Antagonism of Selected Bacterial Strains to *Stemphylium vesicarium* and Biological Control of Brown Spot of Pear Under Controlled Environment Conditions

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ABSTRACT

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Over 410 strains of *Erwinia herbicola* and *Pseudomonas fluorescens* were isolated from aerial plant parts and roots of several crops, and of these strains, 7% inhibited germination of conidia and mycelial growth of *Stemphylium vesicarium*. Among the inhibitory strains, only four were highly effective against brown spot of pear in a detached leaf assay. An inverse relationship was observed between disease severity and inhibition of conidial germination on the leaf surface. The most efficient strains were *P. fluorescens*, which exhibited chemotaxis toward germinating conidia and produced antifungal compounds in solid and liquid culture. Pear plants treated with *P. fluorescens* EPS288, the most active antagonist, and subsequently inoculated with conidia of *S. vesicarium*, showed significantly ($P < 0.001$) lower disease incidence (57% less) and severity levels (88% less) than nontreated controls under conditions conducive to brown spot disease development. A spontaneous rifampicin-resistant mutant of strain EPS288 survived relatively well under moist conditions but survived less well under a moist-dry-moist schedule.

Brown spot of pear (*Pyrus communis* L.) is an aerial fungal disease, caused by *Stemphylium vesicarium* (Wallr.) E. Simmons, that occurs on leaves, on fruit, and, occasionally, on twigs of pear (39). The disease is important in Mediterranean pear-production areas in Europe (7,39,50).

Environmental conditions, such as temperature, relative humidity, and wetness duration are important factors influencing disease development. The optimum temperatures for radial growth of mycelium and conidial germination are 21 and 23°C, respectively (34). Significant disease levels on plants exposed to a controlled environment or on trees under orchard conditions are found after wetting periods of more than 6 to 10 h at temperatures of 20 to 25°C (33). Cultivar type and fruit or leaf age are host parameters that also affect brown spot disease of pear. Susceptibility of fruits and leaves decreases logarithmically with age in the very susceptible cultivars Passe Crassane and Conference (32).

Disease control is achieved with fungicides applied on a 7- to 15-day schedule, and under standard production conditions, as many as 10 to 25 fungicide applications per season are needed for acceptable disease control (7,34,39,50). A forecasting model for predicting infection periods and timing fungicide applications has reduced fungicide use and improved control efficacy (33,34).

Because chemical control of brown spot depends on frequent applications of fungicides, biological control may offer an alternative or complementary method for disease management. Several bacterial epiphytes of plants, such as the species *Pseudomonas fluorescens* and *Erwinia herbicola*, produce multiple antibiotics against plant-pathogenic fungi and bacteria (17,19) and have been useful as biocontrol agents of some diseases in the phyllosphere and rhizosphere (2,6,22). However, there are no reports on antagonism of these bacteria against *S. vesicarium* and their potential use as biocontrol agents of brown spot of pear.

In this study, we report on isolation and characterization of strains of *P. fluorescens* and *E. herbicola* that are antagonistic to *S. vesicarium*. One of the most efficient antagonists was evaluated for efficacy of biological control under controlled environment conditions conducive to expression of brown spot disease. We also describe a preliminary characterization of mechanisms involved in inhibition of *S. vesicarium*.

MATERIALS AND METHODS

Isolation, characterization, and origin of the bacterial strains. Putative bacterial antagonists were isolated from 190 samples taken from aerial parts and roots of several plants from 24 species (7 families) and 14 locations in northeastern Spain during the spring and autumn of 1993 and 1994. Plant material included leaves, flowers, buds, and roots. Roots were washed gently with sterile distilled water to remove soil debris. Buds and flowers were cut into small pieces before extracting epiphytic bacteria. Epiphytic bacteria were extracted from plant material by stirring at 200 rpm for 30 min in buffered peptone water (0.1% Bacto peptone in 30 mM potassium phosphate buffer). The extract was diluted serially in potassium phosphate buffer (70 mM, pH 7.0), and a 10-µl drop of each dilution was spotted onto the surface of King's medium B agar (KB) (24) or Miller and Schroth (MS) agar plates amended with mannitol (30). Plates were incubated for 48 h at 25°C.

P. fluorescens was preliminarily identified in KB agar as fluorescent colonies under ultraviolet light (366 nm) and positive for the oxidase test. *E. herbicola* was preliminarily identified as typi-

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cal red-orange colonies with a darker central area in MS medium. Species identification was performed according to *Bergey's Manual of Systematic Bacteriology* (25) with the following additional tests: Gram stain, motility, oxidative/fermentative metabolism, arginine dihydrolase, nitrate reduction, levan production, indol, gelatin hydrolysis, methyl-red, Voges-Proskauer, tobacco hypersensitivity, and ice-nucleation activity. Over 500 bacterial isolates were initially obtained in the current study. A more complete identification of the strains that were antagonistic to *S. vesicarium* was performed by the analytical profile index (API) system (API, Vercieu, France). Strain CU135 of *E. herbicola* also was used (S. V. Beer and Y. Ophir, Department of Plant Pathology, Cornell University, Ithaca, NY). For long-term preservation, bacteria were stored in 20% glycerol at –70°C.

Preparation of *S. vesicarium* inoculum. Strain EPS26 of *S. vesicarium*, which is highly virulent, was used for inoculations. Inoculum was prepared as previously described (33). The fungus was grown in petri dishes on V8 agar at 20°C under a photoperiod of 12 h of fluorescent light at 150 $\mu E\ m^{-2}\ s^{-1}$. Conidial suspensions were prepared from 8- to 10-day-old cultures in a diluted solution of Tween 20 in distilled water (1 drop of Tween 20 per liter) and maintained at 4°C to prevent germination during manipulations. Conidial concentration was assessed with a hemacytometer and adjusted to 10^6 conidia per ml. Conidial germination was assessed after 2 h of incubation at 20°C in a hemacytometer chamber. Suspensions with less than 90% conidial germination were discarded.

In vitro antagonism assay. Screening for in vitro antagonism on agar media was performed with agar overlays of glucose-asparagine (GA) (53) and Luria-Bertani (LB) (28). GA agar amended with iron (50 μM $FeCl_3$) was used to prevent siderophore production in bacteria that showed antagonism on GA agar. Overlays were prepared by mixing in agar 0.1 ml of a conidial suspension of 10^6 conidia of *S. vesicarium* per ml. Bacterial colonies of the putative antagonists were transferred to the surface of the overlay agar plates with toothpicks, colonies were incubated at 25°C, and inhibition zones were assessed after 3 days.

An agar diffusion test was used for screening antagonists. Bacteria were streaked on one edge (2 cm wide) of GA and LB agar petri dishes (9 cm wide) and incubated for 48 h at 25°C. After growth of the bacteria, 5-μl drops of a conidial suspension of 10^6 conidia per ml were deposited 1.5 cm from the edge of the bacterial mass, and the percentage of conidial germination was assessed after 12 h. Drops of the conidial suspension were spotted in LB and GA agar plates without bacteria and used as nontreated controls. Conidia of *S. vesicarium* can produce up to six germ tubes per conidium during germination. Also, antifungal compounds decrease the number of germ tubes per conidium and the proportion of conidia with germ tubes. Therefore, conidial germination (G) was calculated according to the formula: percent $G = T_i \times 100/C_i$, where T_i and C_i were the mean number of germ tubes per conidium at a given distance from the bacterial antagonist and in the nontreated control, respectively.

Production of antifungal compounds in liquid culture and assay of activity. Antifungal compounds were produced in LB broth amended with heat-inactivated conidia. Bacterial strains were streaked onto solid LB medium and incubated at 25°C. Dishes were flooded with 10 ml of LB broth, and suspensions were adjusted to an absorbance of 0.8 at 620 nm. A 2.5-ml aliquot was mixed with 100 μl of a heat-inactivated conidial suspension (10^6 conidia per ml) and incubated at 100 rpm and 22°C. Conidia were inactivated for 10 min in a water bath at 100°C. In preliminary trials, these conditions were suitable for production of antifungal compounds against *S. vesicarium* in *P. fluorescens* EPS288. After 36 h of incubation, cultures were centrifuged at 3,500 rpm for 15 min, the supernatant was filtered through a 0.45-μm pore filter (Millipore Corp., Bedford, MA) and used for antifungal activity assessment. The pellet was extracted with 1 N NaOH at 100°C for 10 min and used for protein determination with Coomassie blue (4).

Antifungal activity was determined by a conidial germination inhibition assay. Culture filtrates were diluted serially into flat-bottom microwell plates to a final volume of 200 μl. The contents of each microwell were mixed with 20 μl of a conidial suspension (10^6 conidia per ml) and incubated for 6 h at 25°C. After incubation, the number of germ tubes per conidium was determined with a microscope at 400×. Conidial germination was calculated as for the agar diffusion test by dividing the mean number of germ tubes per conidium in each dilution of the filtrate by the corresponding numbers in the nontreated control and was expressed as a percentage. For each bacterial isolate, conidial germination obtained for the different dilutions of the supernatant was transformed into probits and regressed against the reciprocal of dilution and the effective dose (ED_{50}) determined according to Finney (11). Antifungal activity corresponded to the reciprocal of the dilution necessary to inhibit 50% of conidial germination. Antifungal activity was divided by the total protein concentration of the bacterial culture to obtain units of activity adjusted for culture density.

Chemotaxis assay. A swarm assay (16) was used to determine the capacity of bacterial isolates to move toward conidia of *S. vesicarium*. A 10-μl suspension of 10^6 conidia of *S. vesicarium* per ml was placed close to the edge of a petri dish (9 cm diameter) containing 0.2% agar in 10 mM potassium phosphate buffer (pH 7.0). Plates were incubated for 24 h at 20°C to permit conidial germination. A washed suspension (20 μl) of 10^9 CFU of the bacterial antagonist per ml was placed in the center of the plate. Colony movement was measured in millimeters after 24 h of incubation at 20°C. The radius of the bacterial mass in the direction of the conidia was subtracted from the radius on the opposite side.

Plant material. Self-rooted pear plants of cultivar Conference (CAV clone) obtained by micropropagation (Agromillora Catalana, S.A., Barcelona, Spain) were used. Plants were 2 to 3 years old, were about 1 m tall, and were grown in 20-cm-diameter plastic pots. Plants were left outside the greenhouse during winter for chilling. During early spring, plants were pruned to leave one main shoot and were forced to bud in the greenhouse. Plants were fertilized once a week with a 200 ppm N-P-K solution (20-10-20) and were used when the shoot was about 50 cm tall and had 15 to 20 young leaves, which were the most susceptible to *S. vesicarium* infection. Standard insecticide and miticide sprays were applied.

Inhibition of brown spot of pear on detached leaves. A detached pear leaf assay was used to determine inhibition of conidial infection by antagonistic bacterial isolates. Some of the 31 bacterial antagonists active against *S. vesicarium* in vitro were obtained from the same host plant and sampling area. Therefore, only one representative strain was retained for further analysis, and only 24 strains were used for the detached leaf assay.

Young pear leaves were collected from the shoot tip of Conference pear plants. The leaves were surface-disinfested by immersion in a diluted solution of sodium hypochlorite (1% active sodium hypochlorite) and shaken at 50 rpm for 5 min, washed two times with sterile distilled water, and placed under an air stream to remove excess water. Bacterial suspensions were prepared by resuspending solid LB agar cultures grown at 25°C for 48 h in potassium phosphate buffer (70 mM, pH 7).

To establish optimal conditions for screening, infectivity titration experiments were performed with *S. vesicarium* on leaves treated with different concentrations of bacterial antagonists *P. fluorescens* EPS288 and *E. herbicola* EPS528. The concentrations of *S. vesicarium* were 10^4, 10^5, and 10^6 conidia per ml. The concentrations of *P. fluorescens* EPS288 were 0, 10^6, 10^7, 5×10^7, 10^8, and 10^9 CFU/ml, and for *E. herbicola* EPS501, concentrations were 0, 10^6, 10^7, and 10^8 CFU/ml. Different concentrations of bacteria were obtained by serial dilution of a 10^9 CFU/ml suspension. Leaves were treated with an antagonist by immersion in each bacterial suspension for 10 min. Inoculated leaves were exposed to an air stream to remove excess water and were placed

with the abaxial surface face up in transparent plastic boxes filled with moistened paper towels. Leaves were incubated overnight in the dark at 22°C. Thereafter, 6 10-µl drops of the corresponding conidial suspension were deposited onto the abaxial surface of each leaf and incubated for 3 days at 22°C with a photoperiod of 12 h of fluorescent light (150 µE m^{-2} s^{-1}) in a controlled environment chamber (model PGR-15, Conviron Winnipeg, MB, Canada). Disease levels were assessed for each inoculated drop according to the following scale: 0 = no infection; 1 = pin-point lesions; 2 = pin-point infections that coalesce; and 3 = necrosis affecting more than three-fourths of the drop area. Disease severity for each leaf was calculated as the sum of the ratings for the six inoculation drops and was expressed as a percentage of the maximum attainable severity (18 units). The experimental design consisted of three replicates with three leaves per replicate for each bacterial isolate. Treatments were completely randomized, and the experiment was conducted twice. For routine screening, leaves were treated by immersion for 10 min in each bacterial suspension at a dose of 10^8 CFU/ml and were inoculated with a conidial suspension of *S. vesicarium* of 5×10^4 conidia per ml.

Conidial germination on leaf surfaces was assessed by direct observation under a microscope. Leaf segments of inoculated areas were cut 24 h after inoculation with conidia, stained with lactophenol blue, mounted on microscope slides, and observed at 200× under a microscope. The number of germ tubes per conidium was determined, and conidial germination was calculated as for the agar diffusion test by dividing the mean number of germ tubes per conidium in treated leaves and in the nontreated control and was expressed as a percentage.

Survival of bacterial antagonists on whole plants. Survival of strain EPS288 was studied with a spontaneous rifampicin-resistant mutant. The mutant, EPS288Rif, was similar phenotypically to the parental strain in biocontrol efficacy, colony morphology, antifungal production, and API20 profile. The strain was sprayed at 10^8 CFU/ml onto pear plants with an airbrush atomizer (Badger 250, Sagola, Spain) attached to a pressure source operating at 30 kPa. Spraying was done until runoff on both sides of the leaves. Treatments consisted of continuous moisture and a moist-dry-moist schedule (1 day moist-2 days dry-1 day moist). Moist conditions were obtained by covering plants with plastic bags with the inside sprayed with water. Dry conditions were 50% relative humidity (RH). Plants were incubated in a controlled environment chamber (model PGR-15, Conviron) at 22°C with a photoperiod of 12 h of fluorescent light (150 µE m^{-2} s^{-1}). Population levels of strain EPS288Rif were assessed on leaves immediately after inoculation and after the treatment conditions. The procedure used for population level assessment was as described for isolation of bacterial antagonists, except KB agar amended with 100 µg of rifampicin per ml was used. The experimental design had three replicates with two plants per replicate. The experiment was conducted twice.

Controlled environment plant trials. Cultivar Conference pear plants were prepared according to the methods used for the detached leaf assay. Plants were treated with a bacterial suspension of 10^8 CFU of *P. fluorescens* EPS288 per ml or with a solution of the fungicide captan (Belpron C-50) at 2 g a.i./liter. Plants treated with distilled water were used as nontreated controls.

Treatments were applied with an airbrush atomizer until runoff on both sides of the leaves. Treated plants were covered with plastic bags with the inside sprayed with water and incubated for 12 h under fluorescent light (150 µE m^{-2} s^{-1}) at 22°C in the controlled environment chamber. Plastic bags were removed, and plants were inoculated with a suspension of 5×10^4 conidia of *S. vesicarium* per ml according to the treatments. Inoculated plants again were placed in plastic bags and incubated for an additional 24 h at 22°C in the dark to permit conidial germination and infection. Plants were removed from plastic bags and placed in the greenhouse. Greenhouse mean temperatures were 23°C (18 to 26°C) with 70% RH (60 to 80% RH). The experimental design had three replicates with three plants per treatment. Treatments and replicates were completely randomized. The experiment was conducted three times.

TABLE 1. In vitro antagonism against *Stemphylium vesicarium* on different solid media and inhibition of brown spot on detached pear leaves after treatment with selected bacteria (*Erwinia herbicola* or *Pseudomonas fluorescens*) isolated from different hosts and plant parts

Treatment	Origin		In vitro antagonism on solid media[y]			Disease severity (%)[z]	
	Host	Plant part	GA	GA-Fe	LB	Trial 1	Trial 2
Nontreated control	...	...	...	...	...	99 a	96 a
E. herbicola EPS417	Pear	Buds	d	–	–	81 b	79 a–e
E. herbicola EPS494	Apple	Buds	++	–	+	78 bc	75 a–d
E. herbicola EPS468	Quince	Buds	d	–	–	76 b–d	87 ab
P. fluorescens EPS539	Pear	Buds	+	+	d	75 b–d	74 b–d
P. fluorescens EPS317	Tobacco	Roots	+	+	+	72 cd	91 ab
E. herbicola EPS495	Peach	Flowers	++	–	+	71 c–e	74 b–e
P. fluorescens EPS383	Veronica	Flowers	+	+	+	67 d–f	90 ab
E. herbicola EPS513	Apple	Buds	d	–	–	63 ef	85 a–c
P. fluorescens EPS210	*S. subvillosus*	Roots	d	–	–	59 f–h	64 de
E. herbicola EPS452	Nectarine	Flowers	+	–	–	54 gh	...
E. herbicola EPS528	Pear	Buds	+	+	+	53 hi	...
E. herbicola EPS501	Peach	Buds	++	++	+	50 h–j	64 c–e
E. herbicola CU135	Apple	Flowers	+	+	+	...	60 d–g
P. fluorescens EPS301	Grass	Roots	+	+	+	44 i–k	39 fg
P. fluorescens EPS381	Veronica	Flower	+	+	d	44 jk	...
P. fluorescens EPS356	Pear	Buds	+	+	+	40 kl	29 g
E. herbicola EPS403	Pear	Buds	d	d	–	37 k–m	34 g
E. herbicola EPS482	Pear	Buds	d	–	–	37 k–m	47 e–g
P. fluorescens EPS550	Cherry	Buds	d	d	d	34 l–n	46 e–g
P. fluorescens EPS227	Pear	Roots	d	–	+	29 l–n	...
P. fluorescens EPS373	Pear	Buds	+	+	+	28 no	29 g
P. fluorescens EPS531	Peach	Flowers	+	+	+	20 op	41 fg
P. fluorescens EPS375	Pear	Buds	+	+	+	18 p	34 g
P. fluorescens EPS288	Corn	Roots	+	+	+	15 p	32 g

[y] Antagonism was determined with an agar overlay of the indicator microorganism where bacterial antagonists were inoculated. After 3 days the radius of inhibition on the indicator microorganism was assessed. GA = glucose-asparagine; GA-Fe = GA amended with iron; LB = Luria-Bertani. – = no inhibition; d <= 1 mm; + = 1 to 5 mm; ++ > 5 mm.

[z] Numbers are the means of the disease severity rating of three replications with three leaves each. Values within the same column followed by the same letter do not differ significantly ($P < 0.05$) according to Tukey's mean separation test.

Disease incidence as the percentage of leaves with one or more infections and disease severity as the mean number of lesions per leaf for each plant were assessed 10 days after conidial inoculation. Efficacy was calculated as the relative decrease of disease due to treatment in relation to the nontreated control.

Statistical analysis. Data analysis was performed with the procedures regression (REG), analysis of variance (ANOVA), and Tukey's mean separation test by PC-SAS (SAS Institute, Cary, NC). Severity data in whole-plant trials were transformed to $\log_{10}(S + 0.1)$ to normalize variance before ANOVA. Disease severity as calculated in the detached leaf assay and disease incidence data on whole-plant trials did not require transformation to normalize variance.

RESULTS

Screening bacterial antagonists against *S. vesicarium* in vitro. A total of 410 strains of *E. herbicola* and *P. fluorescens* were isolated from aerial plant parts and roots. Of 193 strains of *E. herbicola*, 10 inhibited *S. vesicarium* on GA agar, and of 217 strains of *P. fluorescens*, 21 inhibited *S. vesicarium* on GA agar. The inhibitory strains of *E. herbicola* belonged to biogroups 1, 2, and 6, and the inhibitory *P. fluorescens* strains belonged to biotypes I and V. Only a set of 24 strains was retained for further analysis, because some of the 31 strains came from the same host plant or sampling area (Table 1). None of the strains were ice-nucleation active or elicited a hypersensitive reaction on tobacco. Of the 24 inhibitory strains on GA, 9 were not active when the medium was supplemented with iron, indicating that its antagonism could be due to siderophore production. Antagonism on LB was less frequent (15 strains) than on nonamended GA. Only 14 strains were antagonistic to *S. vesicarium* on all media tested. No relationship was observed between plant host, plant part of origin, bacterial biotype or biogroup, or antagonism pattern on these media.

Production of extracellular antifungal compounds and chemotaxis of antagonists. Strains EPS288 and EPS375 were the most inhibitory in the agar diffusion test (Table 2). Antifungal activity was detected in cellfree filtrates from liquid cultures grown on LB in all isolates; activities ranged from 14 to 907 units/mg of culture protein. Strains EPS375 and EPS288 of *P. fluorescens* produced antifungal activities that could be diluted 26 and 86 times, respectively, and still retain a capacity for inhibiting 50% of conidial germination. The activity of supernatants produced by the *E. herbicola* strains and *P. fluorescens* EPS381 was significantly lower.

In the chemotactic swarm assay, movement rates ranged from 6 to 16 mm/24 h. Strain EPS288 of *P. fluorescens* showed the strongest tendency to move toward germinating conidia of *S. Vesicarium*. Chemotaxis of *E. herbicola* strains to germinating conidia was not observed, although the strains were motile.

Antagonism on detached leaves. Treatment of detached pear leaves with some bacterial antagonists inhibited infection by *S. vesicarium* (Fig. 1). The effect of different doses of *P. fluorescens* EPS288 and *E. herbicola* EPS528 at three conidial concentrations on disease severity was studied to determine suitable doses for screening antagonists with the detached leaf assay (Fig. 2). Significant inhibition of disease was observed at 10^8 CFU/ml for both bacteria at the three conidial concentrations tested. The highly active *P. fluorescens* strain EPS288 showed maximum efficacy at 10^8 CFU/ml, and no significant increase was observed at 10^9 CFU/ml ($P = 0.156$). Pathogen concentrations of 10^5 conidia per ml developed maximal disease levels (>90%) on leaves not treated with antagonist. However, at this high dose of conidia, antagonists with lower efficiency could not be detected due to a lack of significant decrease in disease levels. Therefore, conditions for screening were selected at 5×10^4 conidia per ml of *S. vesicarium* and a dose of 10^8 CFU of putative biocontrol agent per ml.

The 24 strains of *E. herbicola* and *P. fluorescens* that significantly inhibited *S. vesicarium* on GA agar were compared for their capacity to inhibit brown spot by a detached leaf assay (Table 1). However, we did not determine the number of isolates negative for antagonism in vitro against *S. vesicarium* that were efficient in inhibition of infection on leaves. Differences in disease severity levels among the bacterial antagonists ($P < 0.001$) and between trials ($P < 0.001$) were significant. Separate analysis of both trials indicated greater differences among bacterial antagonists in trial 1 than in trial 2 ($P < 0.001$). Disease levels on leaves treated with the antagonists were higher in trial 2 than in trial 1 ($P < 0.001$). However, levels in nontreated controls were similar in both trials because the inoculated spots had rating values of 3, the maximum attainable. In trial 2, most of the spots on nontreated controls had become necrotic over the entire surface. Differences between trials were attributed to the fact that in trial 2 leaves were younger and more susceptible to *S. vesicarium*, as has been described previously (32). The strong influence of leaf age on susceptibility to infection by *S. vesicarium* resulted in variability in disease severity results. We tried to minimize variability in susceptibility from leaf to leaf within each experiment by selecting leaves of similar physiological age. However, it was more difficult to obtain plant material of similar susceptibility from trial to trial. According to both trials, strains EPS288 and EPS375 were the most effective antagonists in the detached leaf assay.

There was a linear relationship between disease severity and conidial germination on detached leaves treated with bacterial antagonists (Fig. 3). In trial 1, the 24 strains listed in Table 1 were used. In trial 2, only 11 strains chosen to cover the range of disease

TABLE 2. Antifungal activity and chemotaxis of selected bacterial antagonists (*Pseudomonas fluorescens* and *Erwinia herbicola*) against *Stemphylium vesicarium*

Bacterial antagonist	Inhibition of conidial germination (%)		Activity of liquid culture supernatant[w] (units/mg culture protein)	Chemotaxis assay[x] (mm/24 h)
	AD test[u]	DL assay[v]		
P. fluorescens EPS288	86 a[y]	79 a	907 a	16
P. fluorescens EPS375	84 a	81 a	177 b	10
P. fluorescens EPS381	35 bc	72 a	14 d	6
E. herbicola EPS528	30 c	45 b	100 bc	ns[z]
E. herbicola EPS482	12 d	40 bc	80 c	ns
E. herbicola CU135	49 b	28 c	21 d	ns

[u] AD = agar diffusion. Conidia were placed 1.5 cm from the edge of a streaked culture of the bacterial antagonist grown on Luria-Bertani agar for 24 h. Conidial germination was determined after 12 h of incubation at 20°C. Values are the means of three replicate samples in three independent experiments.

[v] DL = detached leaf. Leaves were pretreated with the bacterial antagonist (10^8 CFU/ml) and incubated for 12 h. Drops of conidial suspensions were deposited on the abaxial surface face up, and germination was assessed after 24 h of incubation at 20°C. Values are the means of three replicate samples in three experiments.

[w] Filter-sterilized liquid-culture supernatants were diluted serially, 200 µl was mixed with a conidial suspension (10^6 conidia per ml), and germination was assessed after 12 h of incubation at 20°C. Units are defined as the reciprocal of the dilution that inhibits germination by 50% and were divided by the total protein concentration of 200 µl of bacterial culture. Results are the means of three experiments.

[x] Bacterial antagonists were added to the center of the plate (10 µl at 10^9 CFU/ml), and directional swarming was measured after 24 h. Values are the means of two replications of two experiments.

[y] Values within the same column followed by the same letter do not differ significantly ($P < 0.05$) according to Tukey's mean separation test.

[z] Not significant.

levels found in trial 1 were used. The two experiments gave regression equations that did not differ according to an F test ($P > 0.25$), allowing the data to be pooled. Regression analysis of pooled data gave a significant linear relationship ($R^2 = 0.64$, $P < 0.001$) between disease severity and inhibition of conidial germination on the leaf surface.

Survival of a EPS288Rif mutant on whole plants. There were significant differences in population levels between the continuous moisture and moist-dry-moist schedule treatments ($P < 0.001$). Differences were not significant between trials ($P = 0.093$). Population levels of strain EPS288Rif on wet leaves of plants immediately after inoculation were 8.1 ± 0.3 $\log_{10}$(CFU/g fresh weight). When plants were incubated under continuous moisture for 4 days, population levels were 7.1 ± 0.2 $\log_{10}$(CFU/g fresh weight), whereas levels decreased to 6.1 ± 0.6 $\log_{10}$(CFU/g fresh weight) after a moist-dry-moist schedule.

Biological control of brown spot on whole plants. Disease levels on whole plants treated with *P. fluorescens* EPS288 in comparison with plants treated with captan and nontreated controls are shown in Figure 4. There were significant differences between treatments for both disease incidence and severity ($P < 0.001$). Differences also were significant between trials ($P < 0.001$), but the effect of treatments was consistent. Differences between trials were due mainly to captan treatments ($P < 0.001$). No significant differences in disease incidence were observed between trials for the nontreated controls ($P = 0.073$) or bacterial antagonist treatments ($P = 0.049$).

Pear plants treated with *P. fluorescens* EPS288 showed significantly lower disease incidence (mean values of 38%) than nontreated controls (mean values of 88%) ($P < 0.001$), and the efficacy of the antagonist treatment was 57%. In trials 1 and 3, disease levels were significantly higher ($P < 0.001$) in plants treated with the antagonistic bacteria than in plants treated with the fungicide captan. In trial 2, there were no significant differences ($P = 0.123$) between plants treated with the antagonistic bacteria and plants treated with the fungicide captan. Results on disease severity were similar to disease incidence, but there was higher variability in nontreated control plants. The efficacy of the antagonist for the reduction of disease severity was 88%.

DISCUSSION

Of the bacterial epiphytes isolated and characterized in our study, 7% showed in vitro antagonism against *S. vesicarium*. The number of antagonists was higher among *P. fluorescens* than *E. herbicola*. However, lower percentages of recovery were reported in screening bacterial antagonists for other plant pathogens. Only 0.5% of the bacteria isolated from cucumber leaves were antagonistic to *Colletotrichum lagenarium*, and only 1% of the bacteria isolated from soybean were highly antagonistic to *P. syringae* pv. *glycinea* (26). We obtained a high percentage of antagonists probably because we screened a wide range of plant species, plant parts, and location areas and also because we selected only *P. fluorescens* and *E. herbicola*, which have a high frequency of antibiotic production (12,22).

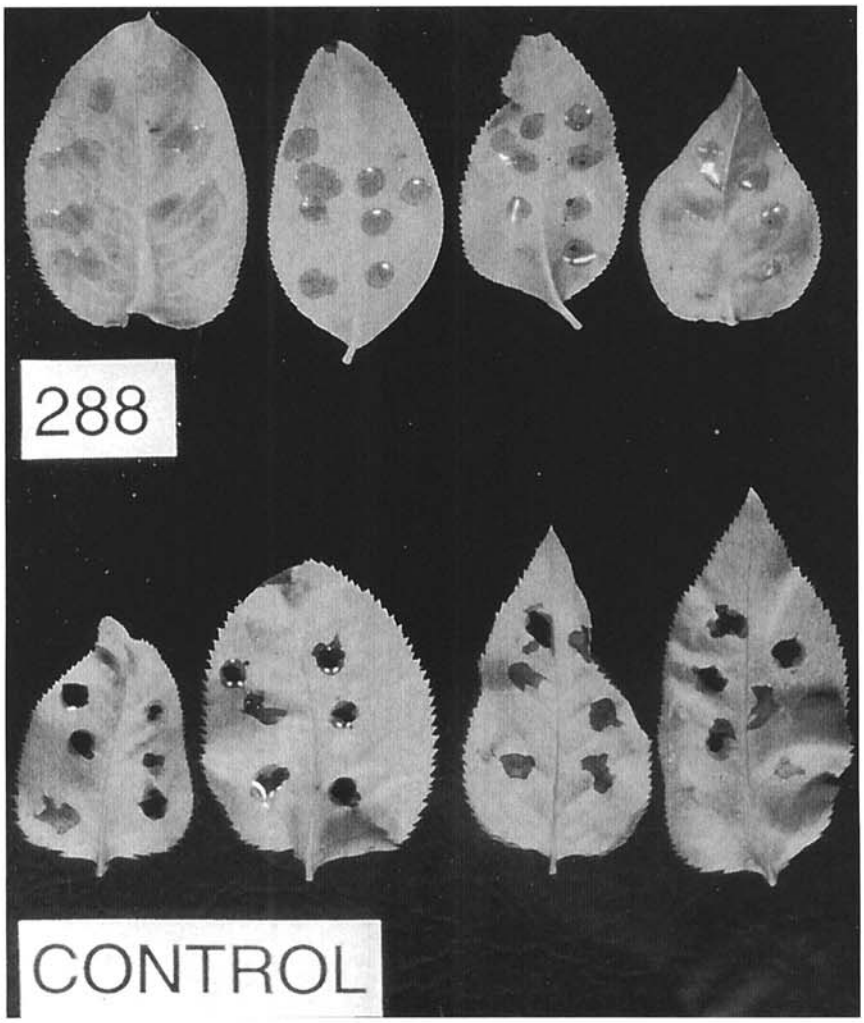

Fig. 1. Control of brown spot caused by *Stemphylium vesicarium* EPS26 on detached pear leaves (cv. Conference) after treatment with *Pseudomonas fluorescens* EPS288 (top row) or no treatment (bottom row). Bacteria were applied to detached leaves at a concentration of 10^8 CFU/ml 12 h before inoculation with conidia.

The in vitro production of antibiotics by bacteria against *S. vesicarium* apparently was related to antagonism on leaves, but antibiotic production was not predictive of efficacy in the detached leaf assay. Not every isolate exhibiting in vitro antagonism on GA or LB agar inhibited leaf infections by *S. vesicarium*. Strains with practically nondetectable antagonistic activity in vitro were low to moderately efficient for reduction of disease severity on detached leaves (e.g., EPS210, EPS482, and EPS513). Nevertheless, the most inhibitory strains on detached leaves (e.g., EPS531, EPS375, and EPS288) produced the highest amounts of antifungal compounds in vitro. Similar results have been reported in other screenings of bacteria for antagonism to plant-pathogenic fungi. A lack of correlation between in vitro antibiosis and biological control was observed during screening of bacterial antagonists against *Bipolaris maydis* on maize (42) and *Ceratocystis ulmi* on elm (36). Conversely, in vitro antibiosis of several bacterial antagonists was correlated with biological control of *Drechslera dictyoides* (3) and *Gaeumannomyces graminis* on wheat (43). However, the role of antibiosis in biological control of plant pathogens depends on the plant-pathogen-biocontrol system, because other mechanisms, such as competition or parasitism, may be implicated (2,6,12,29,38).

The degree of inhibition of brown spot of pear by the bacterial antagonists was directly related to the inhibition of conidial germination on the leaf surface. This finding suggests that our bacterial antagonists inhibited disease by directly affecting conidial germination, the first stage in host infection. The detailed study performed with some of our strains revealed that production of antifungal compounds and chemotaxis were processes associated with the most efficient strains. It is likely that in these cases antibiosis acts in concert with other processes that interfere with germination of conidia.

Several *E. herbicola* and *P. fluorescens* strains that inhibited infection of *S. vesicarium* produced antimicrobial substances in liquid and solid culture. Peptide antifungals, herbicolins A, B, O, and I, are produced by strains of *E. herbicola* (17,23,52). Diverse compounds like phenazine-1-carboxylic acid, 2,4-diacetylphloroglucinol, pyrrolnitrin, pyoluteorin, and oomycin A (19,20,22,29,38, 47) or enzymes such as chitinases (14,44) are produced by strains of *P. fluorescens*. Conditions affecting the synthesis of some antibiotics have been found in *P. fluorescens*, indicating that production is regulated by the catabolite and depends on the type of carbon source, cell density, and temperature, whereas iron, pH, and oxygen concentration had no influence (15,41).

The type of carbon source used in culture media is extremely important for screening antagonism. For instance, glucose is required by *P. fluorescens* HV37A for the synthesis of certain antibiotics against *Pythium ultimum* in potato dextrose agar but inhibits the production of other antibiotics (19). Similar observations were reported for *E. herbicola* in relation to the effect of the type of amino acid on antibiosis against *E. amylovora* on GA (53). Because many of our strains produced antifungal compounds on media rich in glucose (e.g., GA) and amino acids (e.g., LB) or even on media supplemented with iron, it is likely that different types of substances were implicated. However, during the current study no attempt was made to determine the production of antifungal compounds directly on leaves colonized by antagonistic bacteria, and the nature of the compounds produced in vitro was not determined.

The chemotaxis of some antagonists to germinating conidia of *S. vesicarium* suggests that an active phenomenon could be implicated in biological control. Preliminary observations indicate that strain EPS288 interacts with germ tubes of germinating conidia, and rodlike connecting structures can be observed under scanning

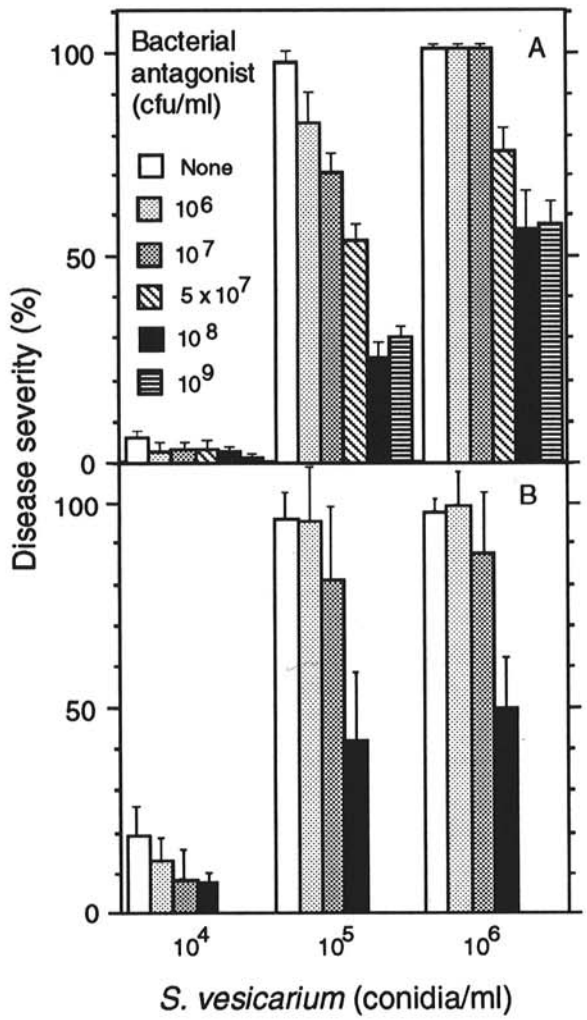

Fig. 2. Infectivity titration of *Stemphylium vesicarium* EPS26 on detached pear leaves after treatment with different doses of **A,** *Pseudomonas fluorescens* EPS288 or **B,** *Erwinia herbicola* EPS528. Bars indicate the confidence interval ($P = 0.05$) for the mean.

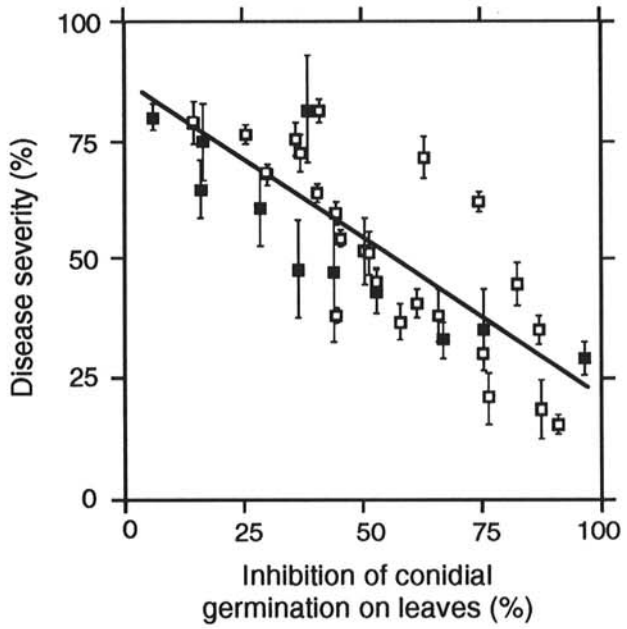

Fig. 3. Relationship between inhibition of conidial germination on detached pear leaves by different antagonistic bacteria and brown spot disease severity. Bacteria were applied at 10^8 CFU/ml 12 h before inoculation with *Stemphylium vesicarium* conidia (5×10^4/ml). The line indicates regression analysis of disease severity on inhibition of germination according to the equation $y = 86.2 - 0.66x$ ($R^2 = 0.64$, $P < 0.001$). Data from two experiments were pooled for regression analysis. Bars indicate the confidence intervals for the mean ($P = 0.05$). Experiment 1 = □, experiment 2 = ■.

electron microscopy (data not shown). Attachment of bacteria and the presence of rodlike connections to fungal surfaces are important in establishing relationships that result in reduction or inhibition of fungal growth (40). All these interacting processes are consistent with studies showing that attachment of *Enterobacter cloacae* to hyphae of *Pythium ultimum* colonizing seeds was directly related to its function as a biocontrol agent (35). Attachment of *Serratia marcescens* to hyphal tips of *Sclerotium rolfsii* and subsequent lysis was implicated in the biological control (37).

The efficacy of biological control depends on the biocontrol agent/pathogen ratio (1,21,31). Data obtained for *P. fluorescens* EPS381 with the detached leaf assay that were fit to mathematical models that relate the densities of the biocontrol agent and pathogen with disease gave an ED_{50} value of ~10^7 CFU/ml and a ED_{50} biocontrol agent/pathogen ratio of ~200 CFU per conidium (31). In greenhouse trials, disease incidence decreased by 57% and disease severity by 88% in plants treated with strain EPS288 at a biocontrol agent dose of 10^8 CFU/ml and a biocontrol agent/pathogen ratio of 2,000 CFU per conidium—10 times the ED_{50} of EPS381 in the detached leaf assay. Also, strain EPS288 was 1.4 times more efficient in biological control than EPS381 in the detached leaf assay. Consequently, some loss of efficacy in whole-plant trials occurs in comparison to detached leaf assays. However, the effective dose for EPS288 in whole-plant trials agreed with the effective doses of 10^7 to 10^8 CFU/ml that were reported in other biocontrol trials of aerial fungal diseases with antagonistic bacteria that were applied as a spray (20,45).

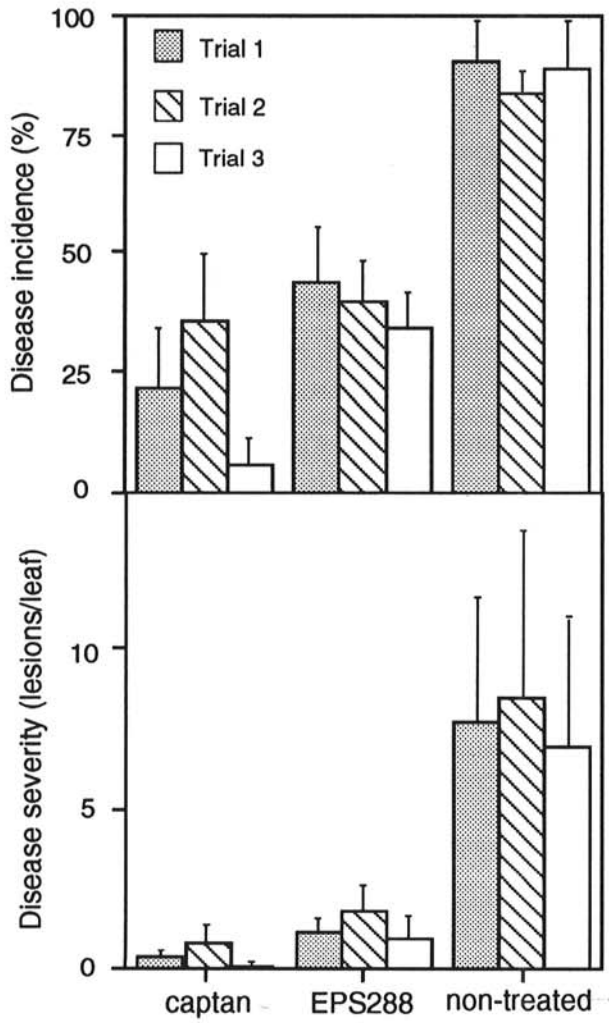

Fig. 4. Disease incidence and severity of brown spot on pear (cv. Conference) treated with captan, *Pseudomonas fluorescens* EPS288, or water and maintained under controlled conditions in the greenhouse. Plants were treated with either a suspension of 10^8 CFU of the antagonist per ml, captan at 2 g a.i./liter, or distilled water and maintained under moist conditions for 12 h. After which, plants were inoculated with a suspension of 5×10^4 conidia of *Stemphylium vesicarium* EPS26 per ml and incubated for an additional 24 h moisture period to permit conidial germination and infection. Plants were placed in the greenhouse for 10 days disease expression.

P. fluorescens strain EPS288 was a soil inhabitant originally isolated from the root system of corn. A mutant of EPS288 resistant to rifampicin survived relatively well under moist conditions in leaves of pear plants but survived less well under moist-dry-moist cycles. In spite of its origin, strain EPS288 was efficient in biological control of brown spot of pear under controlled conditions. However, good performance in greenhouse trials does not presume an ability to exert biological control in the orchard. In the field and in the aerial part of pear trees, survival, colonization and competition with other microorganisms in a changing environment probably are factors limiting the biocontrol potential of the selected strains (2,6,46) because they are not phyllosphere colonizers or they come from plant hosts that differ from pear. However, there are some examples of success in biocontrol contrary to the general belief that only autochthonous microorganisms are suitable for biological control.

For instance, biological control of aerial diseases caused by *Alternaria alternata*, a fungus related to *Stemphylium*, on rice (18), tobacco (13), and postharvest cherries (49) was observed with antibiotic-producing *Bacillus* spp. isolated from different hosts. Epiphytic bacteria isolated from potato leaves colonized and reduced frost injury due to *P. syringae* on corn plants (27), and *E. herbicola* isolated from corn colonized pear flower buds, flowers, and fruitlets (5). Soilborne bacteria colonized pear flowers and were effective in biological control of fire blight (48). Also, growth is not always required for biological control. *Chaetomium globosum*, a soil inhabitant that produces the antibiotic chaetomin, which inhibits germination of spores of *Venturia inaequalis*, was efficient in controlling scab when applied before the pathogen inoculum, and it could produce the antibiotic without extensive growth on leaves (8,9).

Effective biological control and survival of strain EPS288 were seen at 22°C and continuous wetness. These conditions were similar to those required by *S. vesicarium* for growth and disease development (33,34). Conidia of *S. vesicarium* germinate and initiate infections in a short time period when optimal conditions are met (6 to 10 h at 15 to 25°C and continuous wetness or >90% RH). Therefore, independent of whether growth of the antagonist occurs in the plant, the antagonist should be present on trees at effective population levels, e.g., 10^7 CFU/g fresh weight, the carrying capacity of pear leaves (27,51), and during periods of risk of infection and high susceptibility of leaves and fruit to brown spot (32).

Survival of EPS288 during unfavorable periods in the orchard, which coincide with unfavorable conditions for the pathogen, is expected to be a critical factor for success of this antagonist as a biocontrol agent. Low survival may require frequent sprays to restore effective population levels and may be the main limitation for biological control of brown spot of pear, as has been found in other pathosystems on aerial plant parts (2,6,10,45,51).

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