Naturally Occurring Fluorescent Pseudomonads Involved in Suppression of Black Root Rot of Tobacco

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ABSTRACT

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Fluorescent pseudomonads were isolated from tobacco roots grown in soils naturally suppressive to black root rot caused by *Thielaviopsis basicola*. In the suppressive soil, fluorescent pseudomonads could be detected to a depth of 1 m; below 1 m, however, no fluorescent pseudomonads were found and suppressiveness was lost. Heat treatment of the suppressive soil at 60 C for 30 min nullified suppressiveness and fluorescent bacteria could no longer be isolated. Suppressiveness was readily transferred by addition of 5% or more suppressive soil to a conducive soil. Fluorescent pseudomonads could then be isolated following but not prior to this addition. Several strains of these fluorescent pseudomonads were isolated, cloned, and tested for suppressiveness by introducing them into a conducive soil. A highly suppressive strain, CHAo, was chosen for further tests and was identified as *Pseudomonas fluorescens*. Black root rot of tobacco was suppressed in 36 of 39 conducive soil samples by adding strain CHAo at 10^7 cfu/cm^3 of soil. Strain CHAo could not be reisolated from the soil samples that remained conducive.

Fluorescent pseudomonads and *Trichoderma* spp. isolated at random from soils as well as from known suppressive soils have been tested extensively because of their potential to control soilborne pathogens (3,5,7,11,12,14,15,19,23,24,29,30). However, only a few attempts have been made to elucidate their role in natural suppressiveness of soils (2,6,7,21,22,30). Competition between pathogenic and nonpathogenic *Fusarium* spp. has been proposed (1) to explain Fusarium-suppressive soils in France. In soils of similar parent material such as those found in France (13), suppression of black root rot, caused by *Thielaviopsis basicola* (Berk. & Br.) Ferraris, has also been reported (10). These soils are located in a geologically distinct 22-km^2 area (old morainic soil) in Switzerland (27), and the suppressive principle occurs in the rhizosphere (4).

The objective of the present study was to assess the relative importance of fluorescent rhizosphere bacteria and nonpathogenic strains of *T. basicola* in the soils naturally suppressive to black root rot.

MATERIALS AND METHODS

Analyses of the two suppressive soils (MS1 and MS2) and the two conducive soils (MC1 and VC1) are given in Table 1. Soils MS1, MS2, and MC1 are located in the same area at Morens near Payerne, Switzerland. Soil VC1 is located 80 km to the south at Vouvry near Lake Geneva. All four soils are located in western Switzerland. The geology of these soils has been described previously (27). Forty-seven additional soil samples were collected in southwestern Switzerland from an area of more than 1,000 km^2. These samples were selected according to geological characteristics (i.e., morainic soils of the Rhône glacier) and taken either from the surface or from deeper subsurface layers by using sterilized shovels for each sampling. The soil samples were then air-dried for 2 days, sieved through a screen with 0.8-cm openings, and placed in pots of 50-cm^3 or 600-cm^3 capacity with a drainage hole at the bottom. For heat treatment, soils were placed in a 3-cm layer in an autoclave, exposed to moist heat for 30 min, cooled, then placed in pots as above. Heating was controlled by two temperature sensors.

The fluorescent pseudomonads were isolated by transplanting 4-wk-old (four-leaf stage) tobacco plants (*Nicotiana glutinosa* L.) in the various soils. Plants were then grown for 3 wk in a growth chamber. In all tests, the growth chamber was preset at 4,000 lux for 16 hr of light at 22 C followed by an 8-hr dark period at 15 C and 70% RH. Plants were then carefully removed from pots, the roots were separated from adhering soil by gently washing them with distilled water and placing them on King's B medium (16) for 18–24 hr at 27 C. The plates were then examined under UV light (350 nm) and the fluorescent colonies were evaluated. To determine the population density of the bacteria, roots of each plant were washed, blotted, weighed, and added to flasks containing 100 ml of isotonic water (0.9% NaCl) and placed on a rotary shaker for 10 min. Appropriate dilutions were plated on King's B medium and incubated at 27 C. After 24 hr, the colonies of fluorescent pseudomonads on each plate were counted under UV light.

Strain CHAo was identified by Oxi/Ferm Tube (Roche), API 20 E (API-International) and by using the description reported by Stolp and Gadkari (26). The strains were shake-cultured for 15 hr at 27 C in nutrient yeast broth (25 g of nutrient broth, 5 g of yeast extract, and 1 L of distilled water) or grown on nutrient agar (40 g of blood agar, 5 g of yeast extract, and 1 L of distilled water) (25). All media were autoclaved at 121 C for 20 min. For long-term storage, 1.5 ml of a culture grown in nutrient yeast broth for 15 hr was mixed with 1.5 ml of glycerol (87%), incubated for 2 hr at 20 C, and then stored at −80 C.

The bacteria were separated from the liquid medium by centrifugation (10 min, 4,000 *g*) and then resuspended in 1,000 ml of tap water. Eight milliliters of suspension was added to each 100 cm^3 soil samples. This suspension was equivalent to 10^7 cfu/cm^3 of soil and was added 1 day prior to the addition of a highly virulent strain (ETH strain D 127) of *T. basicola*. *T. basicola* was grown on malt agar (15 g of malt extract, 12 g of agar, and 1 L of distilled water) for 3 wk at 25 C in the dark. Endoconidia that developed were suspended in tap water, separated from chlamydospores and mycelia by filtration through glass wool, and added to soil (10^4 endoconidia per cubic centimeter of soil) in the same manner as the bacteria. The soils were then incubated at 20 C and 70% RH and

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TABLE 1. Soil analysis for the naturally occurring suppressive and conducive soils used to study the suppression of black root rot of tobacco caused by *Thielaviopsis basicola*

Soil	pH	Organic matter (%)	$CaCO_3$ (%)	N total (%)	P_2O_5 (μg/g)	K_2O (μg/g)	Mg (μg/g)	Fe (μg/g)	Cu (μg/g)	Zn (μg/g)	Mn (μg/g)	B (μg/g)	Texture
MS1[w]	6.2	2.4	0	0.5	23.5	60	48	76	4.4	1.3	32	0.6	Sandy loam
MS2[x]	6.8	3.0	0	0.6	13.4	53	30	98	3.0	1.2	57	0.6	Sandy loam
MC1[y]	6.3	2.6	0	0.7	30.0	25	112	38	2.1	0.9	31	0.5	Sandy loam
VC1[z]	7.8	1.7	10	0.3	6.2	22	101	32	2.2	1.3	11	0.5	Loamy sand

[w] MS1, naturally occurring suppressive soil from Morens, Switzerland.
[x] MS2, naturally occurring suppressive soil from Morens, Switzerland.
[y] MC1, naturally occurring conducive soil from Morens, Switzerland.
[z] VC1, naturally occurring conducive soil from Vouvry, Switzerland.

TABLE 2. Naturally occurring populations of fluorescent pseudomonads from suppressive (MS1 and MS2) and conducive soils (MC1 and VC1) and the suppression of black root rot caused by *Thielaviopsis basicola*

Soil[y]	Artificial infestation with *T. basicola*	Fluorescent pseudomonads (cfu × 10^4 per g root	Roots infected (%)	Plant fresh weight (g)	Root fresh weight (g)
MS1	−	4 a[z]	0.0 a	3.98 a	1.85 a
	+	6 a	20.4 b	3.65 ab	1.64 a
MS2	−	5 a	0.0 a	3.87 a	1.91 a
	+	4 a	34.6 b	3.44 ab	1.66 a
MC1	−	0 b	0.0 c	4.01 a	1.99 a
	+	0 b	73.6 c	0.89 c	0.12 c
VC1	−	0 b	0.0 a	3.25 b	1.14 b
	+	0 b	66.1 c	0.93 c	0.22 c

[y] Soil samples were taken at 20 cm depth, transferred to a growth chamber and infested with strain D 127 of *T. basicola*. Uninoculated samples served as control. Samples were planted with tobacco seedlings. After 3 wk, the population of fluorescent pseudomonads, percent roots infected, and plant and root fresh weight were determined.
[z] Means in the same column followed by the same letter are not significantly different, $P = 0.05$, according to Duncan's multiple range test. Each value is the mean of three replications and 20 plants per replicate.

TABLE 3. Pathogenicity of isolates of *Thielaviopsis basicola* on tobacco that were isolated from two suppressive (MS1 and MS2) and two conducive soils (MC1 and VC1)

Roots infected (%)[y]	Number of isolates from the soils			
	MS1	MS2	MC1	VC1
0	0[z]	0	0	0
10–25	0	0	0	0
25–50	1	2	2	1
50–75	9	8	7	8
75–100	2	6	5	0
100	0	0	0	0

[y] Pots filled with quartz sand were planted with tobacco and infested with strain D 127 of *T. basicola*. After 2 wk, percent roots infected was evaluated.
[z] Each value is the mean of three replications and 20 plants per replicate.

watered regularly to maintain a matric potential between −1 and −3 bar. After 2 wk, the pots were emptied, and soil from each pot was thoroughly mixed, then returned to the original pots. One tobacco seedling at the four-leaf stage was transplanted into each pot and allowed to grow in the growth chamber for 3 wk. The tobacco was then removed and roots were gently separated from adhering soil by carefully washing them in tap water. Black root rot severity was calculated for each plant as the percentage of root surface infected and darkened by the presence of chlamydospores and assessed on an eight-class scale in which 0% = no disease, 5% = $0\% < x \leq 10\%$ roots infected, 17.5% = $10\% < x \leq 25\%$ roots infected, 37.5% = $25\% < x \leq 50\%$ roots infected, 62.5% = $50\% < x \leq 75\%$ roots infected, 82.5% = $75\% < x \leq 90\%$ roots infected, 95% = $90\% < x \leq 100\%$ roots infected, and 100% = plant dead. The letter x represents the midpoint of the class interval. Severity ratings were based on the average of three replications and 20 plants per replicate.

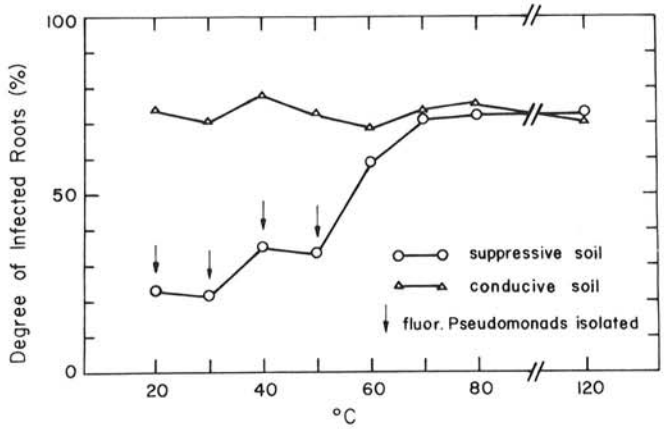

Fig. 1. Effect of moist heat treatment of soil samples for 30 min on suppressiveness to black root rot caused by *Thielaviopsis basicola* and on the presence of fluorescent pseudomonads. The data represent the mean of three replications, each containing 20 plants per replicate.

In all experiments, tobacco seeds were surface disinfected in 70% ethanol for 1 min, immersed in 5% H_2O_2 for 10 min, then rinsed in sterile water. Seeds were sown in quartz sand (particle size range, 0.8–1.2 mm) in 150-cm^3 pots and allowed to germinate in a growth chamber, which was preset at the same conditions given above. Seedlings were watered with Knop's nutrient solution (31) as needed.

Strains of *T. basicola* were isolated from soil by the method of Delon et al (8) or that of Gasser and Défago (10). The strains were grown in the same manner as ETH strain D 127. Pathogenicity was assessed by transplanting one tobacco seedling at the four-leaf stage into a 50-cm^3 pot filled with quartz sand having a particle size range of 1.2–1.5 mm and allowed to grow in a growth chamber for 2 wk. The growth chamber was preset at the same conditions as above. One day after transplanting tobacco seedlings, 4 ml of tap water containing 1.25×10^5 endoconidia per milliliter of water of the various strains were added to each pot. Black root rot severity was assessed after 2 wk.

RESULTS

Fluorescent pseudomonads were isolated readily from soils MS1 and MS2 which are naturally suppressive to black root rot. However, fluorescent bacteria could not be isolated from the conducive soils designated as MC1 and VC1 (Table 2). All 51 isolates of *T. basicola* from both suppressive and conducive soils were pathogenic (Table 3).

In the suppressive soil MS1, the fluorescent pseudomonads were isolated to a depth of 1 m, and black root rot was suppressed on the tobacco grown in all the soils from 0 to 1 m depth. The naturally occurring population of fluorescent pseudomonads was 1×10^4–$5 \times$

10^4 cfu/g fresh root in these soil layers (0–1 m). No fluorescent pseudomonads were detected from soils at a depth greater than 1 m and disease was not suppressed (Table 4). Moist heat treatment of all the soil samples at 60 C and above for 30 min destroyed most or all suppressiveness and fluorescent pseudomonads could no longer be isolated. Some loss of suppressiveness was noticed following a 40 C heat treatment for 30 min; although the fluorescent pseudomonads were still present in these soils (Fig. 1), these differences were not statistically significant. Suppressiveness was established fully by mixing 5, 10, 25, or 50% (v/v) suppressive soil MS1 with the conducive soil VC1 followed by incubation of the mixtures for 4 wk prior to planting. Fluorescent pseudomonads were consistently isolated from roots of tobacco grown in these soil mixtures. The population density of the bacteria was 1×10^4–5×10^4 cfu/g of fresh root (Table 5).

Fifteen isolates of fluorescent pseudomonads were cloned and introduced at 10^7 cfu/cm^3 of soil into the natural conducive soil VC1. Twelve isolates did not suppress disease. Three were about half as effective as the undiluted suppressive soil, and one isolate, designated as strain CHAo, by itself produced nearly as much suppressiveness as that shown by the undiluted suppressive soil (Fig. 2). Isolate CHAo was identified as *Pseudomonas fluorescens* (Trevisan) Migula. When strain CHAo was added at 10^7 cfu/cm^3 of soil to the suppressive MS1 and the conducive VC1 soils that had been heat treated, suppressiveness was restored to the soils, but to a lower extent than that of raw soils. In the heat-treated soil, the population density of strain CHAo after 3 wk was 10^5 cfu per gram of root (Table 6). Strain CHAo was added to 39 conducive and to eight suppressive soil samples at 10^7 cfu/cm^3 of soil. In 36 of the 39 conducive soil samples, strain CHAo induced suppressiveness, and in all eight suppressive soil samples, suppressiveness was slightly increased (Fig. 3). Three soils remained conducive and strain CHAo could not be reisolated.

Addition of strain CHAo to soil samples of conducive soil MC1 and to samples taken at a depth greater than 1 m of the suppressive soil MS1 did not reverse their conductivity and strain CHAo could not be reisolated after 3 wk.

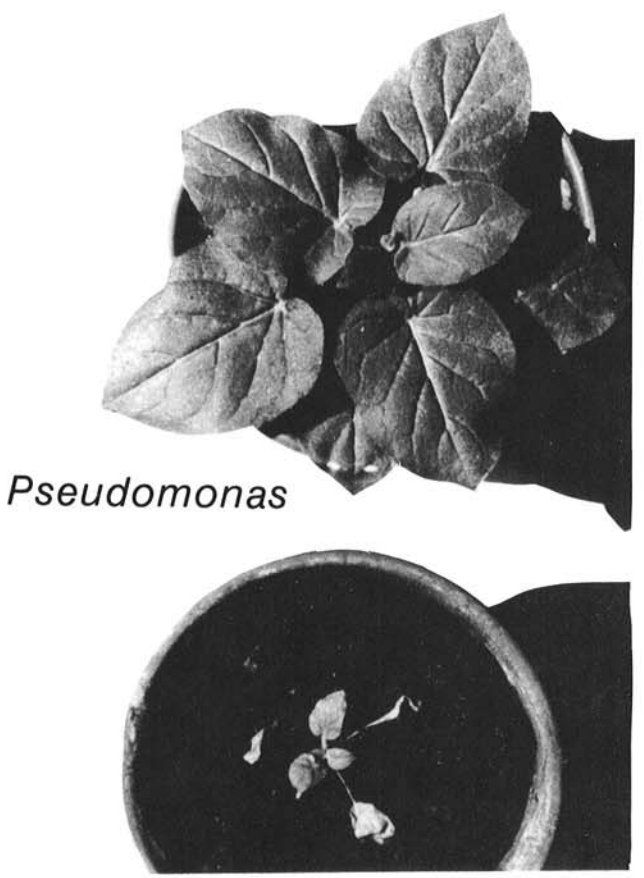

Fig. 2. Influence of strain CHAo of *Pseudomonas fluorescens* on black root rot of tobacco caused by *Thielaviopsis basicola* in a conducive soil: **Top,** infested with strain CHAo of *P. fluorescens* and 24 hr later with *T. basicola*; **Bottom,** infested with strain D 127 of *T. basicola*.

DISCUSSION

In contrast to results obtained by Alabouvette et al (1) with the *Fusarium*-suppressive soils in France, we did not isolate any nonpathogenic strains of *T. basicola*. All isolates of *T. basicola* were pathogenic; therefore, under our conditions, suppressiveness

TABLE 4. Naturally occurring populations of fluorescent pseudomonads at different soil depths and suppression of black root rot caused by *Thielaviopsis basicola* in a suppressive (MS1) and conducive soil (VC1)

Soils	Depth[y] (cm)	Artificial infestation with *T. basicola*	Fluorescent pseudomonads (cfu × 10^4 per g root)	Roots infected (%)	Plant fresh weight (g)	Root fresh weight (g)
MS1	20	–	4 a[z]	0.0 a	4.17 a	1.85 a
	20	+	5 a	20.4 b	3.70 ab	1.68 a
	50	+	3 a	24.3 b	3.42 b	1.65 a
	75	+	0.3 b	30.0 b	3.52 ab	1.51 a
	100	+	0 c	49.5 c	1.16 c	0.34 c
	130	+	0 c	53.3 c	1.03 cd	0.26 c
	160	+	0 c	75.6 c	0.66 d	0.11 c
VC1	20	–	0 c	0.0 a	3.12 b	1.09 b
	20	+	0 c	64.3 cd	0.78 cd	0.16 c

[y] Soil samples were taken at depths from 20 to 160 cm, transferred to a growth chamber and infested with strain D 127 of *T. basicola*. Uninoculated samples served as controls. Samples were planted with tobacco seedlings. After 3 wk, the population of fluorescent pseudomonads, percent roots infected, and plant and root fresh weight were determined.

[z] Means in the same column followed by the same letter are not significantly different, $P = 0.05$, according to Duncan's multiple range test. Each value is the mean of three replications and 20 plants per replicate.

TABLE 5. Transfer of suppressiveness to a conducive soil (VC1) by mixing varying amounts of a suppressive soil (MS1) that contains a naturally occurring population of fluorescent pseudomonads

Ratio of suppressive to conducive soil[x]	Fluorescent pseudomonads (cfu × 10^4 per g root)	Roots infected (%)	Plant fresh weight (g)	Root fresh weight (g)
100:0	4 a[z]	0.0 a	4.01 a	1.83 a
100:0[y]	6 a	20.2 b	3.66 ab	1.69 a
50:50[y]	3 a	19.8 b	3.63 ab	1.62 a
25:75[y]	4 a	21.2 b	3.97 a	1.74 a
10:90[y]	4 a	23.1 b	3.59 ab	1.59 a
5:95[y]	3 a	25.0 b	3.41 b	1.65 a
0:100[y]	0 b	68.2 c	0.64 c	0.14 c
0:100	0 b	0.0 a	3.10 b	1.02 b

[x] Soil samples were taken at 20 cm depth, mixed, and transferred to a growth chamber. Samples were planted with tobacco seedlings. After 3 wk, the population of fluorescent pseudomonads, percent roots infected, and plant and root fresh weight were determined.

[y] Infested with strain D 127 of *T. basicola*. Uninoculated samples served as controls.

[z] Means in the same column followed by the same letter are not significantly different, $P = 0.05$, according to Duncan's multiple range test. Each value is the mean of three replications and 20 plants per replicate.

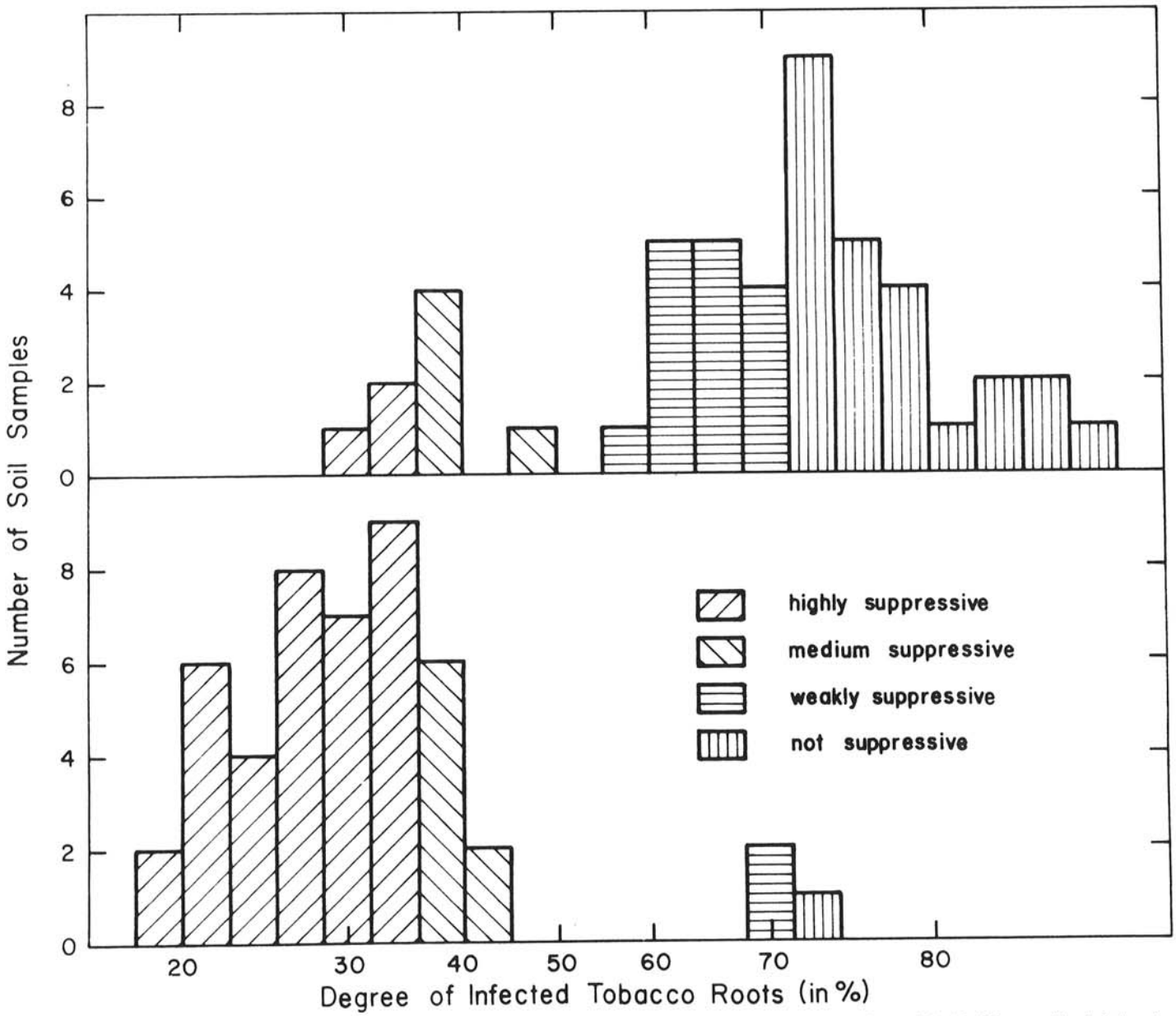

Fig. 3. Influence of strain CHAo of *Pseudomonas fluorescens* on black root rot of tobacco caused by *Thielaviopsis basicola* in 47 different soils. **A,** Infested with strain D 127 of *T. basicola*. **B,** Infested with strain CHAo of *P. fluorescens* and 24 hr later with *T. basicola*. The width of the vertical bar is five percentage points; thus, the actual data value is the midpoint of the width of the bar. The data represent the mean of three replications, each containing 20 plants per replicate.

TABLE 6. Suppression of black root rot caused by strain D 127 of *Thielaviopsis basicola* by addition of strain CHAo of *Pseudomonas fluorescens* to a conducive (VC1) and a suppressive soil (MS1)

Microorganisms added	Roots infected (%)			
	Soil MS1[x]		Soil VC1	
	Raw	Heat treated[y]	Raw	Heat treated
None	0.0 a[z]	0.0 a	0.0 a	0.0 a
P. fluorescens	0.0 a	0.0 a	0.0 a	0.0 a
T. basicola	20.8 b	71.2 d	70.3 d	73.4 d
P. fluorescens and *T. basicola*	18.9 b	36.5 c	28.7 bc	35.1 bc

[x] Soil samples were taken at 20 cm depth, mixed, and transferred to a growth chamber. Samples were planted with tobacco seedlings. After 3 wk, percent roots infected was evaluated.

[y] Moist heat treated for 30 min at 121 C.

[z] Means followed by the same letter are not significantly different, $P = 0.05$, according to Duncan's multiple range test. Each value is the mean of three replications and 20 plants per replicate.

to black root rot does not seem to be related to intraspecific fungal competition.

There is substantial evidence that populations of fluorescent pseudomonads are involved in suppression of black root rot. Their presence in the upper meter of a soil naturally suppressive or in conducive soils made suppressive by transfer of a small amount of suppressive soil correlates with suppressiveness. Their absence in two naturally conducive soils or in suppressive soil made conducive after moist heat treatment or naturally absent below 1 correlates with conduciveness. Fluorescent pseudomonads have been suggested to be important in either naturally or induced (through cultural practice) suppressiveness of soils to disease (6,7,9,21,30). Their role, according to Cook and Rovira (7), was difficult to assess because fluorescent pseudomonads were found also in the naturally conducive soils which were used as controls.

Although many strains of fluorescent pseudomonads were isolated from the suppressive soil, the evidence strongly suggests that strain CHAo of *P. fluorescens* was responsible for the suppression of black root rot we observed. Introduction of strain CHAo into the conducive soil made it suppressive to a degree

similar to that obtained after transfer of 5% suppressive soil (Tables 5 and 6). Reintroduction of strain CHAo into a suppressive soil made conducive by heat treatment restored suppressiveness in the soil, but at a degree slightly lower than that of the original naturally occurring suppressive soil.

Fluorescent pseudomonads, reported by various authors to be antagonists of soilborne pathogens (5,7,9,11,15,17,24,28–30), usually have been isolated from the top 20 cm of soil (7,11,15,17,27). In this report, fluorescent pseudomonads were isolated from one soil naturally suppressive to a depth of 1 m. Therefore, it seems that this soil itself is a favorable environment for fluorescent pseudomonads that induce suppressiveness.

Smiley (24), Cook and Rovira (7), Scher and Baker (20–22), and others (9,15,23,30) reported disease suppression after addition of pseudomonads to infested soil. The introduction of strain CHAo to several naturally conducive soils induced disease suppressiveness, but the levels of suppression differed among different soils. Three soils remained conducive after introduction of strain CHAo and no fluorescent pseudomonads could be isolated. Although fluorescent pseudomonads are known to be common inhabitants of the rhizosphere (18), not every soil has the capacity to support them.

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