Mechanism of Biological Control in a Fusarium-Suppressive Soil

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ABSTRACT

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Metz fine sandy loam soil from the Salinas Valley in California was suppressive to the *Fusarium* spp. which induce wilts of flax and carnation. Suppressiveness to *Fusarium oxysporum* f. sp. *dianthi* was transferred to conducive soil when the Metz fine sandy loam was added in small amounts to steamed greenhouse soil. Aerated steam treatment of the suppressive soil at 54 C for 30 min eliminated the suppressive effect. Lowering of pH values of the Metz fine sandy loam from 8.0 to 6.0 in unit increments eliminated the suppressive effect. Bacteria were isolated from mycelial mats of *F. oxysporum* f. sp. *lini* buried in the suppressive soil and conducive soils. Two isolates from suppressive soil introduced into conducive soil at 10^5 cells per gram of soil significantly reduced disease incidence of Fusarium wilt of flax. The more effective of these isolates inducing suppressiveness was a *Pseudomonas* sp. Viability of this organism was drastically reduced when soil was treated with aerated steam at 54 C. These results suggest that suppressiveness in the Metz fine sandy loam is biological in origin and that control of Fusarium wilt diseases may be accomplished through introduction of appropriate species of bacteria into conducive soil.

In 1975, Toussoun (28) reviewed phenomena associated with Fusarium-suppressive soils. He described two types: (i) the classic type in which pathogenic forms of the fungus are suppressed, and (ii) those in which all fusaria in the soil are affected. We report in this paper studies of a Fusarium-suppressive soil of the classic type (i), and contribute information on the nature of the entity responsible for biological control of vascular wilt diseases.

As early as 1892 (2), and just after the turn of the century (17,20), researchers observed that the prevalence of Fusarium wilt diseases varied among soils, and disease incidence appeared to be dependent on soil texture. Others (19,21,27,31) observed suppressiveness to the wilt Fusaria in soils in various geographical locations; however, a landmark in the study of such soils was the work of Walker and Snyder (30) on the relation of soil type to Fusarium wilt of pea. In experimental plots in a clay soil and a sandy loam from an area where disease was severe, they found that wilt developed in the loam but not in the clay, but were unable to establish the pathogen in the clay soil.

Smith and Snyder (25,26) reported another example of a Fusarium-suppressive soil in the Salinas Valley in California. There, despite long cultivation of a wide variety of crops, Fusarium wilt diseases do not occur. In greenhouse tests, sweet potato wilt was less severe in this suppressive soil than in soil from an adjacent area (where wilt was known to occur) when the soils were infested with the pathogen. The soil from the Salinas Valley was the Fusarium-suppressive soil used in our studies.

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MATERIALS AND METHODS

Properties of the three soils used are given in Table 1. Two of these were conducive to development of Fusarium wilt. One was a Fort Collins clay loam that had been used in other studies (18), and the second was collected from a commercial greenhouse in which carnations (*Dianthus caryophyllus* L.) had been monocultured in ground beds for 20 yr or more. The latter, hereafter referred to as a greenhouse soil, had been heavily amended with organic matter, and fertilizers had been applied routinely (15). Fusarium wilt of carnations was observed in this soil, but the samples taken for experimentation were from an area in which this disease was not observed. Thus, a soil potentially conducive to wilt was obtained, but there was no evidence that it contained the wilt pathogen. Both the Fort Collins clay loam and greenhouse soil were air-dried after collection, sifted through a 4-mm screen, and stored in metal cans until used. The third soil was the Metz fine sandy loam (a Fusarium-suppressive soil) collected from the Salinas Valley in California. After transportation to Colorado, the soil was placed in raised benches in a greenhouse and planted with tomatoes (*Lycopersicon esculentum* Mill. 'Bonny-Best'). Just before experimental use, the amount of suppressive soil required was removed from the benches, air-dried, and sifted through a 4-mm screen.

Fusarium oxysporum Schlecht. f. sp. *dianthi* (Prill. & Del.) Snyder & Hansen was isolated from carnations with symptoms of wilt in a Colorado greenhouse. Diseased flax stem tissue containing *F. oxysporum* Schlecht. f. sp. *lini* (Bolley) Snyd. & Hans. was obtained from C. C. Tu (Taiwan Agric. Res. Inst., Taichung, Republic of China) from which the fungus was isolated in pure

culture. For introduction of these pathogens into soil, 250 g of carnation tissue was autoclaved with 25 ml of distilled water in 1 L flasks plugged with cotton. The pathogens were grown separately in these flasks at 25 ± 1 C for approximately 28 days. Tissue then was removed from the flasks and air dried. This inoculum mix was triturated in a Waring Blendor (Model 5011, Waring Products, New Hartford, CT 06057), passed through a 1-mm screen, and stored in plastic containers until needed. This inoculum, containing macroconidia, microconidia, and chlamydospores, was similar to that used in previous investigations (13).

Two hosts were used in these experiments: Fusarium wilt of carnations has an incubation period of 6 mo or longer. Carnations grown under conventional greenhouse conditions (15) were used in long-term experiments to test hypotheses related to the practical aspects of biocontrol with suppressive soils.

Basic research on biocontrol mechanisms and environmental relationships were done with flax (*Linum usitatissimum* L. 'Taichung, #1'). Symptoms of Fusarium wilt of flax developed in 30 days or less. In this case, the inoculum was added to soil at the rate of 1% (w/w) and evenly mixed in a twin-shell blender (8). After adjustment of the soil matric potential to −0.7 bars, 200-g (dry wt) portions were distributed into plastic pots (11 cm diameter top, 9 cm diamter bottom, and 7 cm high). Soil in each pot was planted with 15 flax seeds at a depth of 1 cm. There were five replications in each treatment. After emergence, the number of plants per pot was thinned to 10 per pot.

Pots were usually placed in growth chambers at 30 ± 2 C with 14 hr of fluorescent illumination daily (approximately 4,000 lx). For large experiments, pots were placed on benches in a room with continuous illumination from neon lamps (approximately 5,000 lx) at 27 ± 2 C. Plants were watered every other day by adding 50 ml per pot of a nutrient solution proportioned at 1:200 from a stock solution containing 11.34 kg KNO_3, 5.44 kg $MgSO_4$, 2.27 kg NH_4NO_3, 110 g $NaBO_3$, 15 g $ZnSO_4$, and 400 ml H_3PO_4 in 94.63 L water.

Every 3 days the number of wilted flax plants was recorded and those with symptoms were pulled and discarded, or randomly selected tissue samples were surface disinfected and plated on Fusarium-selective medium (7) to insure correct diagnosis. Analysis of variance and multiple-range comparisons were employed for processing data.

Heat treatment of soils. Steam-air mixtures were used to test the effect of heat on soil suppressiveness. Steam and air were separately introduced through rubber and glass tubes (inserted through a rubber stopper [41 mm top and 33 mm bottom, #8]) into a 1-L side-armed Erlenmeyer flask. Temperature inside the flask, as measured with a mercury thermometer inserted through the stopper, was adjusted by balancing the amounts of steam and air flowing into the flask. Rubber tubing, attached to the side arm, led the adjusted steam-air mixture directly into the soil. Samples to be steamed were placed in a round copper sieve 21 cm in diameter and 7 cm high with 150-μm openings and covered with aluminum foil. The steam-air mixture was introduced under the foil. Soil temperature was measured by two mercury thermometers periodically placed at different points in the soil mass. Soil was steamed for 30 min after all points had reached the desired temperature.

Measurement and adjustment of soil pH values. The pH of the Fort Collins clay loam (conducive) and the Metz fine sandy loam (suppressive) was lowered by adding 0.1 N H_2SO_4. Both soils originally had a pH of approximately 8.0. This was reduced to pH 7.0 or 6.0. For pH measurement, soil was added 1:2 (w/v) to 0.01 M $CaCl_2$ (22). The pH of 1-g samples of soil from individual replications was measured periodically to ensure that the soil reaction remained constant. During initial experiments, maintaining the soil at pH 6.0 was difficult because the plants were being watered with nutrient solution having a pH of approximately 7.0. After the nutrient solution was adjusted to pH 6.0 with 0.1 N H_2SO_4 a constant soil pH 6.0 was achieved.

Isolation of possible antagonists. Flame-proof nylon screens (2 cm^2) with 1 mm^2 holes were placed on Fusarium-selective medium, five per petri plate. *F. oxysporum* f. sp. *lini* was introduced into the center of each plate. After ~7 days the screens covered with hyphae and spores of the fungus were peeled from the agar surface and buried in either Fort Collins clay loam or Metz fine sandy loam. After 24 hr, the screens were retrieved and placed in two 1-L Erlenmeyer flasks, one for each soil type, and rinsed for 15 min by circulating distilled water into the flasks through tubing inserted in rubber stoppers in the neck of the flasks. The water circulated through the flasks and was flushed out through additional glass tubes in the stoppers. By this rinsing method, most extraneous soil, and microorganisms only coincidently associated with the thallus, were removed from the nylon screens, presumably leaving a high proportion of those intimately associated with the pathogen. Screens were placed on potato dextose agar (PDA). After 24 hr, bacteria and fungi were observed around the nets; bacterial growth was more abundant. Individual colonies of bacteria were streaked on PDA (to ensure purity of culture), transferred to PDA slants, and incubated at 25 ± 1 C. Isolates were washed from the slants with 10% skim milk and lyophilized for storage.

For introduction of the bacteria into soil, lyophilized cultures were rehydrated and shake cultured in nutrient broth for 2 wk. The bacterial cells were removed from the culture medium by centrifugation at 20,200 *g* and rinsed twice in physiological saline solution (0.85% NaCl). These bacterial suspensions in physiological stock solutions were kept in a refrigerator at 10 C until they were introduced into soil. The number of bacterial cells per milliliter in the stock solution was determined by plating serial dilutions on PDA. Bacteria were added to soil by suspending the appropriate amount of stock solution in 100 ml of physiological saline and distributing this evenly into 1,000 g of soil

RESULTS

Effect of adding suppressive soil to conducive soils. Steamed plant growth medium (equal parts of peat, Perlite [Persolite Products Inc., Florence, CO 81226], and Fort Collins clay loam) in raised flats (each 100 × 100 × 18 cm) was infested with *F. oxysporum* f. sp. *dianthi*. Either suppressive or the conducive greenhouse soil was added to the steamed soil in each flat at the rate of 1% by weight, but no additional soil was added in the nontreated controls. Carnations (CSU Pink) were transplanted to these flats

TABLE 1. Properties of Fusarium wilt conducive and suppressive soils[a] used in experiments on biological control of Fusarium wilt of carnation and flax

Soil series name or designation	pH	Conductivity (mmhos/cm)	Lime[b]	Organic matter (%)	NO_3-N (μg/g)	P_2O_5 (μg/g)	K_2O (μg/g)	DPTA[c]-extractable micronutrients	
								Zn (μg/g)	Fe (μg/g)
Fort Collins clay loam	8.2	0.4	High	1.9	4	10	139	16.5	2.5
Extremely modified greenhouse soil	5.9	7.1	Low	5.7	350	70	996	39.7	61.3
Metz fine sandy loam	8.1	1.4	Low	1.3	3	11	308	2.1	4.3

[a] The first two (Colorado) soils were wilt-conducive; the third soil (from the Salinas Valley in California) was wilt-suppressive.
[b] Lime: low = <1%, high = >2%.
[c] DPTA, diethylenetriamine penta-acetic acid extraction.

(30 per flat). There were six replications. Six to 14 mo after transplanting, there was less disease in the suppressive soil than in the control or conducive soil treatments (L. Merrell, *unpulbished*). A second crop of carnations was planted in these same flats. Fusarium wilt symptoms were apparent 8 mo after transplanting. After 17 mo, the incidence of wilt had reached 66% in the control, 50% in the plots treated with the conducive greenhouse soil, and 17% in the suppressive soil treatment (Fig. 1).

The effect of suppressive soil added to greenhouse "ground beds" on development of Fusarium wilt of carnations was studied in a commercial greenhouse. Plots were set up in two adjacent planting beds in which the entire planting of carnations was dead (the preceding crop having been killed by *F. oxysporum* f. sp. *dianthi*). Nontreated suppressive soil was added (1%, w/w) to Fort Collins clay loam (autoclaved 2 hr on each of 2 days in succession), and this mixture was incubated at 25 ± 1 C for 1 mo. This mix was added (600 g/m^2) to plots in the beds after the grower had steamed the beds by using conventional procedures (15). In other plots steamed at the same time, the same amount of nondiluted suppressive soil was added. Additional plots were steamed but not treated with

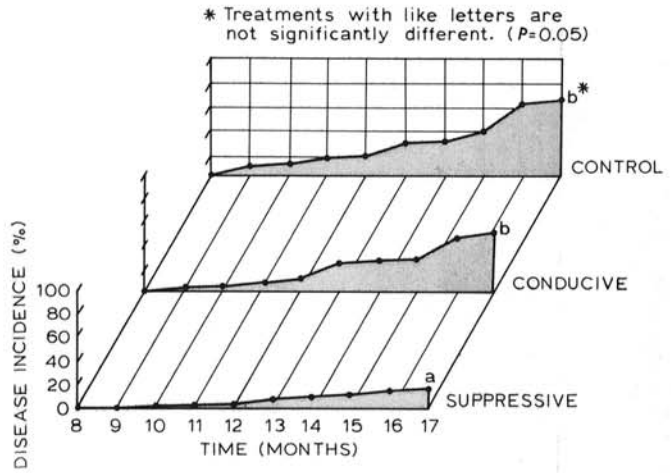

Fig. 1. Incidence of Fusarium wilt of carnations when Metz fine sandy loam (suppressive) or conducive greenhouse soil was added (1:99, w/w) to soil previously steamed and infested with *Fusarium oxysporum* f. sp. *dianthi*. No soil was added to previously steamed and infested soil in the control. There were 30 plants in each of six replications.

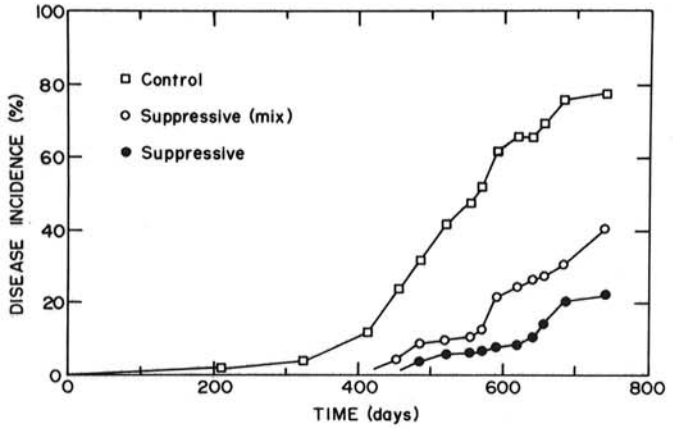

Fig. 2 Incidence of Fusarium wilt of carnations in steamed ground beds in a commercial greenhouse. Suppressive (mix) treatment involved mixing Metz fine sandy loam (suppressive) into autoclaved Fort Collins clay loam (1:99, w/w) and incubating for 1 mo. This incubated mix was added to plots in the ground bed at 600 g/m^2. In the suppressive treatment, nondiluted Metz fine sandy loam was added to plots at the same rate. No soil was added to the controls, but recolonization from adjacent nonsteamed areas certainly occurred. There were 144 plants in each of two replications per treatment.

suppressive soil. All plots were 3 × 1 m wide (in three replications) and there were 1.5-m-long buffers between them. In the 2 yr that carnations (CSU Pink) were observed in these plots, none of the plants in one replication in one of the beds developed symptoms of wilt (suggesting eradication of the pathogen due to steaming). Symptoms of Fusarium wilt were evident in the other two replications, however, and reduction in disease incidence was evident in both suppressive-soil treatments (Fig. 2).

Suppressive soil was blended with conducive Fort Collins clay loam (w/w) at 0, 1, 10, 30, 50, 70, and 100% in the flax wilt system. Disease incidence after 30 days was not significantly different among the 0, 1, 10, or 30% suppressive soil treatments (Fig. 3). The addition of 50 or 70% suppressive soil, however, produced a significant decrease in disease incidence. There was no significant difference between the treatments containing 70 and 100% suppressive soil.

Effect of heat treatment on soil suppressiveness. In a preliminary experiment, the incidence of Fusarium wilt of flax was 18% in nonsteamed suppressive soil after 30 days. When the soil was heated to 57 C or above, suppressiveness was lost and disease incidence increased to over 72% (Fig. 4A). To determine the extinction point of suppressiveness more precisely, increments of temperature for aerated steam treatments were decreased. Suppressiveness was significantly reduced at 54 C (Fig. 4B). Disease incidence in the nonsteamed soil of the former experiment (Fig. 4A) overall was lower than that in the same soil in the latter experiment (Fig. 4B). This gradual loss of suppressiveness was noted after removal of the Metz fine sandy loam from California fields in other experiments during this study.

The effect of soil pH on disease incidence. After the pH values of Fort Collins clay loam (conducive) and Metz fine sandy loam (suppressive) were adjusted in unit increments from 6 to 8, inoculum of *F. oxysporum* f. sp. *lini* was added and flax seeds were planted. In Fort Collins clay loam, change of pH values had no significant effect on disease incidence (Fig. 5A); however, soil reaction significantly affected disease incidence in suppressive soil (Fig. 5B). After 30 days, disease incidence was 38% in the original soil, 61% in the soil adjusted to pH 7.0, and 87% in soil at pH 6.0.

Isolation and reintroduction into soil of possible antagonists. Seven bacterial isolates were obtained from mycelial mats of *F. oxysporum* f. sp. *lini* buried in suppressive soil and four from those

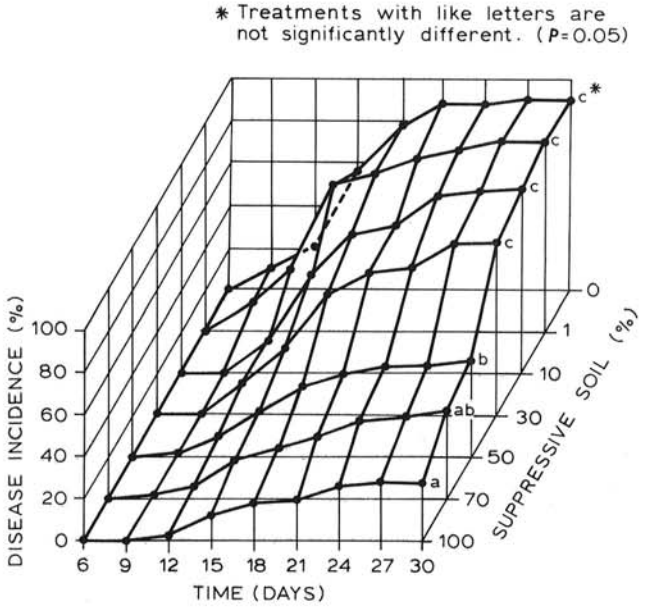

Fig. 3. Effect of adding Metz fine sandy loam (Fusarium-suppressive) on an oven dry (w/w) basis to Fort Collins clay loam (Fusarium-conducive), on the incidence of Fusarium wilt of flax. Soils were infested with *Fusarium oxysporum* f. sp. *lini*. There were 10 plants in each of five replications per treatment.

buried in Fort Collins clay loam (conducive). In preliminary tests, each of these isolates were added at a density of 10^5 cells per gram of soil to previously steamed or nonsteamed Fort Collins clay loam. Disease incidence in flax after 30 days was significantly less in soils to which each of two isolates from the suppressive soil (designated 4A and 5A) was added; the isolates were identified as *Pseudomonas* spp. After this preliminary screening, these isolates and three others from the conducive soil (designated 1B, 2B, and 4B and identified as *Bacillus* spp.) not conferring suppressiveness, were added to steamed and nonsteamed Fort Collins clay loam at ther same population density. No bacteria were added in the control. Addition of isolates 4A or 5A resulted in significantly less disease when treatments were compared with appropriate inoculated controls in steamed and nonsteamed soils (Fig. 6).

Fort Collins clay loam was autoclaved until no microorganisms could be isolated from it on nutrient medium (16,29). Cells of isolate 4A were mixed into this soil and aliquots were exposed to aerated steam at various temperatures for 30 min. Analyses of the densities of viable cells in soil (using dilution plates) after steam treatment yielded the following: 2×10^6 colonies per gram in the nonheated control; 5×10^5 colonies per gram in soil heated to 49 C; 9×10^2 in soil heated to 54 C; and 2×10^1 colonies per gram in soil heated to 80 C.

No antagonism was observed when the bacteria isolated from the mycelial mats were grown in pure culture on PDA adjacent to *F. oxysporum* f. sp. *lini*, except for isolate 2B.

DISCUSSION

Our results confirmed that the Metz fine sandy loam soil was suppressive to the development of Fusarium wilt diseases as reported by others (25). It conferred suppressiveness when added in

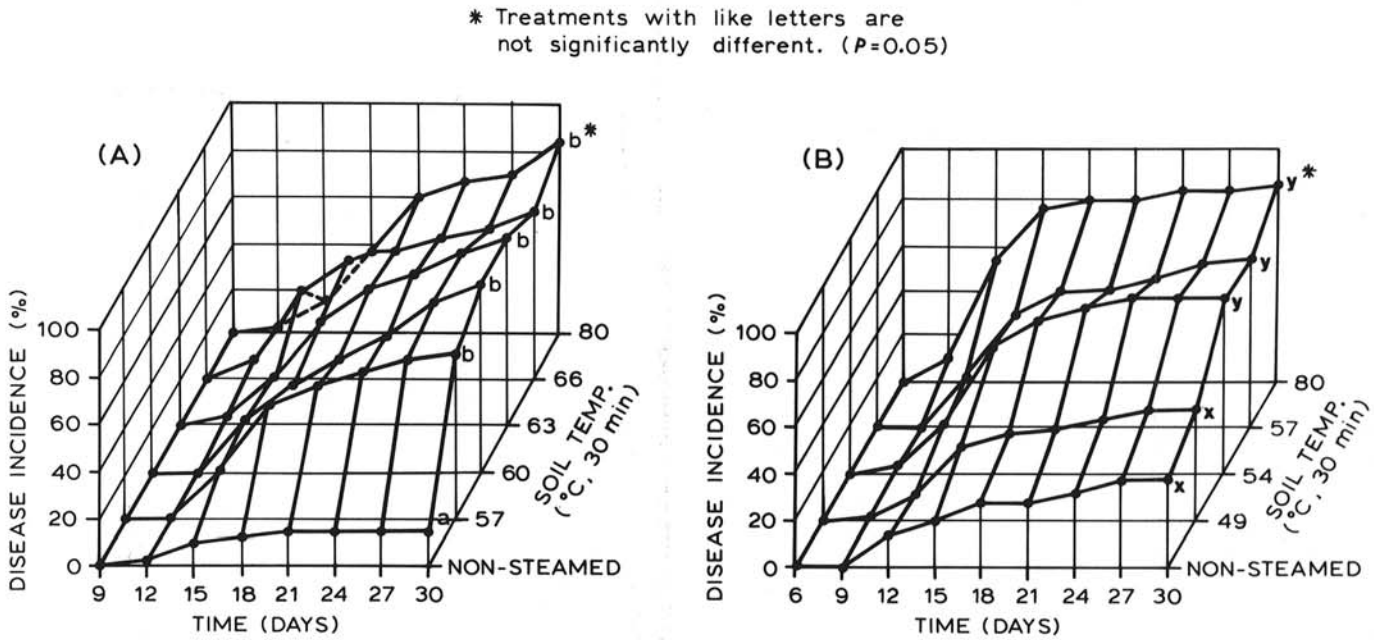

Fig. 4. Incidence of Fusarium wilt of flax when Metz fine sandy loam (suppressive) was heated to various temperatures for 30 min. Soil was infested with *Fusarium oxysporum* f. sp. *lini*. There were 10 plants in each of five replications per treatment. **A,** Preliminary experiment establishing the gross extinction point for suppressiveness; **B,** subsequent experiment in which smaller increments of temperature differential were used.

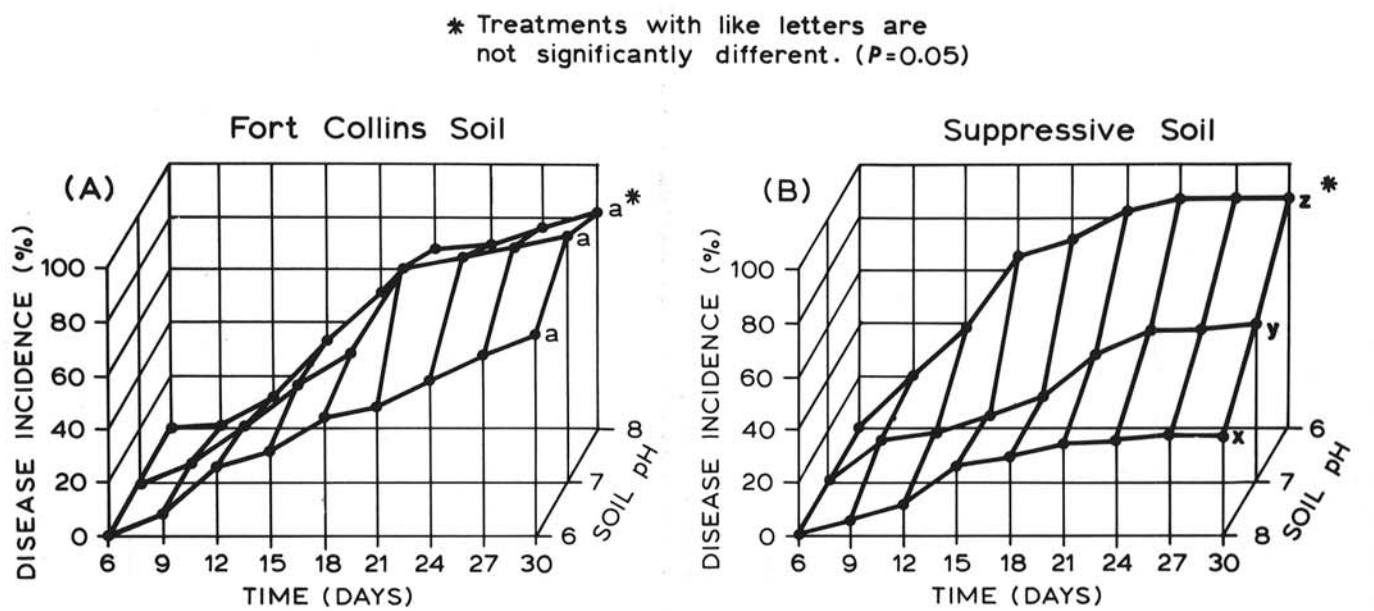

Fig. 5. Effect of soil pH on incidence of Fusarium wilt of flax induced by *Fusarium oxysporum* f. sp. *lini* in **A,** Fort Collins clay loam (conducive) and **B,** Metz fine sandy loam (suppressive). Soil pH was adjusted with 0.1 N H_2SO_4. There were 10 plants in each of five replications per treatment.

small amounts to steamed conducive soil (Fig. 1, 2). This is the first clear evidence that suppressiveness in this system is induced through biological control mechanisms; ie, it suggests that the active factor multiplied in soil in time and, thus, conferred suppressiveness to a previously conducive soil. The soil also induced suppressiveness to development of Fusarium wilt of flax when added in higher proportions to nonsteamed conducive soil (Fig. 3) similar to phenomena observed in a Rhizoctonia-suppressive system (32).

Various lines of evidence suggested that the entity(ies) responsible for suppressiveness might be found among the bacterial flora: (i) Smith (24) observed small colonies of pleomorphic bacteria closely adhering to stunted and lysed germ tubes of chlamydospores of Fusarium wilt pathogens in the Metz fine sandy loam. She observed a large number of colonies of *Arthrobacter* spp. when plate counts were made from suppressive soil following chlamydospore germination, but they were absent or present only in low numbers in similarly treated conducive soil. (ii) When the Metz fine sandy loam was heated to 54 C, suppressiveness was lost. Rouxel et al (21) reported similar results for a Fusarium-suppressive soil and concluded that fungi were responsible for suppression. However, many nonsporeforming bacteria can be killed at relatively low temperatures (3,9,10). The density of *Pseudomonas* spp. in soil declined when it was steamed for 10 min at 60 C (10). Nitrifying bacteria (*Nitrobacter* spp. and *Nitrosomonas* spp.) were inactivated in soil by moist heat for 30 min at 51–57 C (3). *Arthrobacter* spp. did not survive heating to 60 C for 30 min in skim milk (9). Finally, a drastic reduction in density of *Pseudomonas* spp. (isolate 4A which induced suppressiveness when introduced into conducive soil) was observed after treatment of soil with aerated steam at 54 C. (iii) No significant difference in disease incidence of flax wilt occurred in a conducive soil when the pH was lowered from 8.0 to 7.0 or 6.0. Although soil pH has been implicated as a factor in host susceptibility to the Fusarium wilt pathogens (23), our results indicated that hydrogen-ion concentration in soil had no appreciable influence on disease incidence in conducive soil in our flax wilt system within these pH limits. In contrast, change in values of soil pH had a striking effect on disease incidence in the Metz fine sandy loam. At pH 8.0, soil was suppressive; at 7.0, disease incidence significantly increased; and at 6.0, disease incidence was significantly higher than at 8.0 or 7.0. These results also suggested that the biological factor(s) in the Metz fine sandy loam could be bacterial in nature because alkaline soils generally are more favorable to soil bacteria than to some *Fusarium* spp. (4). Many bacteria are more sensitive than most fungi to acidic soil pH (12), and the greater the hydrogen-ion content of the soil, the smaller the size of the bacterial community (1).

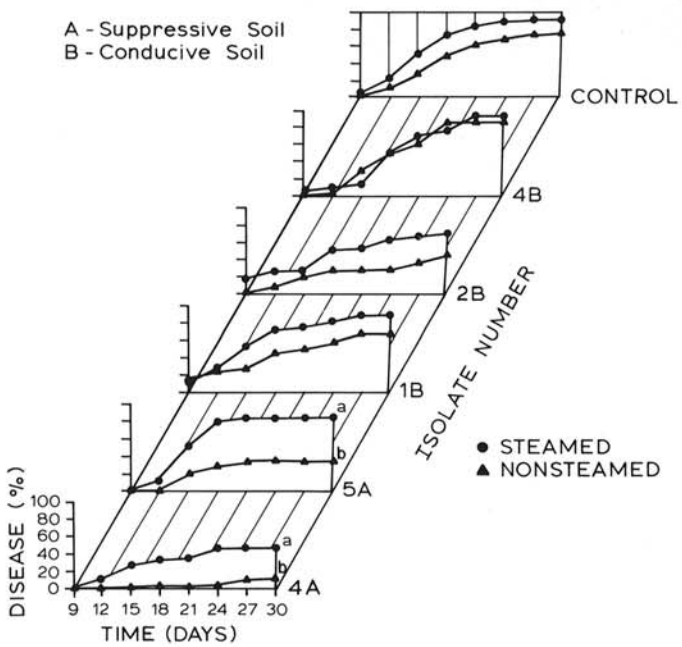

Fig. 6. Effect of addition of bacterial cells to steamed (●) and nonsteamed (▲) Fort Collins clay loam (conducive) at 10^5 cells per gram on incidence of Fusarium wilt of flax. No bacteria were added to soil in the control treatment. All treatments were infested with *Fusarium oxysporum* f. sp. *lini*. The letter "A" in the isolate number signifies that the bacterium was isolated from suppressive soil; "B" indicates isolation from conducive soil. There were 10 plants in each of five replications per treatment. Treatments marked "a" differed ($P = 0.05$) from the steamed control. Treatments marked "b" differed significantly from the nonsteamed control.

Mycelial mats of *F. oxysporum* f. sp. *lini* were introduced into soil to act as "bait" for potential antagonists. Four were isolated from the conducive soil and none of these induced suppressiveness when added to conducive or steamed soils. Of the seven isolated from the Metz fine sandy loam, however, two induced significant decreases in the incidence of flax wilt when added to conducive soil (Fig. 6). Greater suppressiveness by these antagonists was observed in raw soil than in steamed soils; perhaps reflecting greater inoculum potential of the pathogen in the latter soil which did not contain the background antagonism present in all biologically buffered systems (6). Again, the addition of nine other bacteria (associated with mycelial mats of *F. oxysporum* f. sp. *lini*) to conducive soil did not induce suppressiveness. These treatments were necessary "controls" (5) since the addition of massive amounts of thalli, especially to steamed soil, could increase the general level of biological buffering and not necessarily reflect more specific antagonistic interactions.

The results suggest that certain bacteria indigenous to the Metz fine sandy loam that was used in these experiments are responsible for suppression of Fusarium wilt pathogens. The most efficient of these in our tests was identified as a *Pseudomonas* sp.

The principles related to comparative epidemiology of the two suppressive soil systems studied in our laboratory may now be developed in terms of the basic mechanisms involved. For example, the Rhizoctonia suppressiveness induced during radish monoculture developed more rapidly in acid than alkaline soils (18). Fusarium suppressiveness is greater under alkaline conditions (Fig. 5). The characteristics of the biological entities responsible for suppressiveness in each of the cases probably supplies the explanation for these contrasting efficiencies. *Trichoderma* spp. were responsible for suppressiveness in the radish monoculture system and they have greater activity in acid environments. In contrast, the bacteria implicated in biological control in the *Fusarium*-suppressive Metz fine sandy loam should be favored in alkaline environments.

It is significant that candidate antagonists in this study were selected on the basis of their activities in intimate association with the flax wilt pathogen in its soil habitat. Subsequently, the microorganisms inducing suppressiveness, when added to previously conducive soil, had no observable antagonistic properties against the pathogen in vitro. This reinforces the principle that antagonism demonstrated in culture media does not necessarily indicate that antagonism will occur in soil and vice versa (5).

The isolation in pure culture of microbiological entities responsible for the suppressiveness of soils to plant pathogens represents a significant step in plant disease control technology. The difficulties encountered in eradicating Fusarium wilt pathogens by conventional methods, and the failure to develop host resistance to new races of the pathogens (11,14) make biological control with specific agents an attractive and useful addition to the arsenal of plant disease control measures. Through additional research, a commercially feasible means for introduction of these biocontrol agents effective in control of Fusarium wilt diseases to conducive field soils may be accomplished.

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