Inhibition of *Fusarium oxysporum* f. sp. *dianthi* by Iron Competition with an *Alcaligenes* sp.

G. Y. Yuen and M. N. Schroth

Department of Plant Pathology, University of California, Berkeley 94720.
Present address of first author: Department of Plant Pathology, University of Hawaii, Honolulu 96822.
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ABSTRACT

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Alcaligenes sp. strain MFA1, isolated from the roots of carnations grown in a Fusarium wilt-suppressive soil and cultured on low-iron media, produced a siderophore that inhibited microconidium germination and germ tube growth of *Fusarium oxysporum* f. sp. *dianthi*. Germination of chlamydospores of *F. o. dianthi* in soil amended with glucose and asparagine at 0.2 mg/g each to simulate plant exudate was less than 12% in the presence of MFA1 population densities of 10^3 colony-forming units (cfu) per gram of soil in comparison to 28% without MFA1. Inordinately high population densities of MFA1 at 10^9 cfu per gram of soil did not significantly further suppress chlamydospore germination and, in the presence of higher nutrient levels, no suppression was observed. The inhibitory effect of MFA1 on chlamydospore germination in soil was related to siderophore production by the bacterium. The addition of 10^{-3} M $FeCl_3$ to soil reversed the inhibitory effects of the bacterium, and four siderophore-negative mutant strains of MFA1 did not suppress chlamydospore germination, although they survived in soil as well as the parental strain. When carnation seeds were inoculated with a suspension of MFA1 at 10^8 cfu/ml, the frequency of root colonization by *F. o. dianthi* on 10-day-old roots was 24% lower than in untreated controls. The number of colonies of *F. o. dianthi* isolated from roots of rooted cuttings inoculated with MFA1 1 mo after treatment was 55% lower than that of uninoculated controls. The subsequent disease severity of MFA1-treated plants 3 and 4 mo after planting were 45 and 35% less, respectively, than that of control plants, but no significant difference was detected at 5 mo.

Additional key words: antagonism, biological control, *Dianthus caryophyllus*.

A transferable biological factor in Fusarium wilt-suppressive soils reduced the incidence of vascular wilt diseases caused by pathogenic strains of *Fusarium oxysporum* Schlecht (1,10,14,20). This prompted investigations to determine which specific components of the soil microflora might be responsible for disease suppression. Although it is unlikely that any one genus or species of microorganism would be totally responsible for causing a soil to be disease suppressive, various fungi and bacteria have been reported as playing a role in the phenomenon. Saprophytic strains of *Fusarium oxysporum* and *F. solani* were implicated as suppressive agents in Fusarium wilt-suppressive soils of Chateaurenard, France (1). An *Arthrobacter* sp. was reported by Smith (19) to be associated with germinating chlamydospores in Fusarium wilt-suppressive soils, but no evidence was presented for its role in disease suppression. Others (8,11) have noted that *Arthrobacter* spp. may lyse pathogenic *Fusarium* spp. in vitro and suggested that this was of importance in disease control. However, Sneh et al (22), working with an *Arthrobacter* sp. and some chitinolytic bacteria isolated from suppressive soil, could not relate in vitro lytic ability to disease suppression in soil.

Considerable attention has been given to the role of pseudomonads in disease-suppressive soils (2,23). Kloepper et al (6) reported that a wilt-conducive soil was rendered temporarily suppressive to Fusarium wilt of flax when a strain of *Pseudomonas* sp. or its purified siderophore was added. Similarly, Scher and Baker (16) found that strains of *Pseudomonas putida* suppressed the incidence of Fusarium wilt of flax, cucumber, and radish when incorporated into wilt-conducive soil. Inhibition of the wilt pathogen in these two studies was attributed to iron deprivation caused by pseudomonad siderophores, compounds produced in low-iron environments that function in iron transport. Sneh et al (22) subsequently reported that *Pseudomonas* spp. isolated from a wilt-suppressive soil suppressed germination of chlamydospores of *Fusarium oxysporum* f. sp. *cucumerinum* in conducive soil and related the effect to siderophore production.

Alcaligenes Castellani and Chalmers sp. strain MFA1, isolated from the rhizosphere of carnations (*Dianthus caryophyllus* L.) grown in a wilt-suppressive soil, reduced the severity of Fusarium wilt of carnation for up to 4 mo when applied to the roots of cuttings prior to planting (26).

The purposes of the study reported here were to determine the mechanism by which strain MFA1 inhibited disease, to learn whether siderophore production was a factor, and to examine the effect of preplant treatment of carnations with the bacterium on the colonization of root surfaces and tissues by the wilt pathogen. Preliminary results have been reported (24,25).

MATERIALS AND METHODS

Bacteria. Strain MFA1 and other bacteria used in this study are described in Table 1. Inocula for treatment of seed, roots, and soil were prepared from 24-hr cultures grown on King's medium B agar (KBA) (4) at 28 C. The cells were suspended in 0.1 M $MgSO_4$ or water, and the densities were standardized by measurement of optical density and checked by viable plate counts on KBA. Spontaneous mutants of the bacterial strains resistant to rifampicin (Sigma Chemical Co., St. Louis, MO) were used to monitor the populations of these bacteria on plant parts or in soil. Bacteria were detected by dilution platings of root washes and soil suspensions onto KBA supplemented with rifampicin at 100 μg/ml, benomyl (Dupont Co., Wilmington, DE) at 150 μg/ml, and cycloheximide (Sigma Chemical Co.) at 150 μg/ml.

Pathogen inoculum and soil. An orange-colored mutant of *Fusarium oxysporum* f. sp. *dianthi* (Prill et Del.) Snyd. and Hans. strain 987, derived by ultraviolet (UV) mutagenesis, was provided by J. E. Puhalla (13). Its distinctive coloration on agar media, due to constitutive production of carotenoid pigments, facilitated detection of the pathogen in soil and plant tissues. The virulence of the mutant was the same as that of the parent strain.

Delhi sand (subgroup Typic Xeropsamments, pH 4.9) was the soil used throughout the study. It was pasteurized to eliminate carnation pathogens, such as *Pythium* and *Rhizoctonia*, air-dried, and stored for 4 mo at room temperature in open containers. The

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soil was then infested with *F. o. dianthi* by adding a suspension of conidia collected from 7-day cultures grown on potato-dextrose agar (PDA) (Difco Laboratories, Detroit, MI). The infested soil was moistened to field capacity and slowly air-dried, which induced the conidia to form chlamydospores. Populations of *F. o. dianthi* in infested soils were measured by dilution plating of soil suspensions onto Komada's medium (7).

In vitro pathogen inhibition. Inhibition of *F. o. dianthi* by bacteria was tested on various media: KBA, PDA, trypticase soy agar (TSA) (Difco Laboratories), nutrient agar (NA) (Difco Laboratories), yeast dextrose peptone calcium carbonate (YDCP) agar (9), and a medium containing L-asparagine at 2 g/L plus either sucrose, galactose, or glycerol at 10 g/L with 20 g/L agar (ASA). Agar plates with 48-hr colonies of bacteria were sprayed with a suspension of microconidia of *F. o. dianthi* collected from 1-wk PDA cultures that were incubated in the dark. The sprayed plates were incubated at 25 C. Inhibition of *F. o. dianthi* was expressed within 24 hr as a clear zone of ungerminated conidia around bacterial colonies.

To determine if the inhibition of *F. o. dianthi* could be reversed by the addition of iron, the two iron-deficient media, KBA and ASA, were supplemented with various concentrations of $FeCl_3$. Five milliliters of iron-amended KBA also were applied as an overlay onto the surface of KBA and ASA plates before they were sprayed with conidia of *F. o. dianthi*.

Derivation of siderophore-negative mutants. Siderophore-negative (Sid^-) mutant strains of MFA1 were derived by mutagenic treatment with UV or ethyl methane sulfonate (EMS). In UV mutagenesis, MFA1 cells in log-phase growth were suspended in 0.1 M $MgSO_4$ at 10^7 colony-forming units (cfu) per milliliter. Ten milliliters of suspension were irradiated in a 10-cm-diameter glass petri dish with UV at 80 joules/m^2 to obtain 99% kill of cells. The treated suspension was serially diluted and plated on KBA at cell densities that resulted in 10–20 colonies per plate. Following 48 hr of incubation at 28 C, the plates were sprayed with conidia of *F. o. dianthi* and incubated for an additional 12–18 hr. Colonies of MFA1 that did not inhibit *F. o. dianthi* were purified on KBA amended with rifampicin at 100 μg/ml of solution, retested for lack of inhibition, and then tested for siderophore production by methods described below. The morphology and generation times of the mutant strains in King's medium B broth (KBB) were also determined, and only strains similar to the parental-type were retained.

In EMS mutagenesis, log-phase cells of MFA1 in KBB (10^8 cfu/ml) were treated with EMS to obtain 99% kill. After repeated washings with fresh KBB and centrifugation to remove EMS, the treated cells were plated and Sid^- mutants were selected as described above.

Tests for siderophore production. Siderophore production by MFA1 and mutants noninhibitory to *F. o. dianthi* were tested by two procedures. In the first test, the bacteria first were examined for differential inhibition to two isogenic strains, AN193 and AN194, derived from *E. coli* K12 (5). Strains AN193 and AN194 differ only in that AN193 does not produce its native siderophore, enterobactin, while AN194 is a strong producer of enterobactin. KBA plates, which had been spot-inoculated 48 hr previously with MFA1 and Sid^- mutants, were sprayed with the strains of *E. coli*. Inhibition of AN193 but not of AN194 was considered to be an indication of siderophore production. In the second test, MFA1 and Sid^- strains were tested for growth on agar media when stressed for iron by the iron-chelating compound, ethylenediaminedi-*o*-hydroxyphenylacetic acid (EDDHA) (Sigma Chemical Co.). This would require the production of a siderophore having an affinity to iron equal to or greater than that of EDDHA. A 50 mg/ml solution of EDDHA was prepared by the method described by Ong et al (12), filter sterilized, and added in various amounts to molten KBA. The EDDHA-amended KBA, after being poured into plates, was stored at 4 C for 24 hr to allow slow chelation of the iron from KBA. A sterile solution of $FeCl_3$ was also added to half of the EDDHA-amended KBA (final concentration of 10^{-4} M $FeCl_3$) to determine whether growth inhibition was due to iron deprivation or to toxicity of the chelator. Cell suspensions of MFA1 and Sid^- strains were adjusted to uniform optical density and 1 μl of each suspension was streaked onto the agar plates (four strains per plate) with a calibrated inoculating loop. Growth of the strains at 28 C was observed at 24-hr intervals for 4 days.

Chlamydospore germination. To determine the effects of MFA1 and other bacteria on the germination of chlamydospores of *F. o. dianthi* in soil, air-dried Delhi sand infested with 10^6 chlamydospores per g was divided into 1-g samples and contained in wells of plastic "Disposa" trays (VWR Scientific, Inc., San Francisco, CA). A 230-μl bacterial suspension containing glucose and L-asparagine was added to each sample. The nutrients were applied to soil at two concentrations: 0.2 mg each of glucose and asparagine (GA) per gram of soil and 2.3 mg of GA per gram of soil Glucose and asparagine occur in root exudates and stimulate chlamydospore germination (17). The trays containing the soil samples were enclosed in plastic bags to maintain high humidity and incubated at 25 C for 24 hr. Small amounts of soil from each sample were spread onto glass slides, stained with acid fuchsin, and examined according to the method of Schroth and Snyder (17).

The effects of bacteria on chlamydospore germination of *F. o. dianthi* in the vicinity of carnation seeds and roots of seedlings were also determined. Seeds of cultivar White Knight were immersed in bacterial suspensions or water and then planted into 1-g samples of Fusarium- infested soil. Some seeds were germinated in aerated water and after 7 days, when emerging roots were approximately 2 cm long, they were dipped into the bacteria suspensions and transplanted into infested soil samples. The soil samples were each moistened to with 0.23 ml of water and incubated for 24 hr at 25 C while enclosed in plastic bags. The seeds and seedlings were removed and the soil adhering to the ungerminated seeds and roots was washed onto glass slides with 0.5 ml of water, spread thinly, and air-dried. The washed roots were excised and placed on glass slides. Soil smears and roots were then stained and examined. Over

TABLE 1. Characteristics and origin of bacterial strains

Species and strain	Characteristics[y]	Origin
Alcaligenes Castellani and Chalmers sp.		
MFA1	*Fod* antagonist, isolated from roots of carnation grown in wilt-suppressive soil	(26)[z]
UV12	Sid^- mutant from UV mutagenesis of MFA1.	This study
UV14	As above	This study
M12	Sid^- mutant from EMS mutagenesis of MFA1	This study
M22	As above	This study
E. coli (Migula) Castellani and Chalmers K12		
AN193	Mutant derived from AN194 deficient in enterobactin production	J. B. Neilands
AN194	Wild type for enterobactin production	J. B. Neilands
Pseudomonas Migula sp.		
51	Isolated from roots of carnations grown in wilt suppressive soil, nonantagonistic to *Fod* on King's medium B	This study
56	As above	This study

[y] *Fod* = *F. oxysporum* f. sp. *dianthi*, Sid^- = siderophore-negative, UV = ultraviolet, EMS = ethyl methane sulfonate.
[z] Number refers to literature citation.

100 chlamydospores in each soil sample were observed for germination, germ tube length, and lysis. There were four replicate samples for each treatment and each experiment was repeated at least once.

Quantification of root colonization. To determine the effect of MFA1 on colonization of carnation seedling roots by *F. o. dianthi*, four seeds of cultivar White Knight were immersed in a 10^8 cfu/ml suspension of MFA1 or in water, and then planted in glass containers filled with 45 ml of Delhi sand infested with 10^4 of *F. o. dianthi* chlamydospores per gram. There were eight replicates per treatment. After water was added to the soils to 23% water by dry weight, the containers were incubated in a growth chamber for 14 days at 25 C and 12 hr per day of light at 500 μE/m^2/sec. At the end of the incubation period, the roots within each container were removed from soil, pooled, and washed three times by shaking for 30 min in 1% sodium hexametaphosphate. Seventy to 80 cm of washed root segments were aligned on plates of Komada's medium and incubated at 25 C for 3 days. Orange colonies of the pathogen growing on the agar from the roots were presumed to have arisen primarily from individual colonization sites in the root cortex or in the vascular tissues.

Quantification of pathogen density associated with root tissues. Cultivar Improved White Sim carnation cuttings were dipped into a 10^8 cfu/ml suspension of MFA1 or into water and planted in soil infested with *F. o. dianthi*. The plants were maintained in a greenhouse and assessed visually for Fusarium wilt severity on a monthly basis according to conditions and methods previously described (26).

TABLE 2. Effect of *Alcaligenes* strain MFA1 on microconidial germination and germ tube growth by *Fusarium oxysporum* f. sp. *dianthi* (*Fod*) on King's medium B agar (KBA)[z]

Zone of activity	Germination (%) 12 hr	Germination (%) 24 hr	Germ tube length (μm) 12 hr
Inside of MFA1 metabolite diffusion zone	50.0 ± 1.1	69.0 ± 8.5	4.6 ± 3.0
Outside of MFA1 metabolite diffusion zone	95.5 ± 1.1	100.0 ± 0.0	24.5 ± 8.8

[z]MFA1 cultured on KBA for 48 hr and *Fod* microconidia suspension sprayed onto agar surface. Values are means from measurements from three replicate plates incubated at 25 C. Variation is expressed as standard error.

One and 2 mo after planting, root segments were excised from each plant with a 1.5-cm cork borer, washed under running water, and shaken with three volumes of sterile 1% sodium hexametaphosphate. One hundred mg of washed roots were triturated with 50 ml of sterile water in a VirTis 45 Homogenizer (VirTis Research Equip., Inc., Gardiner, NY) for 3 min at medium speed. The mixture was diluted 10-fold with water and 1 ml of each dilution was plated on Komada's medium.

The number of orange mutant colonies of *F. o. dianthi* growing on the agar, a relative measure of the number of pathogen colony-forming units present on the root surface and in root tissues, was counted after 3 days of incubation at 25 C.

RESULTS

Effect of iron on in vitro inhibition of *F. o. dianthi*. Inhibition of *F. o. dianthi* by strain MFA1 occurred only on KBA and ASA which contained relatively low amounts of iron. The pathogen was not inhibited when MFA1 was cultured on the iron-rich media TSA, NA, YDCP, or PDA. Germination of *F. o. dianthi* microconidia in the vicinity of MFA1 colonies on KBA was reduced by 48 and 31% at 12 and 24 hr, respectively, after the conidia were applied to the medium KBA (Table 2). A failure of germ tubes to elongate was also observed in the inhibition zone at 12 hr. However, extensive mycelial growth by 24 hr prevented accurate measurement of single hyphae at this time, and by 48 hr, the zones of inhibition around MFA1 colonies were no longer visible. MFA1 did not inhibit *F. o. dianthi* on KBA and ASA supplemented with 10^{-4} M $FeCl_3$. The fungus was inhibited when it was sprayed onto iron-minus KBA overlays covering MFA1 cultures, but was not affected when sprayed onto iron-supplemented overlays.

In vitro siderophore production by MFA1. MFA1 produced a siderophore when cultured on KBA. It inhibited the growth of strain AN193, the siderophore-minus mutant of AN194, but did not affect strain AN194, the strong siderophore producer. When the medium was supplemented with 10^{-4} M $FeCl_3$, no inhibition of AN193 occurred. MFA1 also grew on KBA amended with EDDHA at a concentration of 200 μg/ml. In contrast, five Sid$^-$ mutant strains of MFA1, derived by treatment with UV and EMS did not inhibit AN193 and did not grow on KBA amended with EDDHA. The Sid$^-$ strains grew on EDDA-amended KBA when the media contained 10^{-4} M $FeCl_3$. These strains did not inhibit *F. o. dianthi* on KBA. No strains which produced a siderophore and

TABLE 3. Germination of chlamydospores of *Fusarium oxysporum* f. sp. *dianthi* in soils as affected by population densities of *Alcaligenes* sp. strain MFA1, UV-killed cells of MFA1, and *Pseudomonas* sp. strains 51 and 56 when incorporated into soil[x]

Treatment: Strain	Initial population densities added to soil (cfu/g)	Chlamydospore germination (%) 0.2 GA	Chlamydospore germination (%) 2.3 GA	Final population densities of bacteria in soil (cfu/g) 0.2 GA	Final population densities of bacteria in soil (cfu/g) 2.3 GA
Untreated control	0	28.1 ± 5.7	46.1 ± 7.6	0	0
MFA1	10^3	9.9 ± 4.4	17.1 ± 4.6	7.2×10^3	1.2×10^4
	10^5	8.2 ± 3.9	18.3 ± 6.8	1.7×10^5	7.4×10^4
	10^7	11.1 ± 6.2	27.6 ± 8.2	6.0×10^6	9.0×10^5
	10^9	6.5 ± 3.6	34.7 ± 12.8	1.4×10^7	1.5×10^7
UV-killed MFA1	10^3	29.2 ± 4.4	43.7 ± 5.4	ND[y]	ND
	10^9	32.3 ± 2.4	38.8 ± 13.8	ND	ND
51	10^3	38.1 ± 8.8	...[z]	8.7×10^3	...
	10^9	40.1 ± 12.1	...	6.6×10^7	...
56	10^3	33.2 ± 4.8	...	3.1×10^3	...
	10^9	24.8 ± 13.0	...	2.8×10^7	...

[x]Bacteria were applied as suspensions to soil. Densities of suspensions were determined by measuring optical density. Final population levels were determined by viable plate counts. cfu = colony forming units. Values are means of assays of four replicate soil samples incubated for 24 hr at 25 C. 0.2 GA = 0.2 mg of glucose and asparagine per gram of soil; 2.3 GA = 2.3 mg each of glucose and asparagine per gram of soil. These nutrients were added to soil to stimulate chlamydospore germination. Variation is expressed as standard error.

[y]Not detected.

[z]No data.

did not inhibit *F. o. dianthi* were derived from MFA1 by UV or EMS treatment.

Inhibition of *F. o. dianthi* chlamydospore germination by MFA1. Strain MFA1 significantly inhibited chlamydospore germination in soil in the presence of glucose and asparagine (Table 3). Germination in response to 0.2 mg GA per gram of soil was 28% in the absence of MFA1 and in the range of 6 to 11% when the soil contained 10^3–10^9 cfu of MFA1 per gram. There were no significant differences in the inhibition of germination among different population densities of MFA1 in the presence of 0.2 GA per gram of soil. When 2.3 mg GA per gram of soil was added, 46% of the chlamydospores germinated with no MFA1; with the addition of MFA1 at increasing population densities, there was a trend towards increased germination. This was reflected in significant differences in germination between 10^3 cfu of MFA1 (17%) and 10^9 cfu of MFA1 (35%) per gram of soil. Pseudomonas strains 51 and 56 isolated from carnation roots and UV-killed cells of MFA1 used as controls had no effect on chlamydospore germination when incorporated into soil at either 10^3 or 10^9 cfu/g.

Population densities of MFA1 detected in soil by viable plate counts (Table 3) were relatively unchanged after 24 hr from initial levels of 10^3 and 10^5 cfu of MFA1 per gram of soil, as determined by optical density measurement. When MFA1 was added to soil at 10^7 cfu/g or greater, population densities detected after 24 hr were 10 to 100-fold lower. Densities of *Pseudomonas* strains 51 and 56 detected after 24 hr were similar to those of MFA1.

Germ tube length and frequency of lysis in soil were measured in three experiments. Germ tube length (57 to 67 μm) did not vary significantly among treatments, and lysis of germ tubes regularly occurred at the same frequency (25–30%) with and without MFA1.

Strain MFA1 substantially inhibited the germination of chlamydospores of *F. o. dianthi* in spermosphere soil and on the rhizoplane when applied as a suspension at 10^3 cfu/ml to seed and seedling roots (Table 4). Chlamydospore germination in the untreated spermosphere and on the untreated rhizoplane were 28 and 43%, respectively; in the presence of MFA1, the germination was reduced to 15 and 14%, respectively. Germination in rhizosphere soils of treated and untreated roots occurred at frequencies too low to enable an assessment of the influence of MFA1.

Relationship of iron competition to inhibition of chlamydospore germination. The inhibitory effect of MFA1 on chlamydospore germination in soil was reversed by iron (Table 5). In the presence of 0.2 mg of GA per gram of soil, the addition of 10^{-3} M $FeCl_3$ greatly reduced the effect of MFA1, resulting in increased chlamydospore germination from 8%, with no $FeCl_3$, to 24%. Addition of 10^{-5} M $FeCl_3$ negated the effect of MFA1 in the presence of 2.3 mg of GA per gram of soil but did not with 0.2 mg of GA per g of soil.

Four Sid^- mutant strains of MFA1 did not affect chlamydospore germination when added at densities of 10^3 and 10^9 cfu/g to soil containing 0.2 mg of GA per gram (Table 6). At the greater population size, a slight inhibition in germination resulted from treatment with mutant strain M22. Population levels of the Sid^- strains detected in the soils at the end of the experiment were similar to those of the wild-type strain.

Inhibition of *F. o. dianthi* root colonization by MFA1. Colonization of carnation seedling roots by *F. o. dianthi* was significantly inhibited ($P = 0.01$) by seed treatment with MFA1. The number of colonies growing from the roots of 14 day-old seedlings placed on Komada's medium was 139 per 100 cm of roots without MFA1 and 105 per 100 cm with MFA1, a reduction of 24%. Two repetitions of this experiment yielded similar results with MFA1 inhibiting pathogen colonization by 25 and 37%.

Colonization and systemic spread of the pathogen in the root system of mature carnation plants was inhibited by a preplant dip of cuttings into a cell suspension of MFA1 (Table 7). The density of

TABLE 4. Inhibition of chlamydospore germination of *Fusarium oxysporum* f. sp. *dianthi* (Fod) in the carnation spermosphere, rhizosphere, and rhizoplane in the presence of *Alcaligenes* strain MFA1[y]

Site	Germination (%)	
	MFA1	Untreated control
Spermosphere soil	15.1 ± 7.0[z]	28.2 ± 0.9
Rhizoplane	14.0 ± 9.3	43.5 ± 1.4
Rhizosphere soil	5.9 ± 6.4	9.6 ± 4.1

[y] MFA1 was applied as a suspension of 10^3 colony-forming units per milliliter to seeds and the roots of 7-day-old germinated seeds prior to transplanting into *Fod*-infested soil.

[z] Values are means of assays of four replicate soil samples incubated at 25 C for 24 hr. Variation is expressed as standard error.

TABLE 5. Chlamydospore germination by *Fusarium oxysporum* f. sp. *dianthi* in soil as affected by *Alcaligenes* strain MFA1 and $FeCl_3$[z]

Treatment	Germination (%)	
	0.2 GA	2.3 GA
No bacteria	30.2 ± 4.0	39.9 ± 12.6
MFA1	8.3 ± 3.4	15.5 ± 8.0
10^{-5} M $FeCl_3$ + no bacteria	30.4 ± 3.4	49.5 ± 3.5
10^{-5} M $FeCl_3$ + MFA1	8.4 ± 0.6	33.1 ± 5.6
10^{-3} M $FeCl_3$ + no bacteria	36.5 ± 5.2	53.0 ± 3.1
10^{-3} M $FeCl_3$ + MFA1	24.0 ± 3.7	40.7 ± 1.7

[z] MFA1 was applied to soil as a suspension at 10^3 cfu/g. Values are means of assays of four replicate soil samples incubated for 24 hr at 25 C. Variation is expressed as standard error. 0.2 GA = 0.2 mg each of glucose and asparagine. 2.3 GA = 2.3 mg each of glucose and asparagine per gram of soil. These nutrients were applied to soil to stimulate chlamydospore germination.

TABLE 6. Chlamydospore germination by *Fusarium oxysporum* f. sp. *dianthi* in soil as affected by *Alcaligenes* strain MFA1 and siderophore-negative (Sid^-) mutant strains of MFA1 when applied to soil at 10^3 and 10^9 cfu/g[z]

Strain	Germination (%)	
	10^3 cfu/g soil	10^9 cfu/g soil
MFA1-wild type	11.5 ± 4.5	6.3 ± 2.5
Sid^- mutants		
M12	30.6 ± 11.5	29.0 ± 4.4
M22	31.4 ± 6.2	23.2 ± 3.4
UV12	29.4 ± 5.1	29.3 ± 4.2
UV14	32.5 ± 1.7	30.7 ± 2.2
Untreated control	30.9 ± 1.7	

[z] Glucose and asparagine each were added to soil at a concentration of 0.2 mg/g to stimulate chlamydospore germination. Values are means of four replicate assays of soil samples incubated 24 hr at 25 C. Variation is expressed as standard error. Strains M12 and M22 were derived by mutagenesis with ethane methyl sulfonate, and strains UV12 and UV14 by ultraviolet mutagenesis.

TABLE 7. Inhibition of *Fusarium oxysporum* f. sp. *dianthi* (*Fod*) at carnation root surfaces and in root tissues, and delay in wilt symptoms by treatment of carnations with *Alcaligenes* strain MFA1[w]

Months after planting	*Fod* colonies per 100 mg root[x]		Wilt severity rating[y]	
	MFA1	Control	MFA1	Control
1	47**[z]	104	0	0
2	254	270	0.4	1.0
3	···	···	1.8*	2.5
4	···	···	2.5*	3.9
5	···	···	3.8	4.0

[w] Roots of cuttings were dipped into a suspension of MFA1 (10^8 cfu/mg); plants were grown in potting mix infested with *Fod* (10^3 propagules per gram).

[x] Roots were washed, triturated, and plated on Komada's medium. Values are means of eight replicate assays.

[y] Mean of eight plants rated on scale from 0 (symptomless) to 4 (dead).

[z] Asterisks (*, **) indicate difference from control is significant at $P = 0.05$ and $P = 0.01$, respectively.

F. o. dianthi on and in the roots 1 mo after treatment, represented by the number of pathogen colonies growing from triturated root tissue on agar, was less than 50% of that without the bacterial treatment. Assay of roots 2 mo after treatment revealed greater amounts of pathogen mycelia in both treated and untreated plants, but no significant difference in colonization was obtained. Treatment with MFA1 resulted in a delay in the subsequent development of Fusarium wilt symptoms. Ratings of the plants 3 and 4 mo after planting revealed reductions in Fusarium wilt severity of 28 and 36%, respectively, in response to MFA1-treatment, but no significant difference was detected at 5 mo. Similar results were obtained upon repeating this experiment.

DISCUSSION

The germination of chlamydospores of *F. o. dianthi* in soil and in the carnation spermosphere and rhizoplane was significantly inhibited by *Alcaligenes* sp. strain MFA1 when it was present in soil, on seeds, or on seedling roots. Chlamydospore germination in the rhizosphere was too low to detect significant differences resulting from MFA1. The inhibitory effect of MFA1 was related to production of a siderophore that limits the availability of iron, which (presumably) is necessary for chlamydospore germination. This apparently resulted in reduced colonization and infection of carnation roots by *F. o. dianthi*.

Germination of chlamydospores of *F. oxysporum* is less in wilt-suppressive soils than in conducive soils (1,3,15,18,19,21). This effect presumably results from the activity of specific soil microflora. Sneh et al (22) reported that *Pseudomonas* spp. isolated from a wilt-suppressive soil reduced the chlamydospore germination of *F. oxysporum* f. sp. *cucumerinum* to a greater extent than did chitinolytic bacteria when added to conducive soil. Iron competition, by the elaboration of siderophores, was suggested as a mechanism by which pseudomonads inhibited chlamydospore germination (22) and conferred disease suppressiveness (6,16). This was demonstrated by the reversal of the inhibitory effects of the bacteria when iron was incorporated into the soil and by the simulation of the inhibitory effects of the bacteria through the addition of iron chelators to soil. In each of these studies, the bacteria produced siderophores in vitro, and in one (6) a purified siderophore added to conducive soil reduced the incidence of Fusarium wilt of flax. The actual production of microbial chelators in soil, however, was not demonstrated.

Siderophore production by MFA1 appears to be a principal factor in the inhibition of *F. oxysporum* in vivo. Several mutant strains of MFA1, selected for absence of siderophore production in vitro, did not inhibit chlamydospore germination when incorporated into soil even though they colonized soil to the same extent as the wild type.

Competition for carbon and nitrogen did not appear to be a major factor in the inhibitory effect of MFA1 on *F. o. dianthi*. High populations of two strains of *Pseudomonas* applied to soil as controls did not reduce chlamydospore germination. Moreover, the incorporation of MFA1 into soil at a density as low as 10^3 cfu/g provided an initial antagonistic population sufficient to inhibit chlamydospore germination. This population seems realistic since MFA1 colonizes carnation roots at densities ranging from approximately 100 to 10^6 cfu/cm (26). A surprising result was that, in the presence of high levels of GA, an artificially high population density of MFA1 in soil (10^7 to 10^9 cfu/g) was less inhibitory than a lower densisty (10^3 cfu/g). Sneh et al (22) observed the opposite with a *Pseudomonas* sp. in that chlamydospore germination decreased with increasing bacterial densities. The diminished effect of high MFA1 densities observed in our study may be related to the death of MFA1 cells, as the incorporation of high densities of bacteria into soil was always accompanied by a decrease in population sizes within 24 hr. However, there is no complete explanation for the increase in germination since it did not occur in the presence of the lower amounts of GA.

Preplant application of the *Alcaligenes* sp. to the roots of carnations in this study inhibited colonization on root surfaces by *F. o. dianthi* and delayed the establishment of the pathogen on and in root tissues, which effectively increased the incubation period preceding symptom development by 1–2 mo. In a previous study (26), treatment of carnation cuttings with MFA1 and other antagonistic bacteria resulted in a similar delay in disease development. These effects may have been caused by the inhibition of *F. o. dianthi* during the prepenetration phase. This would be influenced by the population sizes of bacteria at susceptible sites along the root. The incidence of infection and systemic invasion by the pathogen increases once antagonist populations decline or when susceptible roots advance beyond the zone of high antagonist concentration. Populations of *Alcaligenes* sp. strain MFA1 and other antagonistic bacteria on the root systems of carnations grown in certain soils are detectable only up to 5 mo (26). For these reasons, the commercial use of biological controls for diseases such as Fusarium wilt of carnation where the host plant has a long susceptible period of growth will depend on the development of methods to sustain high population of antagonistic microflora for a year or more.

LITERATURE CITED

1. Alabouvette, C., Rouxel, F. and Louvet, J. 1979. Characteristics of Fusarium wilt-suppressive soils and prospects for their utilization in biological control. Pages 165-182 in: Soil-Borne Plant Pathogens. B. Schippers and W. Gams. eds. Academic Press, London. 686 pp.
2. Cook, R. J. and Baker, K. F. 1983. The Nature and Practice of Biological Control of Plant Pathogens. The American Phytopathological Society, St. Paul, MN. 539 pp.
3. Hwang, S. F., Cook, R. J., and Haglund, W. A. 1982. Mechanisms of suppression of chlamydospore germination for *Fusarium oxysporum* f. sp. *pisi* in soils. (Abstr.) Phytopathology 72:948.
4. King, E. O., Ward, M. K., and Raney, D. E. 1954. Two simple media for the demonstration of pyocyanin and fluorescin. J. Lab. Clin. Med. 44:301-307.
5. Kloepper, J. W., Leong, J., Teintze, M., and Schroth, M. N. 1980. Enhanced plant growth by siderophores produced by plant growth-promoting rhizobacteria. Nature 286:885-886.
6. Kloepper, J. W., Leong, J., Teintze, M., and Schroth, M. N. 1980. Pseudomonas siderophores: A mechanism explaining disease-suppressive soils. Curr. Microbiol. 4:317-320.
7. Komada, H. 1975. Development of a selective medium for quantitative isolation of *Fusarium oxysporum* from natural soil. Rev. Plant Protec. Res. 8:114-125.
8. Koths, J. S. and Gunner, H. B. 1967. Establishment of a rhizosphere microflora on carnations as a means of plant protection in steamed greenhouse soils. Proc. Am. Soc. Hortic. Sci. 91:617-626.
9. Leben, C., Schroth, M. N., Hildebrand, D. C. 1970. Colonization and movement of *Pseudomonas syringae* on healthy bean seedlings. Phytopathology 60:677-680.
10. McCain, A. H., Pyeatt, L. E., Byrne, T. G., and Farnham, D. S. 1980. Suppressive soil reduces carnation disease. Calif. Agric. 35(5):9.
11. Mitchell, R., and Hurwitz, E. 1965. Suppression of *Pythium debaryanum* by lytic rhizosphere bacteria. Phytopathology 55:156-158.
12. Ong, S. A., Peterson, T., and Neilands, J. B. 1979. Agrobactin, a siderophore from *Agrobacterium tumefaciens*. J. Biol. Chem. 254:1860-1865.
13. Puhalla, J. E. 1984. A visual indicator of heterokaryosis in *Fusarium oxysporum* from celery. Can. J. Bot. 62:540-545.
14. Scher, F. M., and Baker, R. 1980. Mechanism of biological control in a Fusarium-suppressive soil. Phytopathology 70:412-417.
15. Scher, F. M., and Baker, R. 1982. Use of fluorescence microscopy for comparison of *Fusarium oxysporum* chlamydospore germination in conducive and suppressive soil. (Abstr.) Phytopathology 72:931.
16. 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.
17. Schroth, M. N., and Snyder, W. C. 1961. Effect of host exudates on chlamydospore germination of the bean root rot fungus, *Fusarium solani* f. *phaseoli*. Phytopathology 51:389-393.
18. Smith, S. N. 1975. Association of *Arthrobacter* with banana wilt fusaria in suppressive soils. (Abstr.) Proc. Am. Phytopathol. Soc. 2:79.
19. Smith, S. N. 1977. Comparison of germination of pathogenic *Fusarium oxysporum* chlamydospores in host rhizosphere soils conducive and suppressive to wilts. Phytopathology 67:502-510.
20. Smith, S. N., and Snyder, W. C. 1971. Relationship of inoculum density and soil types to severity of Fusarium wilt of sweet potato. Phytopathology 61:1049-1051.

21. Smith, S. N., and Snyder, W. C. 1972. Germination of *Fusarium oxysporum* chlamydospores in soils favorable and unfavorable to wilt establishment. Phytopathology 62:273-277.
22. 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.
23. Weller, D. M., and Cook, R. J. 1983. Suppression of take-all of wheat by seed treatment with fluorescent pseudomonads. Phytopathology 73:463-469.
24. Yuen, G. Y., and Schroth, M. N. 1983. Reductions in *Fusarium oxysporum* infection and systemic colonization of carnation from treatment with rhizobacterium MFA1. (Abstr.) Phytopathology 73:963.
25. Yuen, G. Y., and Schroth, M. N. 1984. Iron competition by *Alcaligenes* sp. reduces Fusarium chlamydospore germination. (Abstr.) Phytopathology 74:806.
26. Yuen, G. Y., Schroth, M. N., and McCain, A. H. 1985. Reduction of Fusarium wilt of carnations with suppressive soils and antagonistic bacteria. Plant Dis. 69:1071-1075.