Dose-Response Relationships in Biological Control of Fusarium Wilt of Radish by *Pseudomonas* spp.

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ABSTRACT

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The dose-response relationships in the suppression of Fusarium wilt of radish by *Pseudomonas putida* strain WCS358 and *P. fluorescens* strain WCS374 were investigated. The sole mechanism involved in the suppression of Fusarium wilt of radish by strain WCS358 is siderophore-mediated competition for iron(III), whereas strain WCS374 suppresses this disease by induction of systemic resistance. The efficacy of siderophore-mediated disease suppression and induced resistance was highly dependent on the level of disease incidence. Both mechanisms of biological control were effective over a wide range of disease incidences. The absolute disease reduction reached a maximum of approximately 30% at an average level of disease incidence of 50% for both mechanisms. The rhizosphere population density of strain WCS358 and of strain WCS374 was an important determinant of their efficacy to suppress Fusarium wilt of radish. Regression analysis demonstrated significant nonlinear asymptotic relationships between the rhizosphere population densities of strain WCS358 or strain WCS374 and the level of suppression of Fusarium wilt of radish. At moderate to relatively high levels of disease incidence for both mechanisms, a threshold population density of the bacterial strain of approximately 10^5 CFU per gram of root is required for a significant suppression of Fusarium wilt of radish. When rhizosphere population densities of both strains dropped below this threshold level, a relatively small decline in the population density had a major effect on their efficacy to suppress Fusarium wilt of radish. Increasing the rhizosphere population density of both strains to levels higher than the threshold level did not result in a significant improvement of disease suppression.

Biological control of soilborne plant diseases by application of specific microorganisms to seeds or planting material has been studied extensively over the last 2 decades. Among the plant growth-promoting rhizobacteria (PGPR), the fluorescent *Pseudomonas* spp. have received much attention. Certain strains of fluorescent pseudomonads have been shown to suppress various plant diseases caused by soilborne pathogens, including deleterious microorganisms (29,41). Under field conditions, however, most biological control agents, including strains of *Pseudomonas* spp., are too variable in their performance to be used successfully as a common practice in agriculture and horticulture. Variability in plant growth promotion and disease suppression by strains of *Pseudomonas* spp. has been reported in field trials conducted with sugar beet (31), wheat (40), potato (3,6,30), and radish (18).

Multiple factors could account for this variability given the complex interactions between the *Pseudomonas* sp. strain, the pathogen, the plant, and the environment (29,39). The major factors reported in the literature are related to rhizosphere colonization and the expression of the genes involved in disease suppression. Recently, Johnson (13) defined various epidemiological parameters that may govern the efficacy of biological control of plant diseases. In the theoretical relationships he proposed, the degree of disease suppression by a biocontrol agent depends on the density of the agent, the density of the pathogen, the efficiency of the biocontrol agent to suppress the pathogen, and the proportion of the pathogen population that is potentially affected by the biocontrol agent. The importance of the rhizosphere population density of *Pseudomonas* strains has been emphasized by Bull et al. (5), who demonstrated a positive relationship between the population size of antibiotic-producing *P. fluorescens* strain 2-79RN$_{10}$ on seminal roots of wheat and the level of suppression of take-all. However, the epidemiological parameters that govern the efficacy of biological control by introduced *Pseudomonas* strains are still poorly understood for most other host-pathogen systems, as well as for other mechanisms of disease suppression. Understanding these parameters is essential to improve the efficacy of fluorescent *Pseudomonas* spp. as biocontrol agents in agriculture and horticulture.

In this paper, dose-response relationships in the suppression of Fusarium wilt of radish by *P. putida* strain WCS358 and *P. fluorescens* strain WCS374 were investigated. The mechanism involved in the suppression of Fusarium wilt of radish by *P. putida* strain WCS358 was siderophore-mediated competition for iron (this study), whereas *P. fluorescens* strain WCS374 suppresses this disease by induction of systemic resistance (19). The efficacy of both mechanisms of disease suppression is described for various levels of disease incidence and for different rhizosphere population densities of these *Pseudomonas* strains. The implications of the relationships described in this study for biological control under field conditions are discussed.

MATERIALS AND METHODS

Bacterial strains and growth media. Wild-type strains *P. putida* WCS358 and *P. fluorescens* WCS374 are plant growth-promoting rhizobacteria isolated from the roots of potato (9).

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Strain WCS358 produces a specific siderophore, designated pseudobactin 358, which consists of a dihydroxyquinoline chromophore linked to a linear nonapeptide (33). JM218 is a Tn*5* transposon mutant of strain WCS358 defective in the production of pseudobactin 358, and is resistant to kanamycin and streptomycin (24). Leeman et al. (19) demonstrated that strain WCS358 does not induce resistance in radish against Fusarium wilt. For the preparation of bacterial suspensions, strains were cultured on King's medium B (KB) agar plates (15) for 48 h at 27°C, harvested in 0.01 M $MgSO_4$, and washed twice by centrifugation (10 min, 5,000 × *g*). The density of the bacterial strains in the suspensions was determined spectrophotometrically at 660 nm.

Preparation of inoculum of *Fusarium oxysporum* f. sp. *raphani*. *F. oxysporum* Schlechtend.:Fr. f. sp. *raphani* J. B. Kendrick & W. C. Snyder strain WCS600, which causes Fusarium wilt of radish, was isolated from naturally infected radish plants (*Raphanus sativus* L. cv. Saxa Nova) on Komada agar plates (16) and subcultured every 2 months on potato-dextrose agar (PDA) plates (39 g of PDA [Difco Laboratories, Detroit] per liter). For the bioassays, an aerated 2% malt extract (Difco Laboratories, Detroit) medium was seeded with PDA agar plugs (diameter, 0.5 cm) overgrown with *F. oxysporum* f. sp. *raphani*. After 9 days of growth at 22°C, cultures were filtered through glass wool to remove mycelial mats. Microconidia in the filtrate were pelleted by centrifugation (10 min, 5,000 × *g*) and washed twice with 0.01 M $MgSO_4$. The conidial density in the inoculum was determined by direct observation using a hemocytometer. The initial density of *F. oxysporum* f. sp. *raphani* in soil or peat was determined by dilution plating of soil or peat suspensions on Komada agar plates (16).

Biocontrol assay with *P. putida* strain WCS358. Suppression of Fusarium wilt of radish by *P. putida* strain WCS358 was studied in a soil bioassay. Potting soil was mixed with quartz sand in a 2:1 ratio (vol/vol). This mixture was sieved (5-mm mesh) and autoclaved twice (20 min, 120°C, 0.1 MPa) with a 24-h interval. Autoclaving did not lead to sterile conditions, but reduced the number of indigenous microorganisms from 5×10^8 CFU to approximately 10^4 CFU/g of soil, thereby allowing *F. oxysporum* f. sp. *raphani* to readily infect radish plants. Conidial suspensions of *F. oxysporum* f. sp. *raphani* were introduced into the soil mixture at densities ranging from approximately 10^4 to 10^5 conidia/g. After introduction of *F. oxysporum* f. sp. *raphani*, the soil was incubated for 5 days in the dark at 24°C for 16 h, followed by an 8-h period at 20°C.

For the application of bacterial strains, serial dilutions of bacterial suspensions (2×10^9 CFU ml^{-1}) were introduced (20 ml/600 g) into autoclaved potting soil mixed with quartz sand (2:1, vol/vol). The bacterized soil was stored for 12 h at 5°C. For the bioassay, *F. oxysporum* f. sp. *raphani*-infested soil, bacterized soil, and quartz sand were thoroughly mixed in a 1:2:4 ratio (wt/wt/wt). The pH of this soil mixture was 6.9. Subsequently, polyvinylcarbonate pots (height, 11 cm; diameter, 14 cm) were filled with a layer (8-cm) of 750 g of the final soil mixture on top of a bottom layer (3-cm) of hydrogranules. Radish seeds (*Raphanus sativus* L. cv. Stellar, moderately resistant to Fusarium wilt) were sown in the soil mixture at a depth of 1 cm, and radish plants were grown in a growth chamber under controlled conditions with a 16-h light period (irradiance 200 $mE/m^2/s$) at 24°C and 70% relative humidity, followed by an 8-h dark period at 20°C and 70% relative humidity. Plants were watered twice per week, once with 200 ml of tap water and once with 200 ml of a nutrient solution containing (per liter) 0.51 g of KNO_3, 0.16 g of KH_2PO_4, 0.084 g of $Mg(NO_3)_2.6H_2O$, 1.2 mg of $Na_2B_4O_7$, 0.5 mg of $MnSO_4$, and 0.5 mg of $NaMoO_4$. The composition of this nutrient solution was based on a chemical analysis (ReLab Den Haan B.V., Wateringen, the Netherlands) of the soil used in these bioassays.

Biocontrol assay with *P. fluorescens* strain WCS374. The relationships in suppression of Fusarium wilt of radish by strain WCS374 were determined using data of rockwool bioassays conducted by Leeman et al. (19) and by P. A. H. M. Bakker and P. G. J. Vogel (*unpublished data*). In this type of assay, serial dilutions of a suspension of strain WCS374 were delivered in talcum on the root tips of 5-day-old radish seedlings, whereas the pathogen was delivered in peat on the root base 1 or 2 days later. Strain WCS374 and *F. oxysporum* f. sp. *raphani* remained at spatially separated locations on the root system throughout the experiments. Therefore, direct interaction between WCS374 and *F. oxysporum* f. sp. *raphani* (e.g., via competition or antibiosis) was excluded in this assay (19). A time lapse of at least 1 day between bacterization and pathogen inoculation is required for a significant induction of resistance in radish against Fusarium wilt (19). Radish plants (*Raphanus sativus* L. cv. Saxa Nova, moderately resistant to Fusarium wilt) were grown in the greenhouse with a 16-h light period at 22 to 28°C and 70% relative humidity, followed by a dark period at 22°C and 70% relative humidity. Plants were watered twice per week with tap water and once per week with half-strength Hoagland solution (12).

Disease severity. Disease symptoms in radish after infection with *F. oxysporum* f. sp. *raphani* are yellowing and wilting of the leaves followed by general chlorosis, necrosis, and plant death. A distinct internal symptom is brownish discoloration of the vascular tissue, which is caused by the oxidation of phenols as a reaction to the presence of the pathogen (26). In the bioassays conducted with strains WCS358 and WCS374, the number of diseased plants was determined by examination of cross sections of the lower and upper part of the taproot for brownish discolorations of the vascular system. In the bioassays with strain WCS358, the percentage of diseased plants was determined for six replicates of 10 plants each after approximately 27 days of plant growth. In the bioassays with strain WCS374, the percentage of diseased plants was determined for 10 to 12 replicates of three plants each after approximately 24 days of plant growth.

Enumeration of introduced bacterial strains. In the bioassays conducted with strain WCS358, population densities of the applied bacterial strains were determined in the soil and the rhizosphere. For the preparation of soil suspensions, 2 g of soil was sampled at random from each pot, suspended in 10 ml of sterile 0.1 M $MgSO_4$, and shaken vigorously for 60 s in glass test tubes containing 2.5 g of glass beads (diameter, 0.17 mm). For the preparation of rhizosphere suspensions, plants were harvested from the polyvinylcarbonate pots after loosening the soil surrounding the root system. Loosely adhering soil was removed from the roots by gentle shaking. Root segments (0.3- to 0.5-g) were sampled at random from a depth of 0 to 8 cm from the stem base, suspended in 5 ml of sterile 0.1 M $MgSO_4$, and shaken vigorously for 60 s in glass test tubes containing 2.5 g of glass beads (diameter, 0.17 mm).

Aliquots (0.1-ml) of serial dilutions of soil and rhizosphere suspensions were mixed homogeneously through selective agar media (0.4-ml, 40°C) in 24-well tissue culture plates on a reciprocal shaker. After incubation for 24 h at 27°C, the developed colonies of the applied bacterial strains were enumerated by immunofluorescence colony-staining (IFC) according to the method described by Van Vuurde and Roozen (35) and modified by Leeman et al. (17). For IFC, antibodies specifically directed against strain WCS358 were conjugated to fluorescein isothiocyanate (FITC). Population densities of wild-type strain WCS358 were determined by IFC in modified KB agar (KB^+) (9) supplemented with 300 µM of pseudobactin 358 (28), whereas Tn*5* mutant JM218 was enumerated by IFC in KB^+ agar supplemented with streptomycin (200 µg/ml) and kanamycin (200 µg/ml). Total population densities of indigenous *Pseudomonas* spp. were determined in KB^+ agar after staining of the developed colonies for 20 min with 400 µl of ethidium bromide (1 mg/ml of demineralized water). Stained colonies were enumerated microscopically using a Zeiss Axioskop 20 (Carl Zeiss, Inc., Oberkochen, Ger-

many) with fluorescence-illumination. For each treatment, microbial enumerations were performed on six replicates of one plant each. Soil population densities of strains WCS358 and JM218 were determined at the beginning of the bioassay, and the rhizosphere population densities were determined after approximately 15 days of plant growth.

In the bioassays conducted with strain WCS374, rhizosphere population densities of strain WCS374 were similarly determined by IFC in KB^+ agar using WCS374-specific FITC-conjugated antibodies (17); rhizosphere population densities were determined 2 and 24 days after application of strain WCS374 to the root tips. Ten replicates of one plant each were used.

Data analysis. Rhizosphere populations of introduced bacterial strains approximate a lognormal distribution along the root system (22). Therefore, the numbers of colony-forming units were transformed to $\log_{10}(\text{CFU} + 1)$ prior to statistical analysis. Transformed values and percentages of diseased plants were analyzed by analysis of variance followed by Fisher's least significant difference test (SAS Institute Inc., Cary, NC). For different bioassays, the relative disease reduction by strain WCS358 or strain WCS374 was calculated by the equation $R = [(C - W)/C] \times 100$, where R represents the relative disease reduction, C the average percentage of diseased plants in the control treatment, and W the percentage of diseased plants in the bacterial treatment. Regression analysis (SAS Institute Inc., Cary, NC) was performed to determine the relationship between the absolute and relative disease reduction by the bacterial strains, their rhizosphere population density, and the average level of disease incidence in the ap-

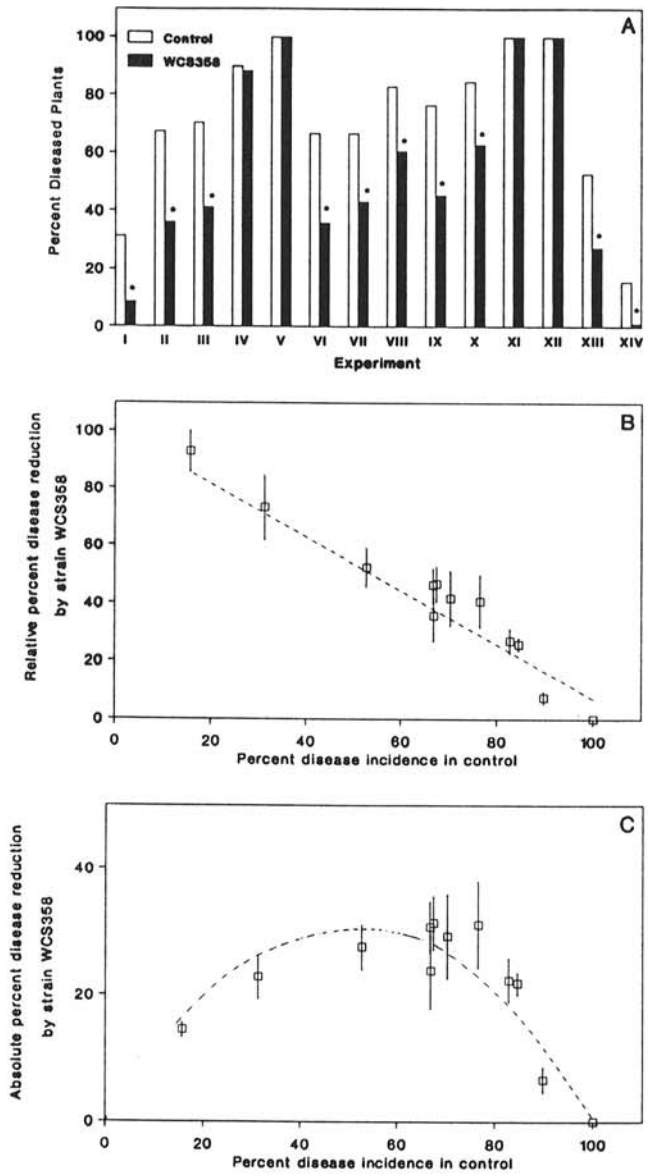

Fig. 1. A, Incidence of Fusarium wilt of radish after introduction of *Pseudomonas putida* strain WCS358 into soil infested with *Fusarium oxysporum* f. sp. *raphani*. For each bioassay, an asterisk indicates a significant difference from the control ($P \leq 0.05$). **B,** Relationship between the relative percent disease reduction by strain WCS358 and the average level of disease incidence in the control. Regression analysis demonstrated a significant relationship ($Y = -0.93X + 100$ [$r = 0.98$, $P = 0.0001$, $n = 6$]). **C,** Relationship between the absolute percent disease reduction by strain WCS358 and the average level of disease incidence in the control. Regression analysis demonstrated a significant relationship ($Y = -0.0126X^2 + 1.27X$ [$r = 0.91$, $P = 0.0001$, $n = 6$]). Mean values of six replicates of 14 bioassays are presented. Error bars represent the standard error.

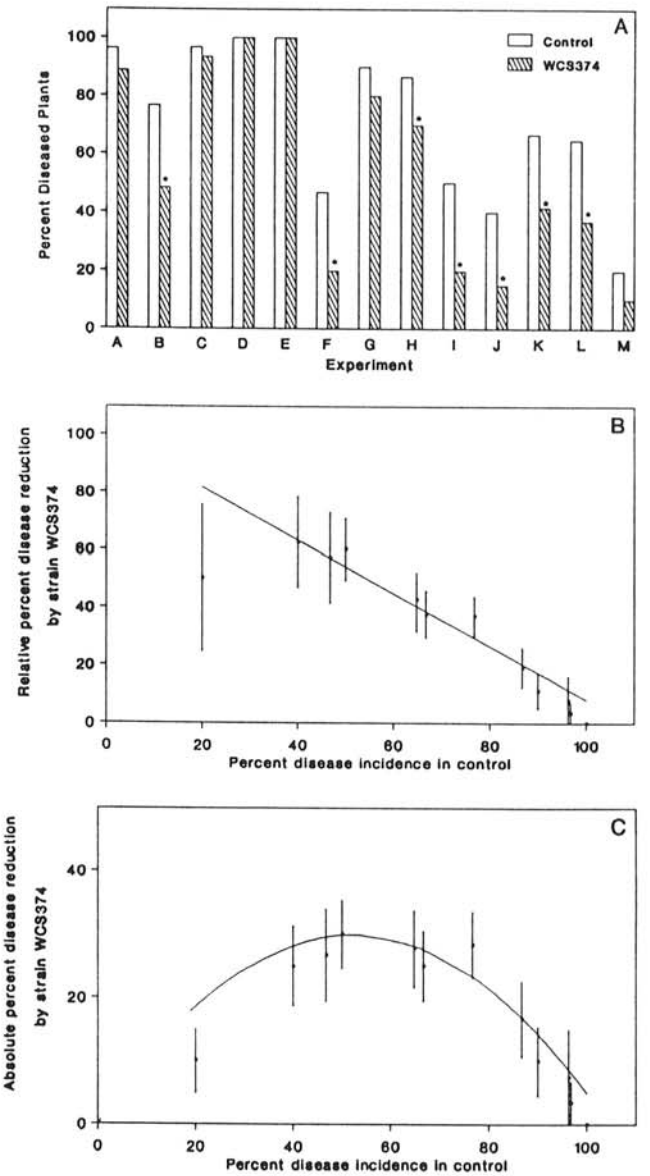

Fig. 2. A, Incidence of Fusarium wilt of radish after application of *Pseudomonas fluorescens* strain WCS374 onto the root tips of radish seedlings. For each bioassay, an asterisk indicates a significant difference from the control ($P \leq 0.05$, $n = 10$). **B,** Relationship between the relative percent disease reduction by strain WCS374 and the average level of disease incidence in the control. Regression analysis demonstrated a significant relationship ($Y = -0.92X + 100$ [$r = 0.90$, $P = 0.0001$, $n = 10$]). **C,** Relationship between the absolute percent disease reduction by strain WCS374 and the average level of disease incidence in the control. Regression analysis demonstrated a significant relationship ($Y = -0.011X^2 + 1.15X$ [$r = 0.77$, $P = 0.0001$, $n = 10$]). Mean values of 10 replicates of 13 bioassays are presented. Error bars represent the standard error.

propriate controls. In all regression analyses, intercepts with the Y-axis (dependent variable) were tested for differences from zero by performing *t* tests. When the intercepts were not significantly different from zero, regressions were forced through the origin. Experiments were repeated at least once.

RESULTS

Siderophore-mediated suppression of Fusarium wilt by *P. putida* WCS358. Prior to analyzing the relationships in the suppression of Fusarium wilt of radish by strain WCS358, we first determined the mechanism involved. Therefore, the efficacy of strain WCS358 was compared to that of its siderophore-deficient mutant JM218. Both strains were introduced into soil at an initial density of log 7 CFU/g of soil. No bacteria were introduced in the control treatment. The initial density of *F. oxysporum* f. sp. *raphani* was log 4.0 CFU/g of soil. After 27 days of plant growth, 73% of the plants in the control treatment were diseased. Strain WCS358 significantly reduced the number of diseased plants to 43%, whereas its mutant JM218 was not effective (67% diseased plants). No significant difference was observed between the rhizosphere population density of strain WCS358 and that of mutant JM218 after 15 days of plant growth. Both strains established a population density of approximately log 6.3 CFU/g of root. Similar results were obtained when the experiment was repeated.

Suppression of Fusarium wilt by strain WCS358 at different levels of disease incidence. In Figure 1A, the results of 14 bioassays with strain WCS358 are presented. In all 14 bioassays, strain WCS358 was introduced into the soil at an initial density of log 7.0 CFU/g of soil. No bacteria were introduced in the control treatment. The initial density of *F. oxysporum* f. sp. *raphani* was log 4.6 CFU/g of soil in experiments I to V, log 4.0 CFU/g of soil in experiments VI to XII, and log 3.6 CFU/g of soil in experiments XIII and XIV. After 15 days of plant growth, strain WCS358 established a rhizosphere population density of approximately log 6.3 CFU/g of root in all 14 experiments. In the controls, the average level of disease incidence varied between the experiments from approximately 15 to 100% (Fig. 1A). Significant disease suppression by strain WCS358 was observed in 10 out of the 14 experiments. In these 10 experiments, the average disease incidence in the controls did not exceed 85%. No disease suppression was evident in the other four experiments, in which the average disease incidence ranged from 89 to 100% (Fig. 1A). When the disease suppression by strain WCS358 was expressed either as a reduction relative to the control (Fig. 1B) or as an absolute disease reduction (Fig. 1C), significant relationships between disease incidence and disease reduction emerged. When disease incidence in the controls increased from 15 to 100%, the relative disease reduction by strain WCS358 decreased linearly from approximately 90 to 0% (Fig. 1B). The absolute disease reduction, however, increased from 14% to a maximum of 32% at an average level of disease incidence of approximately 50% in the controls, and then decreased again to 0% as disease incidence in the controls increased to 100% (Fig. 1C). Regression analysis showed a significant quadratic relationship between the absolute disease reduction by strain WCS358 and the average level of disease incidence (Fig. 1C).

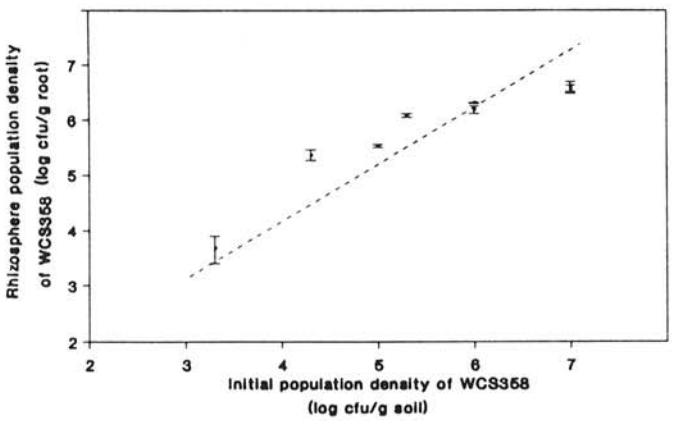

Fig. 3. Relationship between the initial soil population density of *Pseudomonas putida* strain WCS358 and its rhizosphere population density determined after 15 days of plant growth. Regression analysis demonstrated a significant linear relationship: $Y = 1.04X$ ($r = 0.99$, $P = 0.0001$, $n = 6$). Error bars represent the standard error.

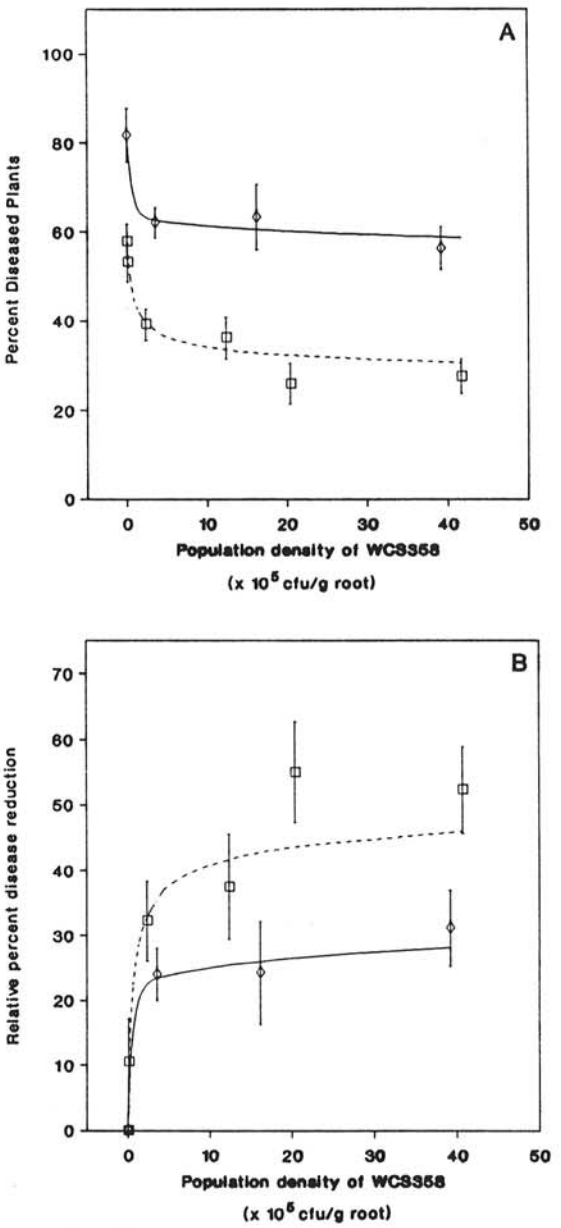

Fig. 4. A, Relationship between the rhizosphere population density of *Pseudomonas putida* strain WCS358 after 15 days of plant growth and the incidence of Fusarium wilt of radish. At a moderate level of disease incidence (- - -), regression analysis demonstrated a significant relationship ($Y = -4.83\log(X) + 62.54$ [$r = 0.74$, $P = 0.0001$, $n = 6$]). At a relatively high level of disease incidence (———), regression analysis demonstrated a comparable relationship represented by the equation: $Y = -3.52\log(X) + 82.00$ ($r = 0.60$, $P = 0.0021$, $n = 6$). **B,** Relationship between the relative percent disease reduction by strain WCS358 and its rhizosphere population density after 15 days of plant growth. At a moderate level of disease incidence (- - -), regression analysis demonstrated a significant relationship ($Y = 6.94\log(X)$ [$r = 0.91$, $P = 0.0001$, $n = 6$]). At a relatively high level of disease incidence (———), regression analysis demonstrated a comparable relationship represented by the equation: $Y = 4.27\log(X)$ ($r = 0.82$, $P = 0.0001$, $n = 6$). Mean values and standard errors of six replicates are given.

Suppression of Fusarium wilt by strain WCS374 at different levels of disease incidence. In Figure 2A, the results of 13 rockwool bioassays conducted with strain WCS374 are presented. In this type of assay, disease suppression was mediated via induced resistance only. In all 13 bioassays, strain WCS374 was applied to the root tips at an initial density of approximately log 7.5 CFU/g of root. No bacteria were introduced in the control treatment. The initial density of *F. oxysporum* f. sp. *raphani* applied to the root base was log 5.3 CFU/root system in experiments A to E, log 4.6 CFU/root system in experiments F to J, log 4.0 CFU/root system in experiments K and L, and log 3.5 CFU/root system in experiment M. Twenty-four days after bacterization, strain WCS374 established a rhizosphere population density of approximately log 6.4 CFU/g of root in all 13 experiments. In the controls, the average level of disease incidence varied between the experiments from approximately 20 to 100% (Fig. 2A). Significant disease suppression was observed in 7 out of the 13 experiments. In these seven experiments, the average disease incidence in the controls ranged from approximately 40 to 80%. Strain WCS374 did not significantly suppress Fusarium wilt when disease incidence in the control was 20% or when it ranged from 80 to 100% (Fig. 2A). When disease suppression by strain WCS374 was expressed as a reduction relative to the control (Fig. 2B) or as an absolute disease reduction (Fig. 2C), the efficacy of strain WCS374 was again highly dependent on the level of disease incidence. As was demonstrated previously for siderophore-mediated disease suppression by strain WCS358, the relative disease reduction via induced resistance by strain WCS374 decreased linearly to 0% when the average disease incidence in the controls increased from 20 to 100% (Fig. 2B). The absolute disease reduction by strain WCS374 increased from 10% to a maximum of 30% and then decreased again to 0% (Fig. 2C). Interestingly, the absolute disease reduction by strain WCS374 reached a maximum of 30% at an average level of disease incidence of approximately 50% (Fig. 2C), which was similar to the results described above for strain WCS358.

Relationship between population densities of WCS358 and siderophore-mediated suppression of Fusarium wilt. The relationship between population densities of WCS358 and disease suppression was investigated by introducing strain WCS358 into the soil at different initial densities. In the control treatment no bacteria were introduced. The results of two bioassays, which differ in the level of disease incidence, are presented in Figures 3 and 4. In the bioassay with a moderate level of disease incidence (58%), the initial density of *F. oxysporum* f. sp. *raphani* was log 3.6 CFU/g of soil and the initial densities of strain WCS358 were 0 (control), log 3.3, 4.3, 5.3, 6.0, and 7.0 CFU/g of soil, respectively. In the bioassay with a relatively high level of disease incidence (82%), the initial density of *F. oxysporum* f. sp. *raphani* was log 4.0 CFU/g of soil and the initial densities of strain WCS358 were 0 (control), log 5.0, 6.0, and 7.0 CFU/g of soil.

The results of both bioassays demonstrated that an increase in the initial soil population density of strain WCS358 led to an increase in its rhizosphere population density after 15 days of plant growth (Fig. 3). Increasing the rhizosphere population density of strain WCS358 up to approximately 10^5 CFU/g of root resulted in a decrease in the percentage of diseased plants (Fig. 4A). For both disease incidences, regression analysis demonstrated significant nonlinear asymptotic relationships between the rhizosphere population density of strain WCS358 and the percentage of diseased plants (Fig. 4A). Comparison between the results of both bioassays also demonstrated that, at similar rhizosphere population densities, the relative disease reduction by strain WCS358 was greater at a moderate level of disease incidence than at a relatively high level of disease incidence (Fig. 4B).

Relationship between population densities of WCS374 and suppression of Fusarium wilt via induced resistance. The relationship between population densities of WCS374 and disease suppression was investigated by applying strain WCS374 onto the root tips of 5-day-old radish seedlings at densities of 0 (control) and approximately log 4, 5, 6, and 7 CFU/g of root. An increase in the initial rhizosphere population density of strain WCS374, determined 2 days after bacterization, to approximately 10^5 CFU/g of root led to a decrease in the percentage of diseased plants (Fig. 5A). Also, for this type of bioassay, regression analysis demonstrated a significant nonlinear asymptotic relationship between the initial rhizosphere population density of strain WCS374 and the percentage of diseased plants (Fig. 5A). Similarly, a significant relationship was apparent between the initial rhizosphere population density of strain WCS374 and the relative percent disease reduction (Fig. 5B). No such relationships could be demonstrated between disease reduction and the rhizosphere population density of strain WCS374, determined 24 days after bacterization. The different initial rhizosphere population densities of strain WCS374 resulted in a similar rhizosphere population density of log 6.7 CFU/g of root 24 days after bacterization.

DISCUSSION

Various mechanisms are involved in the suppression of plant diseases by selected strains of fluorescent *Pseudomonas* spp., including antibiotic production (8,14,32), siderophore production (4,21,30), and induced resistance (19,25,34,37,42). The major biocontrol activity associated with siderophores of fluorescent pseudomonads is iron(III) depletion of the target pathogen. This study demonstrated that the sole mechanism in suppression of Fusarium wilt of radish by *P. putida* strain WCS358 was the production of the siderophore pseudobactin 358. Strain WCS358 does not induce resistance in radish against Fusarium wilt (19). These results confirm and extend the observations of Bakker et al. (3), and of Lemanceau et al. (20) and Duijff et al. (7), who demonstrated that siderophore-mediated competition for iron(III) was the sole mechanism involved in potato plant growth promotion and in suppression of *F. oxysporum* f. sp. *dianthi* in carnation by strain WCS358, respectively.

It is apparent from this study that the efficacy of siderophore-mediated suppression of Fusarium wilt of radish by strain WCS358 was highly dependent on the level of disease incidence. Interestingly, for *P. fluorescens* strain WCS374, which induces systemic resistance in radish against Fusarium wilt (19), similar relationships existed between the average level of disease incidence and disease reduction. These results clearly demonstrated that, when *Pseudomonas* strains WCS358 or WCS374 were applied inundatively (i.e., artificially in high concentrations), they were effective over a wide range of disease incidences. However, for both mechanisms there appeared to be a maximum to the extent of disease suppression. Under the given experimental conditions, the absolute disease reduction reached a maximum of approximately 30% at an average level of disease incidence of 50% for strain WCS358, as well as for strain WCS374. According to Johnson (13), the magnitude of disease suppression may be influenced by various biological factors, including the existence of a refuge that may protect some of the pathogen propagules from the influence of the biocontrol agent, and the degree to which the spatial distributions of the pathogen and the biocontrol agent readily coincide. These biological factors possibly played a role in siderophore-mediated disease suppression by strain WCS358. For induced resistance by strain WCS374, however, other factors seemed to determine the magnitude of disease suppression.

In field trials, the absolute reduction of Fusarium wilt of radish by strain WCS374 ranged from 0 to 25% during 4 consecutive years (18). In view of the relationship between disease suppression by strain WCS374 and the level of disease incidence, the seasonal changes in the incidence of Fusarium wilt of radish and its heterogeneous distribution in commercial greenhouses (18)

offer an explanation for the variability in biological control by strain WCS374 under field conditions. Surprisingly, the different levels of disease incidence obtained in this study occurred in several bioassays with the same initial density of *F. oxysporum* f. sp. *raphani*. Variability in disease incidence of Fusarium wilt of radish under field conditions has been ascribed to seasonal influences and the use of radish cultivars differing in susceptibility to Fusarium wilt (18). Seasonal influences also may have played a role in the bioassays conducted with strain WCS374 in the greenhouse. However, the variability in disease incidence in the bioassays with strain WCS358, which were performed in growth chambers under controlled conditions with only one moderately resistant radish cultivar, indicated that other yet unknown factors were involved. This phenomenon is currently under investigation.

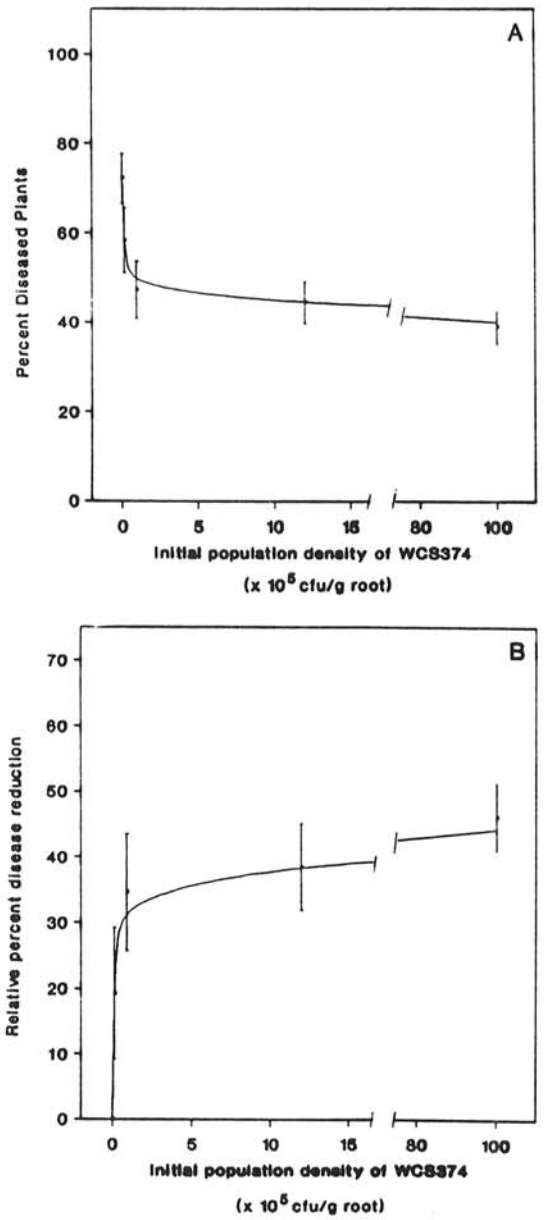

Fig. 5. A, Relationship between the initial rhizosphere population density of *Pseudomonas fluorescens* strain WCS374, determined 2 days after bacterization, and the incidence of Fusarium wilt of radish. Regression analysis demonstrated a significant relationship ($Y = -4.77\log(X) + 73.40$ [$r = 0.52$, $P = 0.0001$, $n = 12$]). **B,** Relationship between the relative disease reduction by strain WCS374 and its initial rhizosphere population density determined 2 days after bacterization. Regression analysis demonstrated a significant relationship ($Y = 6.32\log(X)$ [$r = 0.77$, $P = 0.0001$, $n = 12$]). Mean values and standard errors of 12 replicates are given.

In addition to the level of disease incidence, the rhizosphere population density of strain WCS358 was an important determinant of its efficacy to suppress Fusarium wilt of radish. These results supported the common assumption that efficient root colonization by strains of fluorescent *Pseudomonas* spp. is critical to the suppression of soilborne plant pathogens. For the induction of systemic resistance in radish against Fusarium wilt, however, only the initial rhizosphere population density of strain WCS374 appeared to be an important determinant of the efficacy of disease suppression. For both mechanisms of disease suppression, regression analysis demonstrated significant nonlinear asymptotic relationships between the rhizosphere population densities of strain WCS358 or strain WCS374 and the level of suppression of Fusarium wilt of radish. From the asymptotic nature of these relationships, it can be concluded that at levels of disease incidences ranging from 55 to 85%, for both strains WCS358 and WCS374, a threshold population density of approximately 10^5 CFU/g of root is required for significant suppression of Fusarium wilt of radish. When rhizosphere population densities of strains WCS358 or WCS374 dropped below this threshold level, a relatively small decline in their population density had a major effect on their efficacy to suppress Fusarium wilt of radish. Moreover, increasing the rhizosphere population density of strains WCS358 or WCS374 to levels higher than the threshold level did not lead to a significant improvement of disease suppression. Because these asymptotic relationships are logarithmic functions, they become inverse linear relationships when the population densities of strains WCS358 or WCS374 are arranged on a logarithmic scale (data not shown). A comparable relationship has been described for suppression of take-all of wheat by antibiotic-producing strain *P. fluorescens* 2-79RN$_{10}$ (5). For biocontrol agents other than fluorescent *Pseudomonas* spp., which use mechanisms of hyperparasitism (1,11), competition for nutrients, and induced resistance (23), comparable relationships also exist between the dose of the biocontrol agent and the level of disease suppression.

Field trials have demonstrated that population densities of introduced pseudomonads decrease with time. Initial population densities of *P. fluorescens* 2-79 (38) and *P. putida* WCS358 (3) of 10^7 and 10^5 CFU/g of root, respectively, gradually decreased to approximately 10^3 CFU/g of root during 3 to 4 months of plant growth. Moreover, population densities of introduced strains of fluorescent *Pseudomonas* spp. decline substantially with increasing distance from the inoculum source (2,3,22). Given the asymptotic nature of the relationships between the rhizosphere population density and the level of disease suppression, inefficient root colonization by strains of fluorescent *Pseudomonas* spp. may offer an additional explanation for the inconsistency of biological control encountered under field conditions. Considering the rhizosphere population densities of introduced bacterial strains reported in field trials, the development of cultural practices and delivery systems that favor the establishment and biocontrol activity of introduced bacterial strains along the essential parts of the root system will provide a significant contribution to the improvement of biological control under field conditions. Also, the introduction or amplification of specific genes involved in biocontrol (10,36) and the application of mixtures of biocontrol agents (20,27) are promising strategies to improve biological control. We expect that the amplification of pseudobactin 358 production in strain WCS358 will lower the threshold rhizosphere population density that is required for significant disease suppression, whereas the combination of strains WCS358 and WCS374 also will improve the extent of disease suppression.

In conclusion, it can be stated that the efficacy of the suppression of Fusarium wilt of radish by *Pseudomonas* spp. strains WCS358 and WCS374, which use mechanisms of siderophore-mediated competition for iron(III) and induced resistance, respectively, depends on the level of disease incidence and the rhizosphere population density of the bacterial strains. The similar-

ity between the relationships described for siderophore-mediated competition for iron(III) and induced resistance support the theoretical relationships proposed by Johnson (13), which are intended to include various mechanisms involved in biological control.

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