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Microbial Antagonism at the Root Level Is Involved in the Suppression of Fusarium Wilt by the Combination of Nonpathogenic *Fusarium oxysporum* Fo47 and *Pseudomonas putida* WCS358

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ABSTRACT

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Two biological control agents, nonpathogenic *Fusarium oxysporum* Fo47 and *Pseudomonas putida* WCS358, were evaluated for suppression of Fusarium wilt of flax grown in nutrient solution and for suppression of the population density and metabolic activity of the causal organism *F. oxysporum* f. sp. *lini* strain Foln3GUS on root surfaces. Due to the presence of an introduced *gusA* reporter gene construct in Foln3GUS, the pathogen expressed β-glucuronidase activity that was related to its carbon metabolism. At a Fo47 to Foln3GUS inoculum ratio of 100:1, both the population density of the pathogen and the β-glucuronidase activity on and in flax roots were reduced by the nonpathogenic strain, and Fusarium wilt was suppressed. At a Fo47 to Foln3GUS inoculum ratio of 10:1, Fo47 decreased the severity of Fusarium wilt to a smaller extent and it also reduced β-glucuronidase activity without reducing the density of Foln3GUS on flax roots. At a nonpathogenic to pathogenic Fusarium strains ratio of 10:1, the addition of *P. putida* WCS358 further suppressed Fusarium wilt and the density of the pathogen at the root level, whereas a mutant of WCS358 deficient in pseudobactin production had no significant effect. Iron availability to WCS358 on flax roots, assessed by ice-nucleation activity conferred from a transcriptional fusion (*pvd-inaZ*) of an ice-nucleation reporter gene to an iron-regulated promoter, was sufficiently low to allow pseudobactin production. *P. putida* WCS358 did not reduce the severity of Fusarium wilt of flax when inoculated without Fo47, and it did not improve disease suppression achieved by high inoculum doses of Fo47 (a Fo47 to Foln3GUS ratio of 100:1). Together, these data provide evidence that (i) suppression of Fusarium wilt of flax by Fo47 is related to reductions in the population density and metabolic activity of the pathogen on the root surface; (ii) WCS358 can enhance the biological control activity of Fo47, but this enhancement depends on the population of Fo47 relative to the pathogen; and (iii) pseudobactin contributes to suppression of Fusarium wilt by the combination of Fo47 and WCS358 on roots in which conditions are conducive to pseudobactin production by the bacterium.

Natural suppressiveness of some soils to Fusarium wilts has been associated with resident populations of fluorescent pseudomonads and nonpathogenic *Fusarium oxysporum* (17,34). Some strains of nonpathogenic *F. oxysporum* and fluorescent pseudomonads can suppress Fusarium wilt when applied to roots or seeds of various crop plants (3,9,10,22), but biological control by individual strains can be inconsistent (35,37). Recognizing that natural suppressiveness is not caused by a single microbial population, several workers have tested combinations of nonpathogenic *F. oxysporum* and fluorescent pseudomonads for suppression of Fusarium wilt. Certain strains of fluorescent *Pseudomonas* spp. can enhance the biological control of Fusarium wilt achieved by nonpathogenic *F. oxysporum* (4,19,21,23,33).

Natural suppressiveness to Fusarium wilts has been related to reduced saprophytic growth of pathogenic *F. oxysporum* (20). The conjoint activities of nonpathogenic *F. oxysporum* and fluorescent *Pseudomonas* spp. in suppressing Fusarium wilt could be caused by reducing saprophytic growth of the pathogen through carbon and iron competition. This hypothesis was tested previously with a combination of *P. putida* WCS358 and nonpathogenic *F. oxysporum* Fo47, which suppresses Fusarium wilt of carnation more effectively than either antagonistic microorganism inoculated alone (23). Enhanced disease suppression by the microbial combination was related to the production by *P. putida* WCS358 of the siderophore pseudobactin (synonym: pyoverdine) (23). Strain WCS358 enhanced biological control by Fo47, whereas a Sid$^-$ mutant of WCS358, deficient in pyoverdine production, did not enhance biocontrol by Fo47 (23). Both purified pyoverdine and nonpathogenic *F. oxysporum* Fo47 reduced mycelial growth of a pathogenic *F. oxysporum* due to iron and carbon competition, respectively (24).

The aim of the current study was to evaluate at the root level the role of direct antagonism in suppression of Fusarium wilt of flax by Fo47 and WCS358, inoculated individually and in combination. The effect of the biocontrol agents on the density and activity of a *gusA*-marked derivative of the pathogen was evaluated on roots of flax grown in solution culture. Ice-nucleation activity (INA) expressed by WCS358*pvd-inaZ*, which contains an ice-nucleation reporter gene *inaZ* under the control of an iron-regulated promoter involved in pyoverdine synthesis, was assessed to determine if iron availability on flax roots was sufficiently low for pyoverdine production by *P. putida*.

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MATERIALS AND METHODS

Microorganisms and culture conditions. Nonpathogenic *F. oxysporum* Fo47 was isolated previously from the Châteaurenard soil

(France) naturally suppressive to Fusarium wilt; the efficacy of Fo47 in reducing the severity of Fusarium wilt has been demonstrated (2,13,21). Foln3 is a virulent strain of *F. oxysporum* f. sp. *lini.* Strain Foln3GUS is a derivative of Foln3 containing a fusion of a promoterless *gusA* reporter gene to a promoter of *gpd*, the gene encoding glyceraldehyde-3-phosphate dehydrogenase (GPD) from *Aspergillus nidulans*. GPD catalyzes the second step of glycolysis, and the activity of the *gpd* promoter relates to the carbon metabolism of the fungus. Therefore, β-glucuronidase activity conferred from the *gusA* construct in Foln3GUS indicates the carbon metabolism of the transformed strain (8,12). The pathogenicity of Foln3GUS does not differ from that of the wild-type strain (8). *F. oxysporum* Fo47 and Foln3GUS are single-spore isolates; they were cryopreserved by freezing conidial suspensions at –80°C in 50% glycerol. *F. oxysporum* strains were grown in malt extract liquid medium (10 g $liter^{-1}$) at 25°C. After 7 days of growth, cultures were filtered through glass wool to remove mycelial mats. Microconidia left in the filtrate were pelleted by centrifugation (5,000 × *g*, 10 min) (model GR20-22; Jouan, Saint-Herblain, France) and rinsed three times in 0.01 M $MgSO_4$. The conidial densities of suspensions were determined by direct observation on a haemocytometer and adjusted by dilution.

P. putida WCS358 was isolated from the rhizosphere of potato plants (14) and is suppressive to Fusarium wilt (9,10,19,23). Strain JM218 is a siderophore-negative (Sid^-) mutant of WCS358, which was obtained following Tn*5* mutagenesis (5,27). Strain WCS358*pvd-inaZ* is a derivative of the strain WCS358 containing a transcriptional fusion (*pvd-inaZ*) of the promoterless *inaZ* gene, which confers INA, cloned downstream of an 8-kilobase (kb) *Eco*R1 fragment involved in pyoverdine (*pvd*) production and uptake by *P. syringae* (25). Cultures of bacterial cells, grown in Luria-Bertani broth medium (30), either supplemented with kanamycin (200 mg $liter^{-1}$) for strain JM218 or not supplemented for strain WCS358, were stored at –80°C in 50% glycerol. Bacterial inoculants were produced on King's medium B agar (KB) plates (16) at 25°C for 48 h. Bacteria were scraped from the medium and suspended in 0.01 M $MgSO_4$, pelleted by centrifugation (5,000 × *g*, 10 min), and washed three times. The bacterial densities of the suspensions were measured by direct observation on a haemocytometer and adjusted by dilution.

Treatments and plant growth conditions. Flax seeds (*Linum usitatissimum* L., cv. Opaline) were sterilized in a 1.25% solution of NaOCl for 20 min and pregerminated for 30 h on malt agar medium at 25°C. Two germinated seeds were placed on a wire netting floating at the surface of a nutrient solution (30 ml) in a glass tube (200 × 22 mm) and grown for 2 days before microbial inoculation. The nutrient solution consisted of a 1,000-fold dilution of a commercial nutrient stock solution (Hydrokani AO; Hydro Agri, Saint Brice Courcelle, France) to which $Ca(NO_3)_2 \cdot 4H_2O$ (0.65 mM) and 2 μM 50% ferrated form of ethylenediamine-di-(*o*-hydroxyphenylacetic acid) (EDDHA) (Sigma Chemical Co., St. Louis) were added separately. This nutrient solution was buffered with 100 mM *N*-2-hydroxyethylpiperazine-*N′*-2-ethanesulfonic acid (HEPES) (pH 7) Sigma Chemical Co.), filter-sterilized, and aerated with filter-sterilized air.

Inoculation was performed by dipping the roots, located on the wire netting, in the appropriate microbial suspension for 1 h. All plants were infested with a conidial suspension of Foln3GUS (10^3 conidia per ml). Control plants were only inoculated with Foln3GUS. Protected plants were also simultaneously inoculated with Fo47, WCS358, or JM218. The biocontrol agents were inoculated, either in combination or separately, together with the pathogen. The density of the bacterial suspensions used for the inoculation was 10^5 cells per ml. To obtain different inoculum ratios of Fo47 to Foln3GUS (10:1 and 100:1), two different densities of Fo47 were used for inoculation (10^4 and 10^5 conidia per ml). Plants were grown in a growth cabinet, previously sterilized with a 12% solution of NaOCl on a cycle of 16-h light (25°C) and 8-h dark (23°C), and harvested 10 days after microbial inoculation. Disease severity was assessed by recording the number of wilted leaves on two plants from each of nine replicates. This way of measuring the disease severity was chosen in order to record early manifestation of the symptoms because the gnotobiotic conditions only allow a short period of plant growth. The results were expressed as the percentage of wilted leaves.

Root colonization by the microorganisms. Root colonization by *P. putida* and *F. oxysporum* was evaluated by counting the number of CFU from four replicates of two plants each. From each plant, 50 mg of fresh root tissue were placed in a sterile glass tube containing 5 ml of 0.01 M $MgSO_4$ and 1 g of glass beads (0.18 mm in diameter). Tubes were shaken for 30 s on a vortex mixer (Scientific Industries Inc., Bohemia, NY). A sample (100 μl) from each appropriate dilution was plated on KB (14) to evaluate the population size of *P. putida*, and on Komada (18) to evaluate the population size of *F. oxysporum.* Dilutions also were plated on Bacto peptone agar (Difco Laboratories, Detroit) in order to check the gnotobiotic conditions. The CFU were counted after incubation of plates for 48 h at 27°C. Colonies of Foln3GUS and Fo47 on the same agar plate were distinguished by the β-glucuronidase activity of Foln3GUS. Plates were sprayed with a 0.1% solution of 5-bromo-4-chloro-3-indolyl-β-glucoronide (X-gluc) (Sigma Chemical Co.) in phosphate buffer (0.1 M, pH 7) and incubated for one more day. The blue-stained colonies, indicating β-glucuronidase activity, were identified as Foln3GUS.

β-glucuronidase activity of pathogenic *F. oxysporum* Foln3GUS. The β-glucuronidase activity of *F. oxysporum* Foln3GUS was measured on root extracts using a fluorimetric assay (14). Per treatment, four replicates, each consisting of the whole root system from two plants, were frozen in liquid nitrogen, ground with a pestle in a chilled mortar, and diluted 10-fold (wt/vol) in an extraction buffer (0.1 M phosphate buffer, pH 6.8; 0.5 mM β-mercaptoethanol; 0.5 mM EDTA, and 171 mM NaCl). The root extracts were homogenized (2 min) with a high-speed homogenizer (Ultra-turax T8; IKA, Staufen, Germany), centrifuged (5,000 × *g*, 10 min), and the supernatants stored at –80°C.

Extraction buffer (450 μl), supplemented with 0.44 mg of 4-methylumbelliferyl glucuronide (MUG) per ml (Sigma Chemical Co.), was added to 50 μl of each root extract. After 90 min of incubation at 37°C in the dark, 200 μl was removed and the reaction was stopped by adding 1.8 ml of 0.2 M Na_2CO_3. Fluorescence of the samples, resulting from the catalysis of MUG to 7-hydroxy-4-methylcoumarin (MU), was measured with a fluorescence spectrophotometer (model SFM 25; Kontron, Montigny Le Bretonneux, France) at 455 nm after excitation at a wavelength of 365 nm. MU concentrations were normalized for the density of Foln3GUS and results were expressed as micromolar MU per log (CFU). For each replicate, results are the mean of three assays.

Expression of an iron-regulated promoter by WCS358. INA of WCS358*pvd-inaZ* was assessed to determine whether the conditions on flax roots were favorable to the expression of pyoverdine biosynthesis and uptake genes by *P. putida* WCS358. The *pvd-inaZ* construct (25) consisted of an 8-kb *Eco*RI fragment containing pyoverdine biosynthesis and uptake genes from *P. syringae* cloned 5′ to a promoterless ice-nucleation gene (*inaZ*) in pVSP61, a derivative of plasmid pVS1. pVS1 is stably maintained at six to eight copies per cell in *Pseudomonas* spp. (15). *pvd-inaZ* was introduced into WCS358 by conjugation with an *Escherichia coli* donor strain, as described previously (25).

INA expressed by WCS358*pvd-inaZ* on flax roots was measured from four replicates, each comprised of two root systems inoculated with either WCS358*pvd-inaZ* or WCS358. The whole root systems (about 150 mg fresh weight) were shaken with a vortex mixer (Scientific Industries Inc.) in 5 ml of 0.01 M $MgSO_4$ with 0.5 g of glass beads (0.18 mm in diameter) for 30 s. The number of ice nuclei in the resulting suspensions was estimated by the droplet freezing assay (25). Bacterial population

densities were determined by enumerating CFU as described before. INA of the samples was expressed as log (ice nuclei per cell) (26).

To ensure that the *pvd-inaZ* fusion did not alter pyoverdine production, pyoverdine concentrations in cultures of WCS358*pvd-inaZ* and WCS358 were compared. The strains were grown in the previously described nutrient solution supplemented with EDDHA (2 μM, chelated with iron at 0, 50, or 100%), sucrose (1%), casamino acids (1%), and HEPES buffer (100 mM, pH 7). Each experimental treatment was replicated in three different Erlenmeyer flasks. After incubation for 24 h at 25°C, cell densities were assessed by measuring the absorbance of the suspensions at 600 nm. Pyoverdine concentration was evaluated by measuring the absorbance of the supernatant at 400 nm, after centrifugation and adjustment of the pH to 7, according to the method described by Meyer and Abdallah (29). Absorbance measurements were performed three times for each flask. Pyoverdine concentration was normalized for the density of bacterial cells, and results were expressed as absorbance 400 nm/absorbance 600 nm.

In a culture medium, the relationship of pyoverdine production to INA expressed by WCS358*pvd-inaZ* was determined. WCS358-*pvd-inaZ* was grown for 48 h at 25°C with shaking (200 rpm) in RSM, a medium designed to approximate the nutritional composition of the rhizosphere (6). RSM was amended with various concentrations of ferric citrate (10^{-4} to 10^{-6} M) to increase iron availability. WCS358 and WCS358*pvd-inaZ* cultured in RSM containing $>10^{-4.3}$ M ferric citrate produced a noncharacterized pigment that absorbed at 400 nm, the wavelength commonly used to detect pyoverdines in culture supernatants. Therefore, concentrations of pyoverdine produced by WCS358*pvd-inaZ* in culture media varying in iron content were determined by high-performance liquid chromatography (HPLC) (model 2690; Waters Corporation, Milford, MA), using conditions similar to those described by Nowak-Thompson and Gould (31). Ferric citrate was added to culture supernatants to a final concentration of 10^{-3} M, and the mixture was incubated for 60 min. Solids were removed by centrifugation, and 100 μl of the supernatant was submitted to HPLC analysis. The column was a Nova-Pak C18 radial compression cartridge (2.5 × 10 cm, 6 μm particle size) (Waters Corporation) eluted with 90% 20 mM NH_4HCO_3/10% MeOH, pH 6.5, at a flow rate of 1.5 ml min^{-1}. Several peaks that absorbed at 400 nm were detected following HPLC analysis of culture supernatants of WCS358, and such peaks typically represent multiple pyoverdines or breakdown products of pyoverdines that are commonly detected in cultures of *Pseudomonas* spp. (1). Therefore, we used the following procedure to estimate the pyoverdine concentration in cultures of WCS358 varying in iron content. Pyoverdine concentrations in cultures grown in RSM containing 10^{-6} M ferric citrate (an iron concentration in which the interfering purple compound was not detected) were estimated from the absorbance of crude supernatants, using the published extinction coefficient of 1.9×10^{-4} mol^{-1} cm^{-1} (1). A ratio relating absorbance of the crude supernatant to the area under the major HPLC peak absorbing at 400 nm was determined for cultures grown in RSM containing 10^{-6} M ferric citrate. The retention time of this peak (10.5 ± 0.3 min) corresponded to the major peak observed from HPLC analysis of a sample of authentic pyoverdine isolated from WCS358. Pyoverdine concentrations present in supernatants of cultures grown in media with varying ferric citrate concentrations were calculated as the product of that ratio and the area under the peak with a retention time of 10.5 ± 0.3 min from each culture.

Statistical analysis. Populations sizes were logarithmically transformed before analysis. The percentages of wilted leaves, indicating the disease severity, were submitted to angular transformation before analysis. Nontransformed and transformed values were submitted to analysis of variance and then Fisher's least significant difference test. All experiments have been duplicated, and the results shown represent one representative experiment.

RESULTS

Population densities of antagonistic microorganisms. At Fo47 to Foln3GUS inoculum ratios of 10:1 and 100:1, the densities of Fo47 10 days after inoculation were 4.9×10^6 and 1.3×10^7 CFU per g of fresh root, respectively. These population sizes were significantly different, indicating that differences in inoculum dose influenced populations established by Fo47 on flax roots for at least 10 days. The presence of *P. putida* WCS358 or JM218 did

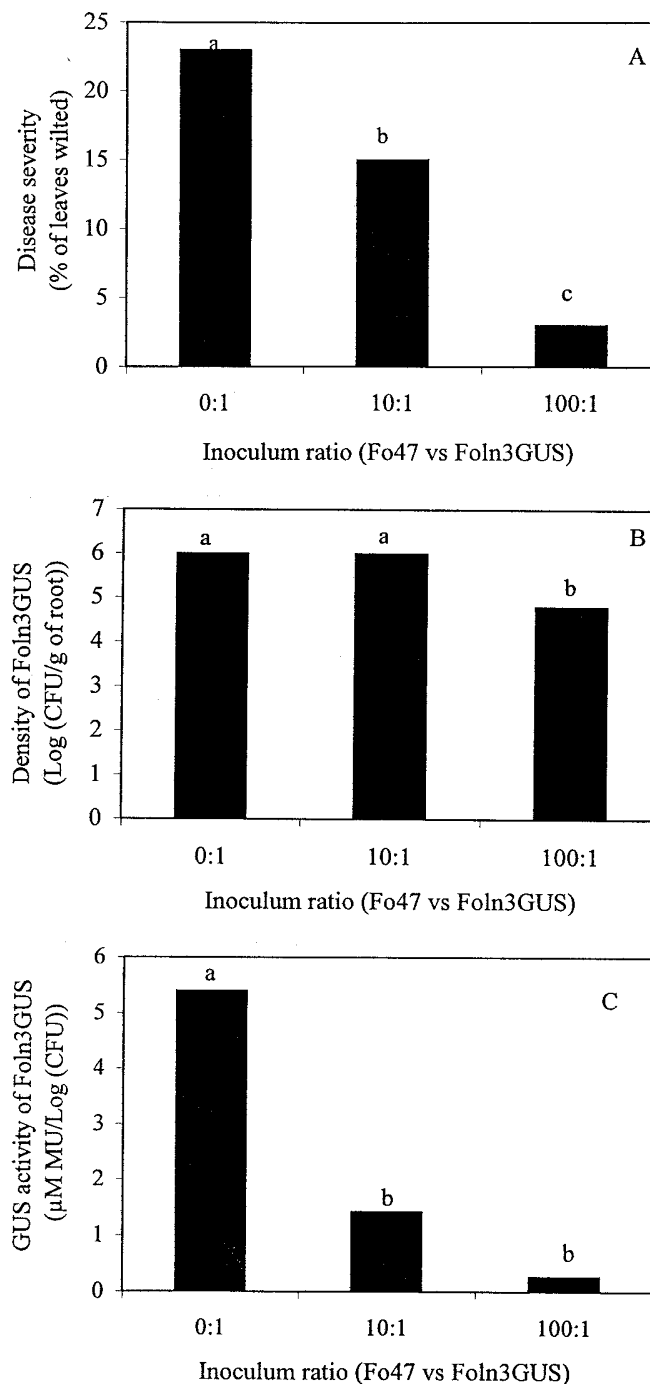

Fig. 1. Effect of inoculum ratios of nonpathogenic *Fusarium oxysporum* Fo47 to pathogenic *F. oxysporum* Foln3GUS on **A,** Fusarium wilt severity (percent wilted leaves); **B,** population size (log [CFU/g of root]); and **C,** β-glucuronidase activity (μM MU/log [CFU]) of Foln3GUS. Means designated with the same letter are not significantly different ($P \leq 0.05$) according to Fisher's least significant difference test. Statistical analyses were performed on transformed values both for population size and disease severity.

not significantly modify population densities of Fo47 on flax roots ($P \le 0.05$) (data not shown).

The influence of pyoverdine production on the population densities of *P. putida* on flax roots was assessed by comparing the densities of the wild-type strain WCS358 with those of mutant JM218. At 10 days after their inoculation, the densities of the bacterial strains WCS358 and JM218 in the absence of the nonpathogenic Fo47 (8.9×10^8 and 1.7×10^9 CFU per g of fresh root, respectively) were not significantly different ($P \le 0.05$). The population sizes of WCS358 and mutant JM218 were not significantly different when inoculated in the presence and in the absence of Fo47 (data not shown).

Effect of nonpathogenic *F. oxysporum* and *P. putida* strains on the severity of Fusarium wilt. Nonpathogenic *F. oxysporum* Fo47 reduced significantly the severity of Fusarium wilt of flax (Fig. 1). There was an improvement in disease suppression achieved by Fo47 when the Fo47 to Foln3GUS inoculum ratio increased from 10:1 to 100:1. To determine the involvement of pyoverdine production in the suppression of Fusarium wilt, bacterial strains WCS358 and JM218 were compared for their ability to suppress Fusarium wilt in the absence or presence of nonpathogenic *F. oxysporum* Fo47. Neither of the bacterial strains inoculated simultaneously with the pathogen (Fo47 to Foln3GUS inoculum ratio equal to 0:1) significantly reduced disease severity (Fig. 2). At the Fo47 to Foln3GUS inoculum ratio of 10:1, the efficiency of the biocontrol achieved by Fo47 was significantly improved by the addition of the bacterial strain WCS358, and the severity of Fusarium wilt was 41.3% lower in the presence of Fo47 + WCS358 than in the presence of Fo47 alone. An increase in disease suppression did not occur when Fo47 was coinoculated with JM218 (Fig. 2). At the highest inoculum ratio of Fo47 tested (100:1), coinoculation with WCS358 or JM218 did not improve the efficiency of control by Fo47, and coinoculation with JM218 even significantly increased the disease severity when compared with Fo47 alone. Nevertheless, the disease severity of the plants inoculated with the combination of Fo47 and the bacterial strains remained significantly lower than that of the infested control.

Effect of nonpathogenic *F. oxysporum* and *P. putida* strains on the population density of strain Foln3GUS. Nonpathogenic *F. oxysporum* Fo47 reduced significantly the densities of Foln3GUS only at the highest inoculum ratio of Fo47 to Foln3GUS (100:1) (Fig. 1). To determine the influence of pyoverdine production on the density of the pathogen, the effects of the strains WCS358 and JM218 on this density was compared in the absence or presence of nonpathogenic *F. oxysporum* Fo47. None of the bacterial strains inoculated simultaneously with the pathogen reduced significantly the pathogen density as compared with the control (Fo47 to Foln3GUS inoculum ratio equal to 0:1) (Fig. 3). At the Fo47 to Foln3GUS inoculum ratio of 10:1, the combination of Fo47 and WCS358 reduced significantly the density of Foln3GUS: the population density of Foln3GUS on flax roots was 0.7 log lower in the presence of Fo47 + WCS358 than in the presence of Fo47 alone. In contrast, decrease in the density of the pathogen did not occur when Fo47 was coinoculated with JM218 (Fig. 3). At the highest inoculum ratio (100:1), the addition of WCS358 or JM218 did not further decrease the density of the pathogen below that caused by Fo47 alone.

Effect of nonpathogenic *F. oxysporum* and *P. putida* strains on the β-glucuronidase activity of Foln3GUS. Nonpathogenic *F. oxysporum* Fo47 reduced significantly the β-glucuronidase activity associated with flax roots colonized by Foln3GUS (Fig. 1). In addition, β-glucuronidase activity was further reduced as the inoculum density of the nonpathogenic Fo47 increased from 10^4 to 10^5 conidia per ml, although nonsignificantly. The addition of either WCS358 or JM218 did not significantly modify the effect of Fo47 on β-glucuronidase activity (Fig. 4).

Transcription of an iron-regulated promoter by *P. putida* in culture and at the root level. Strains WCS358 and WCS358*pvd-inaZ* produced equivalent concentrations of pyoverdine in culture. Furthermore, the pyoverdine synthesis by both strains was reduced with the same magnitude when the iron concentration in the medium was increased (absorbance 400 nm/absorbance 600 nm = 1.5, 1.2, and 1.1 for 0-, 50- and 100%-ferrated EDDHA, respectively). Therefore, the *pvd-inaZ* construct had no detectable influence on pyoverdine production by WCS358 in culture.

INA and pyoverdine production by WCS358*pvd-inaZ* decreased in a parallel fashion as the concentration of ferric citrate in RSM increased (Fig. 5). In cultures of WCS358*pvd-inaZ* grown in RSM amended with ferric citrate concentrations of 5×10^{-5} M or greater, INA was detected, whereas pyoverdine concentrations were below detectable levels. INA was related to pyoverdine production by WCS358*pvd-inaZ* grown in culture over the full range of iron concentrations in which both phenotypes could be detected. On roots of 12-day-old flax plants grown in the presence of 2 μM 50%

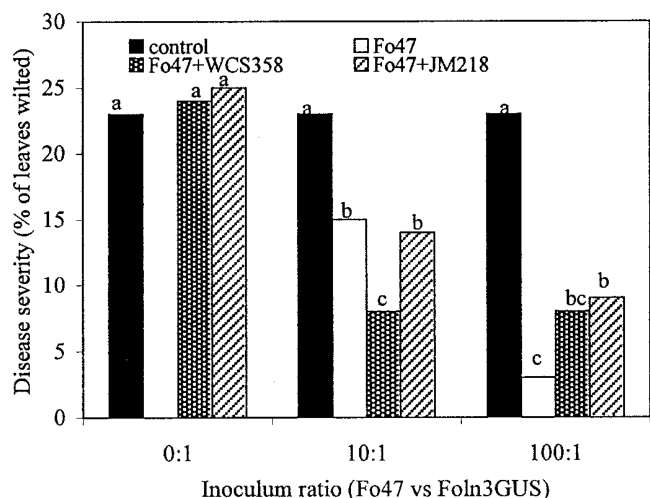

Fig. 2. Effects of *Pseudomonas putida* WCS358 and the Sid⁻ mutant JM218 on Fusarium wilt severity for different inoculum ratios of nonpathogenic *Fusarium oxysporum* Fo47 to pathogenic *F. oxysporum* Foln3GUS. For the same Fo47 to Foln3GUS inoculum ratio, means designated with the same letter are not significantly different ($P \le 0.05$) according to Fisher's least significant difference test. Statistical analyses were performed on transformed values.

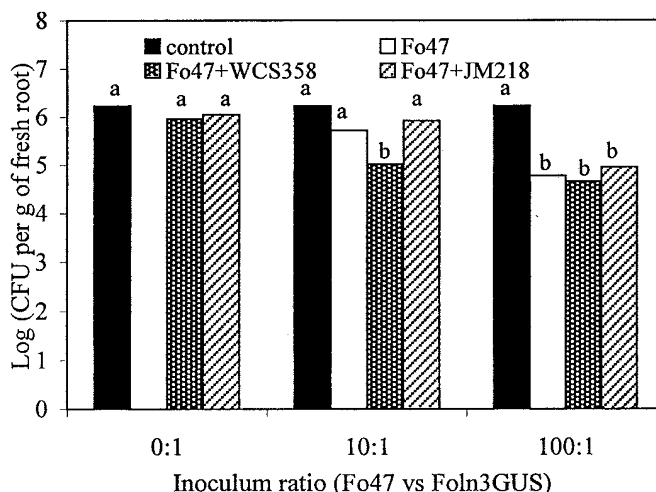

Fig. 3. Effects of *Pseudomonas putida* WCS358 and the Sid⁻ mutant JM218 on the density of Foln3GUS for different inoculum ratios of nonpathogenic *Fusarium oxysporum* Fo47 to pathogenic *F. oxysporum* Foln3GUS. For the same Fo47 to Foln3GUS inoculum ratio, means designated with the same letter are not significantly different ($P \le 0.05$) according to Fisher's least significant difference test. Statistical analyses were performed on transformed values.

ferrated EDDHA, WCS358*pvd-inaZ* expressed an INA of –2.3 log (ice nuclei per cell), whereas WCS358 expressed no INA. INA expressed by WCS358*pvd-inaZ* indicated that iron levels on flax roots were sufficiently low for transcription of *inaZ* from an iron-regulated promoter.

DISCUSSION

This study confirms previous reports of the ability of nonpathogenic *F. oxysporum* Fo47 to suppress Fusarium wilts and that the combination of *F. oxysporum* Fo47 and *P. putida* WCS358 increases disease suppression above that provided by either antagonist alone (3,19,23). These data also provide further evidence that nonpathogenic *F. oxysporum* Fo47 and *P. putida* WCS358 decrease population size and activity of pathogenic *F. oxysporum* on roots, that this decrease contributes to disease suppression, and that pyoverdine production is involved in the antagonism of pathogenic *F. oxysporum* by *P. putida*.

As previously described (3,23), Fusarium wilt was suppressed more efficiently when the ratio of nonpathogenic *F. oxysporum* to pathogenic *F. oxysporum* was increased. This disease suppression was shown to be related to the decreased metabolic activity of the pathogen Foln3GUS on and in roots, as assessed as β-glucuronidase activity. Reductions in β-glucuronidase activity of root tissues can be caused by lower populations of Foln3GUS on or in roots, lower β-glucuronidase activity expressed by individual propagules of Foln3GUS, or a combination of these factors. Both the population size of Foln3GUS and β-glucuronidase activity were assessed; therefore, these different possibilities could be addressed in the current study.

At the Fo47 to Foln3GUS inoculum ratio of 10:1, Fo47 decreased the β-glucuronidase activity expressed by Foln3GUS without reducing the density of the pathogen on flax roots. Therefore, disease suppression by Fo47 could be attributed to a decrease in the metabolic activity of Foln3GUS rather than to a decrease in the number of Foln3GUS propagules associated with flax roots. This result is consistent with the observation made by Olivain and Alabouvette (32), showing that only actively growing hyphae of *F. oxysporum* can penetrate the root. Reductions in the carbon metabolism of the pathogen may result in a decreased number of root infections and consequently reduced disease severity. These reductions could reflect a decrease in the carbon sources available for the pathogen resulting from the carbon competition achieved by Fo47, as previously demonstrated in vitro (24). This analysis could not be made in the previous work of Eparvier and Alabouvette (12), because Foln3GUS could not be distinguished from Fo47 by the antibody used to evaluate root colonization by the fungi; therefore, populations of Foln3GUS were not enumerated and the metabolic activity could not be normalized to the pathogen density.

At the highest inoculum dose of Fo47 (100:1, Fo47 to Foln3GUS), both the density of the pathogen and the β-glucuronidase activity were reduced and disease was less severe than that observed at the lower inoculum dose of Fo47 (10:1, Fo47 to Foln3GUS). The increased efficacy of biocontrol with a greater inoculum dose of Fo47 is consistent with a possible role for competition for carbon and infection sites in suppression of Fusarium wilt (7,24,32).

In the presence of *P. putida* WCS358, suppression of Fusarium wilt of flax by nonpathogenic *F. oxysporum* Fo47, when inoculated at a ratio of 10:1 (Fo47 to Foln3GUS), was increased significantly, as previously described on carnation (23). At this inoculum ratio, enhanced disease control by the microbial combination was related to a significant reduction of the pathogen density but not to a reduction of pathogen activity on or in roots. In contrast, the Sid$^-$ mutant JM218 did not further reduce disease severity or the population density of the pathogen beyond that achieved by Fo47 alone. Because the population sizes of JM218 and WCS358 were similar at the root level, the lack of efficacy of the Sid$^-$ mutant could not be ascribed to reduced fitness of the strain. The conclusion that in situ pyoverdine production contributes to disease suppression by WCS358 was supported by data indicating that iron availability to the bacterium at the root level, assessed by the ice-nucleation reporter gene, was sufficiently low for pyoverdine production. Together, these results provide convincing evidence that WCS358 suppresses Fusarium wilt of flax by inhibiting the saprophytic growth of the pathogen and that pyoverdine produced in situ by WCS358 contributes to this inhibition. Pyoverdine produced by *P. putida* WCS358 is known to inhibit mycelial growth of a pathogenic *F. oxysporum* in culture (24), and the current data indicate that this inhibition can also occur at the root level. These results also confirm those from a previous study, indicating that pyoverdine production contributes to the conjoint activities of WCS358 and the nonpathogenic *F. oxysporum* Fo47 for biological control of Fusarium wilt of carnation (23).

In contrast with the Fo47 to Foln3GUS inoculum ratio of 10:1, at inoculum ratio of 100:1, neither WCS358 nor JM218 modified the density or the activity of the pathogen at the root level. They also failed to improve the control achieved by Fo47. The difference in the effect of WCS358 on disease severity according to the Fo47 to Foln3GUS inoculum ratio could be related to the higher

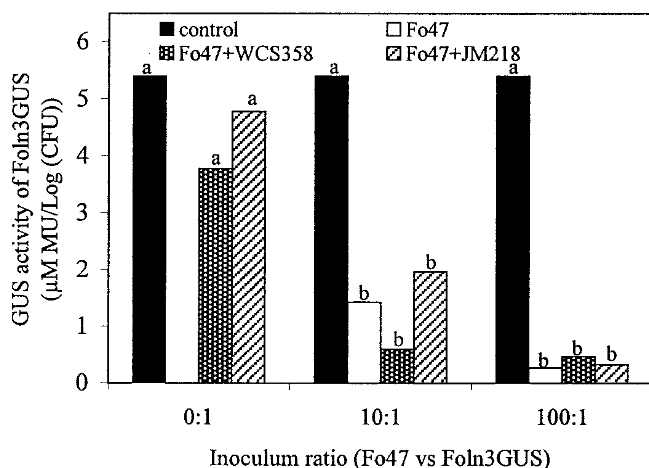

Fig. 4. Comparison of the effects of *Pseudomonas putida* WCS358 and the Sid$^-$ mutant JM218 on the β-glucuronidase activity of Foln3GUS for different inoculum ratios of nonpathogenic *Fusarium oxysporum* Fo47 to pathogenic *F. oxysporum* Foln3GUS. For the same Fo47 to Foln3GUS inoculum ratio, means designated with the same letter are not significantly different ($P \leq 0.05$) according to Fisher's least significant difference test.

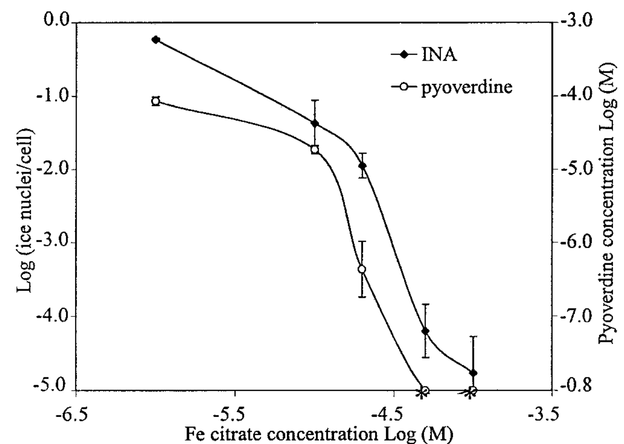

Fig. 5. Relationship between ice-nucleation activity (INA) and pyoverdine production by *Pseudomonas putida* WCS358*pvd-inaZ* grown in RSM amended with increasing concentrations of ferric citrate. Vertical bars indicate standard errors and * indicates pyoverdine below the detection limit.

density of Fo47 at the inoculum ratio 100:1 that would have led to an increased susceptibility of Fo47 to the bacterial antagonism. The Sid⁻ mutant JM218 increased significantly the disease severity when compared with Fo47 alone but nonsignificantly when compared with WCS358 + Fo47. The disease severity in the presence of the microbial combinations, however, remained significantly lower than the infested control.

Besides microbial competition, induction of resistance of the host plant by biocontrol agents is another mode of action that can suppress Fusarium wilt (22,36). Previous studies have demonstrated that nonpathogenic *F. oxysporum* Fo47 and *P. putida* WCS358 can induce systemic resistance of plants against Fusarium wilt (7,11, 36). In addition, siderophores and, more specifically, pyoverdines have been shown to play a role in the induction of resistance of the host plant against different diseases (19,28). In this study, systemic induced resistance could not be excluded, because Fo47 reduced disease severity even in the absence of any significant decrease of the pathogen density (at an inoculum ratio of 10:1 [Fo47 to Foln3GUS]). However, disease suppression was more efficient when the pathogen density was decreased significantly by Fo47 at the highest inoculum ratio and by the combination of Fo47 and WCS358 at an inoculum ratio of 10:1 (Fo47 to Foln3GUS). This observation is in agreement with previous work, indicating that systemic induced resistance does not play a major role in the Fusarium wilt suppression achieved by Fo47 (11). The implication of the antagonism in the disease suppression by the microbial combination is also supported by the fact that *P. putida* WCS358 improved the biocontrol activity of Fo47 only when the bacterial strain enhanced the reduction of the pathogen density achieved by Fo47 alone (ratio 10:1). According to our previous work (24), the strong antagonism associated with the combination of Fo47 and WCS358 at the inoculum ratio 10:1 could be related to an interaction between the competition for carbon and iron performed by Fo47 and WCS358, respectively.

In conclusion, the current study provides evidence that suppression of Fusarium wilt of flax by a *P. putida* and a nonpathogenic *F. oxysporum* is related to a reduction of the pathogen density and activity on roots, and the antagonism achieved by *P. putida* WCS358 is related to pyoverdine production.

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