		Chitinolytic	60
Pseudomonas sp.	712	Fluorescent	
and *Serratia liquifasciens*		Chitinolytic	60
Pseudomonas sp.	712	Fluorescent	
and unidentified	411	Germ-tube lytic	60
Pseudomonas sp.	712	Fluorescent	
and unidentified	711	Germ-tube lytic	60
Pseudomonas sp.	712	Fluorescent	
and *Enterobacter cloacae*		Germ-tube lytic	60
Pseudomonas sp.	346	Fluorescent	
and *Serratia liquifasciens*		Chitinolytic	49
Pseudomonas sp.	381	Fluorescent	
and unidentified	496	Germ-tube lytic	70
Pseudomonas sp.	609	Fluorescent	
and unidentified	710	Germ-tube lytic	55
Pseudomonas sp.	381	Fluorescent	
and unidentified	691	Germ-tube lytic	37
Pseudomonas sp.	346	Fluorescent	
and unidentified	409	Chitinolytic	43
LSD ($P = 0.05$)			23

[a] Initial level of bacterial isolates in soil was 10^8 cfu/g.
[b] Isolates that were not fluorescent, chitinolytic, or germ-tube lytic.
[c] Disease incidence was calculated as percent incidence of the nontreated control which had $65 \pm 13\%$ incidence. Results are the average of three experiments. Each pot contained five plants, treatments were randomized in three replications, one replication per pot.

by a partially purified siderophore of *Pseudomonas* sp. in vitro; again, the effect was counteracted by Fe. Excess Fe also nullified suppressiveness to Fusarium wilt (26). Supplying an excess of available Fe to soils presents certain technical difficulties in that the amount of Fe^{3+} supplied by the equilibrium reaction (above) is determined by soil pH. The addition of Fe^{2+}, however, introduces a new set of parameters which renders Fe available for an extended period of time. These relationships have been reviewed (14,15). Therefore, the results in Tables 6–8 demonstrate that the addition of Fe^{2+} to soil partially counteracted inhibition of chlamydospore germination induced by various bacteria and suggests that competition for this element was involved.

Other micronutrients also partially counteracted inhibition of chlamydospore germination (Table 8). There are a number of hypotheses that might explain this phenomenon. First, all of these elements could be limiting in the system; however, it is unlikely that such a wide variety of ions would all be in short supply and necessary for germination of chlamydospores. These same considerations also devalue an explanation based on the idea that this wide variety of ions could replace Fe in the metabolism of germinating chlamydospores. It is possible that the added microelements depressed multiplication and activity of siderophore-producing bacteria; however, there was no evidence that the elements added to soil at the same concentrations had an effect on cell multiplication of *Pseudomonas* sp. 346 (Table 9). A more likely explanation is based on the lack of specificity for binding of Fe^{3+} by some chelators (14,15). In such systems (unlike EDDHA) other cations may be incorporated and/or substituted for Fe^{3+}. There is no information available regarding specificity of siderophores for binding Fe^{3+} although they are assumed to be Fe-acquiring compounds. If added cations compete for sites ordinarily occupied by Fe in siderophores, however, this may provide an explanation for the observed partial counteraction of germination inhibition by various cations. An explanation for the similar effect of an anion such as MoO_4^{2-} is not evident. It is also possible that certain ions have a detrimental effect on siderophore production.

In the present study, there were considerable differences in siderophore production by a variety of fluorescent *Pseudomonas* spp. isolated from the soil suppressive to *Fusarium*. A direct

correlation ($r = 0.99$) was evident between the level of siderophores produced by the isolates and the inhibition of chlamydospore germination. Some bacterial isolates, including fluorescent pseudomonads, although present in soil in high numbers (high biomass), did not induce soil-suppressiveness to chlamydospores of *Fusarium*. The results indicate that it is not sufficient to merely induce a high biomass in soil to render the soil suppressive to chlamydospore germination. The qualitative composition of the microbial populations in soil determined its level of suppressiveness. This does not support the hypothesis that soil-suppressiveness to *Fusarium* is determined by general "soil biomass" activity (5,8).

Some isolates did not inhibit chlamydospore germination but did significantly reduce disease incidence. For example, *Bacillus* sp. 443 did not produce detectable lytic enzymes, inhibition zones in vitro or inhibit chlamydospore germination in soil. Yet it induced suppressiveness to Fusarium wilt in soil (Table 10). This suggests that other mechanisms than competition for Fe^{3+}, lysis, or antibiosis operate in some systems.

Combinations of lytic and fluorescent bacteria added to conducive soil did not result in suppressiveness that was additive (Table 10). The number of isolates tested in this study, however, was limited; therefore, it is possible that compatible combinations of isolates having various beneficial attributes may induce increased suppressiveness.

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