Control of Fusarium Wilt of Radish by Combining *Pseudomonas putida* Strains that have Different Disease-Suppressive Mechanisms

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ABSTRACT

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Biological control of soilborne plant pathogens in the field has given variable results. By combining specific strains of microorganisms, multiple traits antagonizing the pathogen can be combined and this may result in a higher level of protection. *Pseudomonas putida* WCS358 suppresses Fusarium wilt of radish by effectively competing for iron through the production of its pseudobactin siderophore. However, in some bioassays pseudobactin-negative mutants of WCS358 also suppressed disease to the same extent as WCS358, suggesting that an, as yet unknown, additional mechanism may be operative in this strain. *P. putida* strain RE8 induced systemic resistance against fusarium wilt. When WCS358 and RE8 were mixed through soil together, disease suppression was significantly enhanced to approximately 50% as compared to the 30% reduction for the single strain treatments. Moreover, when one strain failed to suppress disease in the single application, the combination still resulted in disease control. The enhanced disease suppression by the combination of *P. putida* strains WCS358 and RE8 is most likely the result of the combination of their different disease-suppressive mechanisms. These results demonstrate that combining biocontrol strains can lead to more effective, or at least, more reliable biocontrol of fusarium wilt of radish.

Studies on biological control of Fusarium wilts caused by formae speciales of *Fusarium oxysporum* have a long history because available control methods are either inefficient or difficult to apply (1). Also, the concern for environmental protection renewed interest in biological control of soilborne diseases (59). Fusarium wilts particularly can be suppressed through the activity of fluorescent *Pseudomonas* spp. strains and nonpathogenic strains of *F. oxysporum*. The disease-suppressive mechanisms of these biocontrol agents include siderophore-mediated competition for iron (3,46,49), competition for substrate (6), induction of systemic resistance (12,29,44,55,56,58), and production of antibiotics (2,5). Production of chitinolytic enzymes is hypothesized to be responsible for suppression of fusarium wilt of cucumber by *Paenibacillus* sp. and *Streptomyces* sp. strains (53). Unfortunately, due to the complexity of the biotic and abiotic interactions that play a role in biological control, the practical results with biocontrol of soilborne pathogens have been variable (17). Failure of the introduction of antagonistic microorganisms seems to be due to environmental factors resulting in inadequate distribution, insufficient establishment of rhizobacterial strains, or poor expression of their antagonistic activity (50).

One possible approach to improve biological control may be the application of combinations of biocontrol agents (8). By combining microorganisms, multiple antifungal traits can be combined and one may assume that at least one biocontrol mechanism will be functional under the conditions faced by the released biocontrol agents. Moreover, combinations of biocontrol strains are expected to result in a higher level of protection (10), have reduced variability of biological control (15,16), and have potential to suppress multiple plant diseases (21). It has been demonstrated that natural suppressiveness of the Châteaurenard soil in France against Fusarium wilt is based on various mechanisms involving several microbial populations acting alone or together to limit the activity of the pathogen (1). In fact, several authors have demonstrated that combining microorganisms can result in improved plant stand (4,19). Mixtures of biocontrol agents also resulted in enhanced control of several fungal and bacterial plant diseases (20,26,38,43, 48,51,53). Dunne et al. (10) demonstrated that combining phloroglucinol-producing and proteolytic bacteria improved biocontrol of *Pythium*-mediated damping-off of sugar beet. By combining the nonpathogenic *F. oxysporum* strain Fo47 with the bacterial strain *Pseudomonas putida* WCS358, two different disease-suppressive mechanisms acted together to enhance suppression of Fusarium wilt of carnation and flax (9,31,32). In these situations, the pseudobactin siderophore of WCS358 increased the intensity of the antagonism of Fo47 against the pathogenic *F. oxysporum* by making the pathogen more sensitive to competition for carbohydrates by Fo47 (32). Sung and Chung (54) showed that a mixture of bacteria that produce chitinases and antibiotics can effectively suppress rice sheath blight caused by *Rhizoctonia solani*.

Such enhanced disease suppression may involve not only different disease-suppressive mechanisms, but can also result from interactions between the introduced strains that positively influence growth, root colonization and/or activity of the strains. On the other hand, interactions between two or more introduced biocontrol strains can also negatively influence disease control. For instance, siderophore-mediated competition for iron or competition for substrate may limit the colonization or activity of introduced biocontrol strains (24,47). De Boer et al. (7) demonstrated that *P. putida* strain RE8 inhibits growth of *P. fluorescens* strain RS111 in vitro. The combination of these two strains did not improve control of Fusarium wilt of radish, whereas the combination

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of RE8 with RS111-a, a spontaneous mutant of RS111 that was no longer inhibited in growth by RE8, did result in enhanced disease suppression.

The combination of *P. putida* strains WCS358 and RE8 was previously demonstrated to enhance suppression of Fusarium wilt of radish (7). This enhanced suppression is likely to be based on different mechanisms. The disease-suppressive mechanism of strain RE8 is as yet unknown, whereas for strain WCS358, it was demonstrated that pseudobactin production is involved (26,46). In the present study, the involvement of siderophores and induced systemic resistance in disease suppression by RE8 and WCS358 was investigated. Furthermore, the population dynamics of both strains in the rhizosphere were investigated in both the single strain and combination treatments to assess effects on root colonization.

MATERIALS AND METHODS

Microbial cultures. *P. putida* strains WCS358, RE8, and other strains and derivatives used are listed in Table 1. A pseudobactin-negative mutant of RE8 (RE8-30) was obtained by Tn*5* transposon mutagenesis according to Marugg et al. (39) using *Escherichia coli* S17-1 harboring plasmid pSUP2021. A derivative of WCS358, WCS358*pvd-inaZ*, was used to determine iron availability in the rhizosphere (9). The *pvd-inaZ* construct consists of an 8-kb *Eco*R1 fragment containing pyoverdine (*pvd*) production and uptake genes from *P. syringae* cloned 5′ to a promoterless ice nucleation gene (*inaZ*) in plasmid pVSP61 (36). The iron-regulated *pvd* promoter fused to *inaZ* in the *pvd-inaZ* construct initiates transcription of a gene encoding a ferric-pyoverdine receptor protein. *Pvd-inaZ* was introduced in WCS358 by conjugation with an *E. coli* donor strain, as described previously (34). The pathogen used was *F. oxysporum* Schlecht f. sp. *raphani* Kendrick and Snyder strain WCS600 (30). All strains were stored at –80°C in 50% glycerol.

Isolation of a pseudobactin nonproducing mutant of RE8. To perform random mutagenesis of *P. putida* strain RE8, the mobilization system of *E. coli* strain S17-1 and the suicide vector pSUP2021 carrying transposon Tn*5* were used. Cells of donor *E. coli* S17-1 harboring pSUP2021 and recipient *P. putida* strain RE8 were grown in liquid Luria-Bertani (LB) medium (42) at 37°C and liquid King's medium B (KB; 23,) at 28°C, respectively. After overnight growth to late log-phase, suspensions of both strains were mixed in an Eppendorf tube (ratio 1:1) and concentrated by centrifugation (30 s). The mating mixture was carefully resuspended in 50 µl of LB medium and spread on a Millipore filter (0.45 µm; Millipore, Etten Leur, the Netherlands) on LB-agar plates. After incubation for 24 h at 28°C, cells on the filter were suspended in 1 ml of KB medium, and 100 µl portions were spread on KB-agar plates supplemented with 50 µg of kanamycin (Km) per ml (Sigma, St. Louis, MO) and 25 µg of nalidixic acid (Nx) per ml (Sigma) to select for *Pseudomonas* cells that had acquired the transposon and to select against *E. coli*, respectively. Recombinant colonies appeared on the plates after incubation for 48 h at 30°C. Single colonies were then screened for nonfluorescence on KB-agar plates and for nonhalo formation on chrome azurol S medium (CAS) plates (52). When the bacterial colonies sequester iron from the green CAS plates, an orange halo appears around the colonies. Several transconjugant colonies that were nonfluorescent and did not produce a halo on CAS plates were checked for single transposition events by hybridization. Total DNA from Km^r mutants was isolated, digested with *Eco*RV, which has no recognition sites in Tn*5* (22), or *Sal*I, which has one recognition site in Tn*5* (22), and blotted on a positively charged nylon membrane (Boehringer Mannheim, Almere, the Netherlands). The membrane was hybridized with digoxigenin (DIG)-labeled probe against the internal *Hin*dIII fragment of Tn*5* using high stringency conditions. Hybridization was assessed using the DIG-luminescent detection kit (Boehringer Mannheim Biochemica, Almere, the Netherlands).

In vitro interactions between *Pseudomonas* strains. Bacterial suspensions were made by suspending cells cultured for 24 h at 28°C on KB-agar plates in sterile 10 mM $MgSO_4$. Densities of the bacterial suspensions were determined spectrophotometrically at 660 nm. The bacterial strains were adjusted to 10^7 CFU/ml and two droplets of 5 µl were spotted on KB-agar plates, each approximately 1.5 cm from the edge. To determine the influence of iron-regulated metabolites, such as pseudobactins, the same experiment was performed on KB agar supplemented with 200 µM $FeCl_3$. The spot-inoculated plates were incubated at 28°C for 48 h. Subsequently, a suspension of the target strain (10^7 CFU/ml) was atomized over the spot-inoculated plates, and zones of growth inhibition of the target strain around the spot-inoculated strains were measured after an additional incubation period of 24 h at 28°C.

Preparation of inoculum of the antagonists. Bacteria were grown for 24 h at 28°C on KB-agar plates, and suspensions were prepared in sterile 10 mM $MgSO_4$. The bacterial strains were introduced in an autoclaved (2 × 20 min with a 24 h interval) potting soil-sand mixture (46) to a density of approximately 7×10^6 CFU/g of potting soil-sand mixture. When combinations of strains were used, each strain was introduced at a density of 7×10^6 CFU/g. Inoculum for the induced systemic resistance experiments were prepared by mixing the bacterial suspensions with talcum (1:1, vol/wt) to a final density of 5×10^7 CFU/g (29).

Preparation of inoculum of *F. oxysporum* f. sp. *raphani*. *F. oxysporum* f. sp. *raphani* was cultured in aerated 2% malt extract (Difco, Detroit, MI) medium at 22°C. After 14 days of growth, cultures were filtered through sterile glass wool to remove mycelial mats. Microconidia were harvested by centrifugation at 8,000 × *g* for 20 min, resuspended in 10 mM $MgSO_4$, and mixed through a potting soil-sand mixture (12:5, vol/vol) to a density of 3.75×10^5 CFU/g. Infested soil was incubated in polyethylene bags for 3 to 5 days at 20°C before use in the potting soil bioassays to allow colonization of the soil by the pathogen. The inoculum for the induced systemic resistance experiments was prepared as described above, except *F. oxysporum* f. sp. *raphani* was mixed through a peat-sand (1:1, vol/vol) mixture to a density of 3×10^4 CFU/g.

Suppression of Fusarium wilt in potting soil. Effects of single strains and their combinations on Fusarium wilt in radish were tested in a potting soil bioassay (7). For this bioassay, the *F. oxysporum* f. sp. *raphani*-infested soil, bacterized soil, additional autoclaved soil, and nonautoclaved river sand were mixed in order to obtain final densities of 10^4 conidia per g of soil of *F. oxysporum* f. sp. *raphani* and 10^6 CFU/g of soil of each bacterial strain. Per treatment, 9 pots (11 cm high and 14 cm in diameter) were filled with 750 g of the soil-sand mixture, in which 10 radish seeds (*Raphanus sativus* L., cv. Saxa 2*Nova; S&G Seeds B.V.,

TABLE 1. *Pseudomonas* spp. strains used in this study

Microbial culture	Characteristics	Reference
E. coli S17-1 pSUP2021	Cm^r Nm/Km^r Ap^r Mob^+ Tc::Tn*5*	22
P. fluorescens WCS417	Isolated from wheat rhizosphere	25
P. putida RE8	Isolated from radish roots, Nx^r	7
RE8-30	Pseudobactin-negative Tn*5* transposon mutant of RE8, Km^r	This study
P. putida WCS358	Isolated from potato rhizosphere	13
WCS358r	Rifampicine-resistant derivative of WCS358	14
JM213	Pseudobactin-negative Tn*5* transposon mutant of WCS358, Km^r	39
JM214	Pseudobactin-negative Tn*5* transposon mutant of WCS358, Km^r	39
JM218	Pseudobactin-negative Tn*5* transposon mutant of WCS358, Km^r	39
WCS358*pvd-inaZ*	Reporter strain WCS358, containing a *pvd-inaZ* fusion, Km^r	9

Enkhuizen, the Netherlands) were sown. The plants were grown in a climatized greenhouse at 20°C and 70% relative humidity with a photoperiod of 16 h. Plants were watered with tap water once a week, and once a week received half-strength Hoagland solution (18). In those bioassays where addition of iron was required, the solution was supplemented with 10 μM Fe-EDDHA (ethylenediaminodi(*o*-hydroxyphenyl) acetic acid). After approximately 21 days the percentage of diseased plants per pot was scored on the basis of both external wilting and internal browning symptoms (26). The experiment was conducted at least two times for all bacterial treatments.

Suppression of Fusarium wilt by induced systemic resistance. This bioassay was conducted as described by Leeman et al. (29). Radish seeds (cultivar Saxa 2*Nova) were sown in sand and after 5 days, seedlings were transferred to rock wool cubes (Rockwool/Grodan b.v. Roermond, the Netherlands), in such a way that the root system was divided over two cubes. The biocontrol bacteria were inoculated on the lower part of the root system at the root tips, and *F. oxysporum* f. sp. *raphani* was delivered in the peat and sand mixture at the part of the root system near the stem 2 days later. The populations of the biocontrol bacteria and *F. oxysporum* f. sp. *raphani* remained spatially separated throughout the experiment avoiding direct interactions between the biocontrol strains and the pathogen. The plants were grown in a climatized greenhouse as describe above. Plants were watered with deionized water and once a week received half-strength Hoagland solution with iron (Fe-EDDHA, 100 μM) (18). Twenty-eight days after transplanting, plants were scored for both external wilting and internal browning symptoms (26). The experiment was conducted two times.

Root colonization by introduced bacterial strains. In bioassays in which suppression of Fusarium wilt in potting soil was studied, colonization by strains WCS358 and RE8 was determined at two time points. When sampling plants for root colonization, the soil surrounding the root system was loosened and two root systems, randomly chosen from the 10 plants per pot, were harvested. For each treatment, six replicates were used. The samples were suspended in 10 ml of sterile 10 mM $MgSO_4$ and shaken vigorously for 30 s (Vortex shaker at maximum speed) in glass tubes containing 0.5 g of glass beads (0.56 to 0.80 mm in diameter). Aliquots (100 μl) of serial dilutions of these rhizosphere samples were mixed homogeneously with 300 μl selective KB^+ agar at 45°C (KB supplemented with 100 μg of cycloheximide per ml, 40 μg of ampicillin per ml, and 13 μg of chloramphenicol per ml) [13], and amended with 150 μg of rifampin per ml [Sigma] for WCS358r) in 24-well tissue culture plates on a reciprocal shaker to mix the sample through the not yet solidified agar. After incubation for 24 h at 28°C, the number of bacterial colonies that had developed inside the agar was determined. Numbers of strain WCS358r were determined by counting the colonies in KB^+ agar amended with rifampin under the microscope. Colonies of strain RE8 were enumerated by immunofluorescence colony staining (IFC) according to the method described by Van Vuurde (57), as modified by Leeman et al. (28). For IFC, wells were incubated with a 40-fold dilution of a fluorescein isothiocyanate-conjugated polyclonal rabbit antiserum raised against and specific for strain RE8. Colonies were counted using a Zeiss Axioskop 20 (Carl Zeiss, Inc., Oberkochen, Germany) fluorescence microscope. The experiment was conducted three times.

Effects of iron availability on pseudobactin production and ice nucleating activity (INA) of WCS358*pvd-inaZ* in culture. Erlenmeyer flasks (100 ml) with 30 ml of liquid standard succinate medium (SSM) (41), containing different concentrations of Fe-EDDHA, were inoculated in duplicate for each iron concentration with 0.3 ml of a washed suspension of *P. putida* strain WCS358 or reporter strain WCS358*pvd-inaZ* from an overnight SSM culture supplemented with 100 μM Fe-EDDHA. After 48 h of growth at 28°C on a reciprocal shaker at 250 rpm, the numbers of CFU, pseudobactin production by WCS358, and the INA of WCS358*pvd-inaZ* were determined. The numbers of cells in the cultures were determined by plating appropriate dilutions on KB-agar plates using a spiral plater (Spiral Systems, model cu, Cincinnati, OH) and counting colonies after 24 h of incubation at 28°C. Pseudobactin358 production in culture supernatants (pH 7.1) was determined spectrophotometrically using a molar extinction coefficient of 14000/M.cm (pH = 7.1) at 400 nm (45). INA was quantified by the droplet freezing assay described by Lindow (33). The number of frozen droplets was scored after 4 min at –10°C. INA was expressed as ^{10}log (ice nuclei per CFU). The experiment was conducted three times.

Assessment of INA in the rhizosphere. To determine iron availability in the rhizosphere, WCS358*pvd-inaZ* was introduced onto radish roots. Bacteria were mixed with the potting soil and sand mixture to a concentration of 10^6 CFU/g. Radish seedlings were grown for 5 days in river sand before transplanting five seedlings per pot in bacterized soil. For each treatment, 5 replicate pots were used. The seedling roots were sampled at different time intervals after planting. The root systems from each pot were transferred to a glass tube containing 10 ml of 10 mM $MgSO_4$ and glass beads. The tubes were shaken on a vortex for 30 s. After appropriate dilution of the suspension, the INA was determined and related to the number of CFU, determined in 24-microwell plates with KB^+ amended with 50 μg of kanamycin per ml. The experiment was conducted two times.

Data analysis Rhizosphere populations of introduced bacterial strains approximate a log normal distribution along the root system (37). Therefore, the numbers of CFU were transformed to ^{10}log (CFU + 1) prior to statistical analysis (analysis of variance [ANOVA] followed by Fisher's least significant difference [LSD] test).

TABLE 2. Population densities on radish roots of *P. putida* strains WCS358r and RE8 when applied alone or in combination at 13 and 25 days after sowing

	Population density (^{10}log CFU/g of root)[z]			
	13 days		25 days	
Strains	Single	Combination	Single	Combination
WCS358r	6.22 b	5.91 a	6.08 b	5.59 a
RE8	6.14 b	6.47 c	6.37 c	6.31 c

[z] Means at each time point followed by different letters are significantly different according to the protected least significant difference test at P = 0.05 using the analysis of variance model in SPSS.

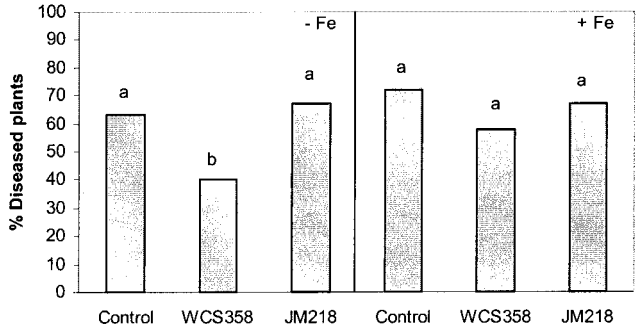

Fig. 1. Suppression of Fusarium wilt of radish in potting soil by *P. putida* strain WCS358 or its pseudobactin-negative mutant JM218. Strains were mixed through soil to a final density of 10^6 CFU/g of soil. The pathogen was mixed through soil to a final density of 10^4 conidia per g of soil. After mixing bacterized soil, *Fusarium*-inoculated soil and sand, radish seeds were sown. In the treatment with Fe (+Fe), plants received nutrient solution amended with Fe-EDDHA (10 μM). Disease was scored 21 days after sowing. Bars with the same letter are not significantly different at $P \leq 0.05$; according to analysis of variance followed by Fisher's least significant difference test. Control treatment is Fusarium alone.

Percentages of diseased plants of two bioassays were pooled after establishing that there was no significant interaction at $P = 0.05$ between experiments and treatments and variances were homogeneous. The pooled data were tested for significance using ANOVA followed by Fisher's LSD test ($P = 0.05$), using SPSS-software (version 10.0 for windows; SPSS Inc., Chicago, IL).

RESULTS

Selection of a pseudobactin negative mutant of RE8. Of 300 transconjugants of RE8, 18 colonies produced no halo or a smaller halo on CAS medium as compared to the wild-type strain. One of these that did not fluoresce on KB and did not produce a halo on CAS medium was used for further study and labeled strain RE8-30. Northern blot analysis revealed that RE8-30 contained a single transposon insertion.

In vitro and in vivo interactions between *P. putida* strains. Interactions between *P. putida* strains WCS358 and RE8 and the pseudobactin-negative mutants of WCS358 (JM218) and RE8 (RE8-30) were studied on KB agar without and with $FeCl_3$ (200 μM). Strain WCS358 inhibited growth of strains RE8 and RE8-30 only on unamended plates. Neither RE8 nor the pseudobactin-negative mutant JM218 inhibited the growth of other strains tested regardless of the iron availability in the medium.

To investigate whether an inhibitory effect of WCS358 on growth of RE8 occurred in the rhizosphere, population densities on radish roots were determined upon application of either the single strains or their combination (Table 2). Surprisingly, the population density of RE8 when combined with WCS358r was significantly higher at 13 days after sowing as compared to the single inoculation. After 25 days, there was no difference for RE8 populations between the single and combination treatments. In two independent bioassays, the population density of WCS358r when combined with RE8 decreased significantly compared to the single strain application. Whereas the population density upon single application was maintained at over 10^6 CFU/g of root, it was decreased in the combination treatment by a factor 2 or 3 at 13 and 25 days, respectively (Table 2).

Disease suppression by WCS358. When no iron was added to the Hoagland nutrient solution for the plants, WCS358 was able to suppress the percentage of diseased plants significantly (Fig. 1). Under these conditions, the pseudobactin-negative mutant JM218 did not suppress disease. When iron was added to the Hoagland solution, WCS358 and JM218 no longer suppressed disease significantly (Fig.1).

In some bioassays, pseudobactin mutants of WCS358 suppressed disease as well as the wild-type strain. In six of 12 bioassays, WCS358 suppressed disease significantly, whereas the pseudobactin mutant did not. In two bioassays, no disease suppression occurred by the wild type or the mutant. Surprisingly, in four bioassays, both WCS358 and the mutants were able to significantly suppress disease to the same extent. In two of these bioassays, different pseudobactin-negative mutants of WCS358 (JM218, JM213, JM214) were tested and all suppressed disease to the same extent as wild-type WCS358 (Fig. 2). Thus, under certain so far unidentified circumstances, pseudobactin-negative mutants of WCS358 can also suppress Fusarium wilt.

It was considered that in the two bioassays in which WCS358 did not significantly suppress disease, the potting soil used might have been relatively high in bio-available iron content, repressing the production of pseudobactin by WCS358. To estimate iron availability for WCS358 in the rhizosphere of radish plants grown in this soil, the reporter strain WCS358*pvd-inaZ* (35) was used. A standard curve of INA as a function of available Fe was determined by growing the reporter strain in liquid medium supplemented with different concentrations of Fe-EDDHA. After 48 h, the presence of Fe-EDDHA did not have a clear effect on bacterial growth, but pseudobactin production decreased with increasing Fe-EDDHA concentration and no production occurred at concentrations of 10.0 μM and higher. In general, INA decreased with increasing Fe-EDDHA concentration. However, the INA in flasks supplemented with 0.1 to 5.0 μM Fe-EDDHA was higher than in control flasks where no iron was added. Above 10.0 μM, INA declined substantially. Thus, at 5.0 μM Fe-EDDHA the INA was still high, whereas pseudobactin production had already decreased (data not shown).

To investigate if the conditions in the radish rhizosphere would favor the production of pseudobactin, the INA of WCS358*pvd-inaZ* was determined in the rhizosphere of plants grown in soil (Fig. 3). The INA was monitored during the first 4 days, as well as at 10 days after planting. In the first 24 h period, the INA level increased 20-fold, after which it decreased again to the same level and remained at that level at 11 days. All values after 1 and 10 days correspond to an in vitro Fe-EDDHA concentration between 5.0 and 10.0 μM. At these concentrations, WCS358 produces little or no pseudobactin. In the first 3 days, the population density of the reporter strain (Fig. 3), as well as that of wild-type strain WCS358 (data not shown), increased approximately 40-fold, after which they decreased again.

Disease suppression by RE8. The disease-suppressive mechanism of RE8 was also investigated. The pseudobactin-negative mutant of RE8 (RE8-30) was tested for its ability to suppress Fusarium wilt. In eight independent bioassays, treatment with RE8-30 always resulted in the same percentage of diseased plants compared to the parental strain RE8. In six of these eight bioassays, both strains suppressed disease (data not shown). Strain RE8 was also tested for induction of systemic resistance in the bioassay described by Leeman et al. (29). In this bioassay, the pathogen and the biocontrol bacterium remained at spatially separated sites on the roots. As negative and positive controls, *P.*

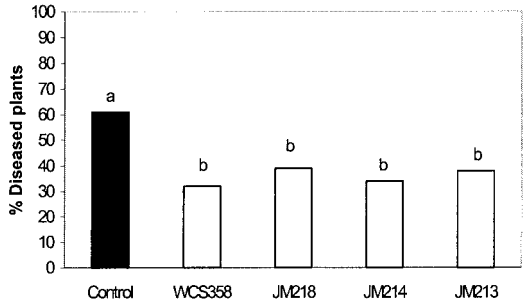

Fig. 2. Suppression of Fusarium wilt of radish in potting soil by *P. putida* strain WCS358 or its pseudobactin-negative mutants JM218, JM214, or JM213. Details as described in the legend to Figure 1. Control treatment is Fusarium alone.

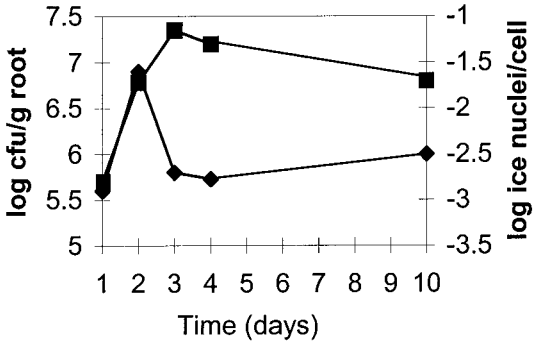

Fig. 3. Ice nucleating activity (^{10}log ice nuclei/cell) (◆) and rhizosphere populations (^{10}log CFU/g root) (■) of WCS358 containing *pvd-inaZ*. Five-day-old radish seedlings were planted in soil mixed with WCS358*pvd-inaZ* (10^6 CFU/g of soil). The numbers of culturable cells and ice nuclei were quantified at 0, 1, 2, 3, and 10 days after planting.

putida WCS358 and *P. fluorescens* WCS417, respectively, were included (29). In this bioassay, both RE8 and WCS417 suppressed disease, whereas WCS358 was ineffective (data not shown).

Disease suppression by the combination of WCS358 and RE8. Disease suppression by the combination of strains WCS358 and RE8 was investigated in the potting soil bioassay. When both single strains suppressed disease in this bioassay, the combination resulted in significantly enhanced disease suppression as compared to the single strains (Fig. 4A). However, when only WCS358 suppressed disease and strain RE8 failed, the combination of RE8 and WCS358 resulted in a disease suppression comparable to that caused by WCS358 alone (Fig. 4B). Conversely, when strain WCS358 was ineffective in lowering the percentage of diseased plants compared to the control (Fig. 4C), but RE8 did reduce disease significantly, the combination resulted in disease suppression comparable to that by the effective RE8.

To check whether the pseudobactin produced by WCS358 is important in the enhanced disease suppression by the combination of WCS358 with RE8, disease suppression by RE8, WCS358, JM218, the combination of WCS358 and RE8, and the combination of JM218 and RE8 were tested. In two bioassays, all single applications including JM218 resulted in a significantly lower percentage of diseased plants. Figure 5 shows a representative experiment. Both combinations resulted in enhanced disease suppression, be it at the border of significance for the combination of WCS358 with RE8.

DISCUSSION

In several bioassays, strain WCS358 was able to suppress Fusarium wilt of radish by effectively competing for iron. The pseudobactin-negative mutant JM218 did not suppress disease, and addition of Fe-EDDHA to the nutrient solution for the plants diminished the disease-suppressive effect of WCS358 (Fig. 1). The involvement of pseudobactin in Fusarium wilt control was demonstrated previously (26,46). Surprisingly, in some bioassays pseudobactin-negative mutants of WCS358 suppressed disease to the same extent as the parental strain (Figs. 2 and 5). It is not clear by which disease-suppressive mechanisms these pseudobactin-negative mutants are able to suppress disease. Unlike for example, *P. fluorescens* strain WCS374 that produces the siderophore pseudomonine in addition to the fluorescent pseudobactin type siderophore (40), WCS358 only produces pseudobactin (39). Induction of systemic resistance can be excluded because WCS358 did not induce systemic resistance nor can its pseudobactin-negative mutant (27). Antibiotic compounds active against *Fusarium* spp. have been identified in in vitro antagonism assays (2,5), but for WCS358 none has been found (45). In vitro antagonism against *F. oxysporum* f. sp. *raphani* could be fully explained by pseudobactin-mediated competition for iron (45). However, it cannot be excluded that WCS358 and/or its pseudobactin-negative mutants produce antibiotic compounds in the rhizosphere. Chitinolytic enzymes, such as were recently demonstrated to be involved in suppression of Fusarium wilt of cucumber (53), are not produced by WCS358 on chitin containing KB medium (J. Folders, *personal communication*). Competition for substrate can be involved in interactions between pseudomonads and pathogenic *Fusarium* strains (11), as was demonstrated also for antagonism between a nonpathogenic and a pathogenic *Fusarium* strain (6,32).

Disease suppression by *P. putida* strain RE8 does not depend on the pseudobactin produced by this strain because the pseudobactin-negative mutant of this strain (RE8-30) suppressed disease to a comparable level as the parental strain. Instead, strain RE8 is able to induce systemic resistance.

When the combination of WCS358 and RE8 was mixed through soil, it resulted in enhanced disease suppression compared to treatments with the single strains (Fig. 4A). This enhanced disease suppression could be the result of pseudobactin-mediated competition for iron combined with induced systemic resistance. However, when JM218 was combined with RE8, those experiments also resulted in enhanced disease suppression, indicating that the pseudobactin of WCS358 does not necessarily play a role in the enhanced disease control by the combination (Fig. 5).

Improved disease control by the combination of WCS358 and RE8 occurred only when each strain by itself was effective in reducing disease. The reason why sometimes treatments with either strain did not result in disease suppression remains unclear. It is unlikely to be due to insufficient root colonization. The population density of WCS358 in the bioassay where it did not suppress disease (Fig. 4C) was 6.3×10^5 CFU/g root at 10 and 21 days after sowing. These population densities are comparable to population densities demonstrated to result in disease suppression (46). Although population densities of RE8 were not measured in the experiment depicted in Figure 4B, results from other bioassays confirmed that in spite of high densities of RE8 reached in the rhizosphere, disease suppression does not always occur. Despite the fact that the combination of strains WCS358 and RE8 resulted in improved and more consistent biocontrol, strain RE8 negatively influenced root colonization by WCS358. When both strains were coinoculated in soil, rhizosphere populations of WCS358 were significantly lower compared to when WCS358 was inoculated alone. While the population of RE8 in the rhizosphere in the combined application was significantly higher after 13 days, after 25 days it was comparable to the single treatment (Table 2). The pseudobactin siderophore of strain WCS358 inhibits in vitro growth of RE8, whereas RE8 does not affect growth of WCS358. Apparently, in these experiments competition for iron in the rhizosphere between WCS358 and RE8 was of no significance for the population densities of both strains. In contrast, in similar experiments when WCS358 was coinoculated with *P. fluorescens* strain WCS374, the latter strain maintained its population density

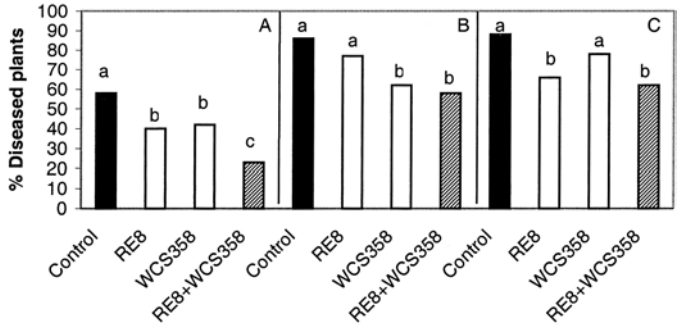

Fig. 4. Suppression of Fusarium wilt of radish by *P. putida* strains RE8, WCS358, and their combination in three independent bioassays showing varying results. Details as described in the legend to Figure 1. Control treatment is Fusarium alone.

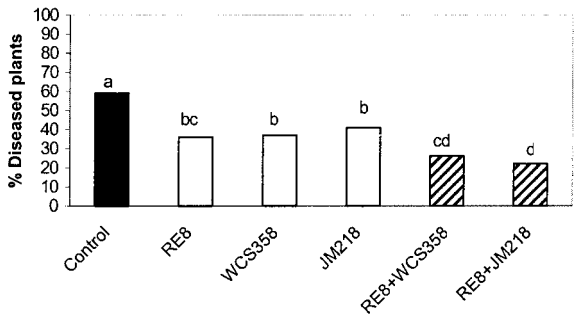

Fig. 5. Suppression of Fusarium wilt of radish by *P. putida* strains RE8, WCS358, JM218, and their combinations. Details as described in the legend to Figure 1. Control treatment is Fusarium alone.

in the rhizosphere only when transformed with the pseudobactin receptor PupA for uptake of ferric pseudobactin 358 (47).

The INA values determined in the rhizosphere (Fig. 3) indicate that the iron availability in the soil used was at the border of iron sufficiency. Therefore, it is likely that at most, only limited competition for iron occurred between strains WCS358 and RE8. It is also possible that WCS358 and RE8 colonize different niches. This stresses again that in vitro observations cannot be generalized and extrapolated to in vivo conditions, as evidenced also by De Boer et al. (7). The population densities of WCS358 that developed in the presence of RE8 were still above the threshold level required for disease suppression (46). This can explain the enhanced effect on disease suppression by the combination.

The improved biocontrol by the combination of WCS358 and RE8 appears to result from combining different disease-suppressive mechanisms. In all bioassays performed, at least one strain suppressed disease, and the combination always resulted in disease suppression. Enhanced suppression of take-all of wheat by a consortium of a nonpathogenic isolate of *Gaeumannomyces graminis* var. *graminis* and a mixture of pseudomonads was reported to be the result of combining different disease-suppressive properties. Here, direct competition for substrates and favored sites in and on the roots by *G. graminis* was combined with antibiotic production by the pseudomonads (8). Dunne et al. (10) similarly demonstrated that combining proteolytic and phloroglucinol-producing bacteria can improve biocontrol of *Pythium*-mediated damping-off of sugar beet. The improved control of Fusarium wilt of carnation by the combination of the nonpathogenic *F. oxysporum* strain Fo47 with *P. putida* WCS358 was the indirect effect of competition for iron that made the pathogenic *F. oxysporum* more sensitive to competition for substrate with the nonpathogenic strain (31,32). Suppression of *Botrytis cinerea* on strawberry was improved by combining the biocontrol agents *Pichia guilermondii* and *Bacillus mycoides* (15). In this case, the yeast effectively competed with *B. cinerea* for nutrients, whereas the bacterium secreted inhibitory compounds and activated the defense systems of the host (16).

Combining biocontrol agents is not only advantageous because it can lead to enhanced levels of disease suppression, it is also advantageous when an application of one of the single strains failed to suppress disease, because the combination still resulted in disease control (Fig. 4B and C). The reason why application of single strains does not always control disease might be related to insufficient root colonization. However, in the present bioassays strains colonized the roots to a similar level in bioassays that did, and in bioassays that did not result in disease suppression. It can be concluded that *P. putida* strains WCS358 and RE8 have different disease-suppressive mechanisms: pseudobactin-mediated competition for iron and/or another yet unknown disease suppressive trait for WCS358, and induction of systemic resistance for RE8. Combining these mechanisms by applying a mixture of the biocontrol strains leads to more effective, or at least more reliable, biocontrol of Fusarium wilt of radish.

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