APPLIED AND ENVIRONMENTAL MICROBIOLOGY, May 1992, p. 1760–1763
0099-2240/92/051760-04$02.00/0

Vol. 58, No. 5

NOTES

Pseudomonas cepacia Suppression of Sunflower Wilt Fungus and Role of Antifungal Compounds in Controlling the Disease

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Received 4 November 1991/Accepted 12 February 1992

In a field experiment, *Pseudomonas cepacia* J82rif and J51rif increased sunflower emergence in the presence of the fungus *Sclerotinia sclerotiorum*. Pyrrolnitrin, aminopyrrolnitrin, and monochloroaminopyrrolnitrin were isolated from J82 and identified by using thin-layer chromatography, high-performance liquid chromatography, and electron impact-mass, UV, and infrared spectroscopy. In growth chamber experiments, two antibiosis-negative mutants were not different from the parent strain in protecting the seeds from the fungus.

Sclerotinia wilt caused by *Sclerotinia sclerotiorum* (Lib.) de Bary is one of the most important diseases of sunflowers (*Helianthus annuus* L.). The pathogen survives in the soil as sclerotia and attacks sunflower roots, resulting in root rot, basal stem canker, and wilt of the plant. Researchers have made only slight progress in breeding for resistance to *Sclerotinia* wilt in sunflowers, and no effective chemical control of this pathogen exists (12). Zazzerini et al. (14) reported that *Bacillus subtilis* isolates suppressed or reduced the ability of sclerotia to germinate.

We report on (i) two strains of *Pseudomonas cepacia* that reduce sunflower wilt in a field experiment and (ii) the role of antibiotics in protecting sunflower seeds against *S. sclerotiorum* in a growth chamber experiment.

P. cepacia isolates J82 and J51 were isolated from the rhizospheres of greenhouse-raised barley and sorghum, respectively, grown in a silty loam soil (pH 6.5) obtained from a cornfield at Jacques Seed Co., Prescott, Wis. These isolates were selected from our strain collection because they showed strong inhibition against *S. sclerotiorum* mycelia and sclerotia on potato dextrose agar (PDA) plates (Difco Laboratories, Detroit, Mich.). Bacteria were cultured overnight in nutrient broth (NB) (Difco) with shaking at 150 rpm at 30°C. For some of the studies, spontaneous mutants, J82rif and J51rif, resistant to 100 μg of rifampin ml^{-1} (Sigma Chemical Co., St. Louis, Mo.), were used.

J82 culture was mutagenized with 20 μg of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine ml^{-1} (Sigma) to give a survival rate of 29.4% by using the method of Adelberg et al. (1). Colonies that did not inhibit the fungus on PDA plates were considered antibiosis-negative mutants.

Sclerotia were collected from infected sunflower plants, surface sterilized, and germinated on PDA. For growth chamber studies, 5-mm^2 disks were cut from the PDA plates described above and placed next to each seed at planting. For field studies, wheat kernels were sterilized, inoculated with mycelial plugs, and incubated at 22°C for 10 days. One infected wheat seed was placed next to each seed at planting.

Field plots were set up in a St. Charles silt loam (pH 6.6) at Arlington, Wis. The field had been in a corn-soybean rotation. Field plot treatments were as follows: (i) uninoculated (no bacteria) plus or minus fungus (wheat seed method); (ii) Captan (*N*-trichloromethylthio-4-cyclohexene-1,2-dicarboximide) plus Apron [*N*-(2,6-dimethylphenyl)-*N*-(methoxyacetyl) alanine methyl ester (Gustafson Inc., Dallas, Tex.)] (treated seed) plus or minus fungus; (iii) J82rif plus or minus (non-fungicide-treated seed); and (iv) J51rif plus or minus (non-fungicide-treated seed). *H. annuus* cv. 449a and *H. annuus* cv. 4818 (Sigco Seed Co., Breckenridge, Minn.) were planted at a rate of one seed per 10 cm (2.5 cm deep) in single, 9-m rows, 0.9 m apart. Each treatment was replicated five times in a randomized complete-block layout.

For growth chamber experiments, a soil mix (pH 6.9) consisting of top soil, peat, perlite, Turface (Aimcor Applied Industrial Materials Corp., Deerfield, Ill.), and ricehulls (2:2:2:1:1) was used. Twenty conetainers (115 cm^3) (Cone-Tainer, R. Leach, Canby, Oreg.) per treatment were used. Each experiment was repeated three times. Growth chamber treatments included the following: (i) uninoculated control (no bacteria or fungus); (ii) uninoculated plus fungus (no bacteria); (iii) J82 plus fungus; (iv) J82 nonproducer (P-5) plus fungus; and (v) J82 nonproducer (P-9) plus fungus. One seed of non-fungicide-treated *H. annuus* cv. 442 (Sigco) was planted per conetainer to a depth of 1.5 cm, and the conetainers were placed in a growth chamber for 3 weeks with a 12-h photoperiod at 22 and 20°C (day and night temperatures, respectively) and watered daily. The light intensity measured 400 microeinsteins $m^{-2}\ s^{-1}$. The percent emergence of the seeds was measured at 14 days after planting for both of the above experiments.

For the field plots, seeds were pretreated prior to planting with peat inoculum by using gum arabic as a sticker (U.S. Biochemical Corp., Cleveland, Ohio) (30% [wt/vol]) to give 2×10^8 cells $seed^{-1}$. Peat inoculum was added at a rate of 10^8 cells $seed^{-1}$ for the growth chamber experiment.

For colonization, 1-g samples (20 replications for the field and 7 replications for the growth chamber) were taken 3 weeks after planting. Dilutions were plated on nutrient agar, nutrient agar plus rifampin (100 μg ml^{-1}), and TB-T (4).

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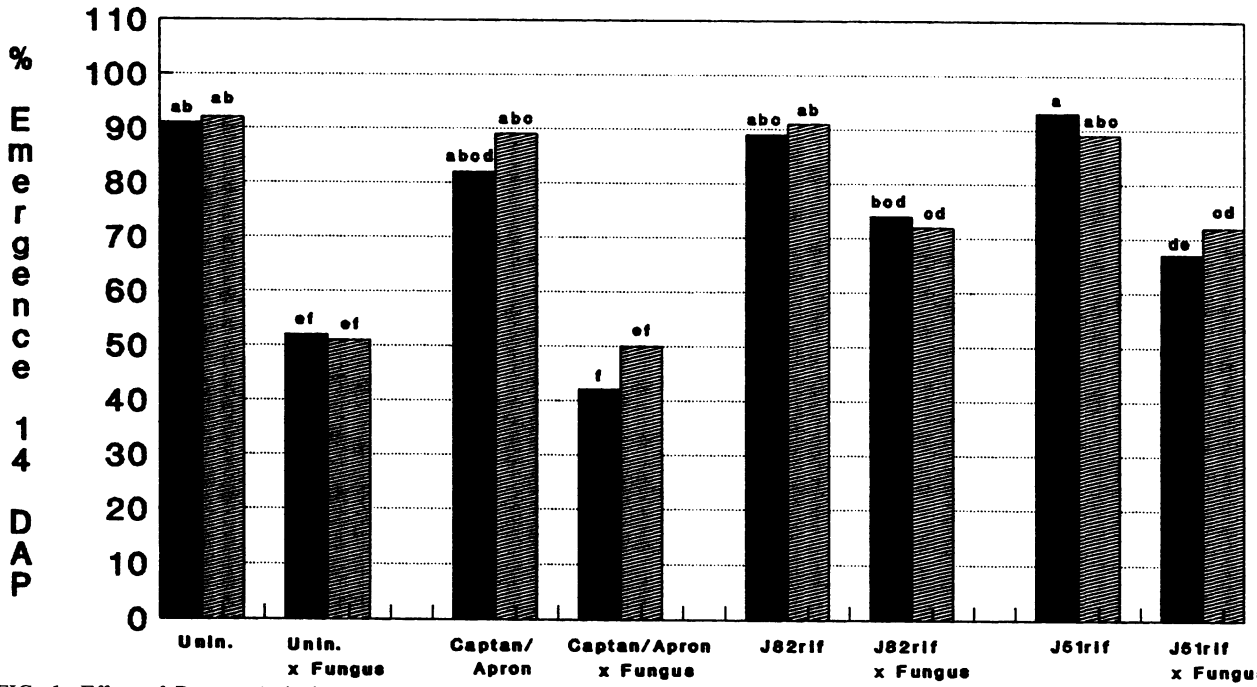

FIG. 1. Effect of *P. cepacia* isolates on sunflower emergence in the presence of *S. sclerotiorum* in a field experiment. Abbreviations: Unin., uninoculated; DAP, days after planting. Symbols: ■, *H. annuus* cv. 449a; ▨, *H. annuus* cv. 4818. Values are the means of five replicates. Means designated with the same letter are not significantly different ($P = 0.05$) according to Duncan's multiple-range test.

Cycloheximide (Sigma; 150 μg ml^{-1}) was used in all media to reduce fungal contamination.

The data were transformed by using the arcsine and subjected to an analysis of variance (11). If a significant F test resulted, mean separations were performed by using Duncan's multiple-range test.

For the isolation of antibiotics, cultures were grown in NB, KMB (6), and PNP (3) for 72 h at 30°C, and the supernatant was used in subsequent purification steps. For high-performance liquid chromatography (HPLC) analysis, a Phenomenex Ultracarb 5 octyldecyl silane column (30 by 250 mm) (Phenomenex Corp., Rancho Palois, Berdes, Calif.) was used. Synthetic pyrrolnitrin (Fujisawa Pharmaceutical Co., Osaka, Japan), and aminopyrrolnitrin (N. Mahoney, U.S. Department of Agriculture, Albany, Calif.) were used as analytical standards. A gradient program was used to separate the phenylpyrrole compounds and consisted of the following: 0 to 5 min, 50% acetonitrile–water; 5 to 25 min, 50 to 100% acetonitrile; and 25 to 35 min, 100 to 50% acetonitrile. The solvent flow rate was 1 ml min^{-1}, and detection was by UV absorption at 252 nm. Fractions which showed antibiotic activity were repurified by HPLC and analyzed by electron impact-mass spectroscopy, UV spectroscopy, infrared spectroscopy, and melting-point determination.

For thin-layer chromatography (TLC) analysis, 0.2-mm thick silica gel sheets (10 by 5 cm) containing a fluorescent indicator (E. M. Science Co., Cherry Hill, N.J.) were used. The sheets were developed with methylene chloride in a saturated atmosphere, and the separated compounds were visualized with a ninhydrin reagent consisting of 0.3 g of ninhydrin, 3 ml of glacial acetic acid, and 100 ml of 2-propanol. The reagent was sprayed over the plate, and the plate was heated to 100°C for 1 min. Both of the standards were also visible by using 252-nm light.

Fractions collected during HPLC separation of the J82 supernatant were dried and reconstituted in 1 ml of H_2O. Five hundred microliters of the above solution was added to 5 ml of molten PDA agar in petri dishes (15 by 60 mm), and the dishes were inoculated with 2.5-mm^2 agar plugs from an *S. sclerotiorum* PDA plate. Inhibition of growth was measured after 1 day at 25°C.

TLC determination of relative amounts of antibiotic compounds produced by J82 and the two mutants was accomplished by quantitative removal of the sample zones corresponding to the R_f distance of the ninhydrin-visualized standards. The separated compounds were eluted from the silica by using acetone, transferred onto antibiotic assay disks (Schleicher and Schuell Inc., Keene, N.H.), and assayed on spore plates of *Neurospora crassa* (M45-846), previously reported to be sensitive to pyrrolnitrin (3) (Eli Lilly Corp., Indianapolis, Ind.). The relative zones of inhibition were measured after 15 h at 22°C.

In the field, seeds treated with J82rif had significantly higher emergence counts than the uninoculated controls on both cultivars ($P < 0.05$) in plots where the fungal inoculum was added. Similar results were obtained in greenhouse studies (data not shown).

In the absence of the fungal inoculum, there was no difference between the treatments (Fig. 1). This lack of disease incidence highlights the difficulties encountered when testing biologicals under field conditions and relying on natural infestations. The numbers of surviving plants throughout the season did not change from the first emergence counts (data not shown). Colonization counts measured from 5.2 to 5.6 and 5.3 to 5.5 log CFU g of $root^{-1}$ for J82rif and J51rif, respectively, on two cultivars 14 days after planting.

Three biologically active compounds were identified by HPLC from cultures of J82 grown in NB and PNP: pyrrolnitrin, aminopyrrolnitrin, and monochloroaminopyrrolnitrin.

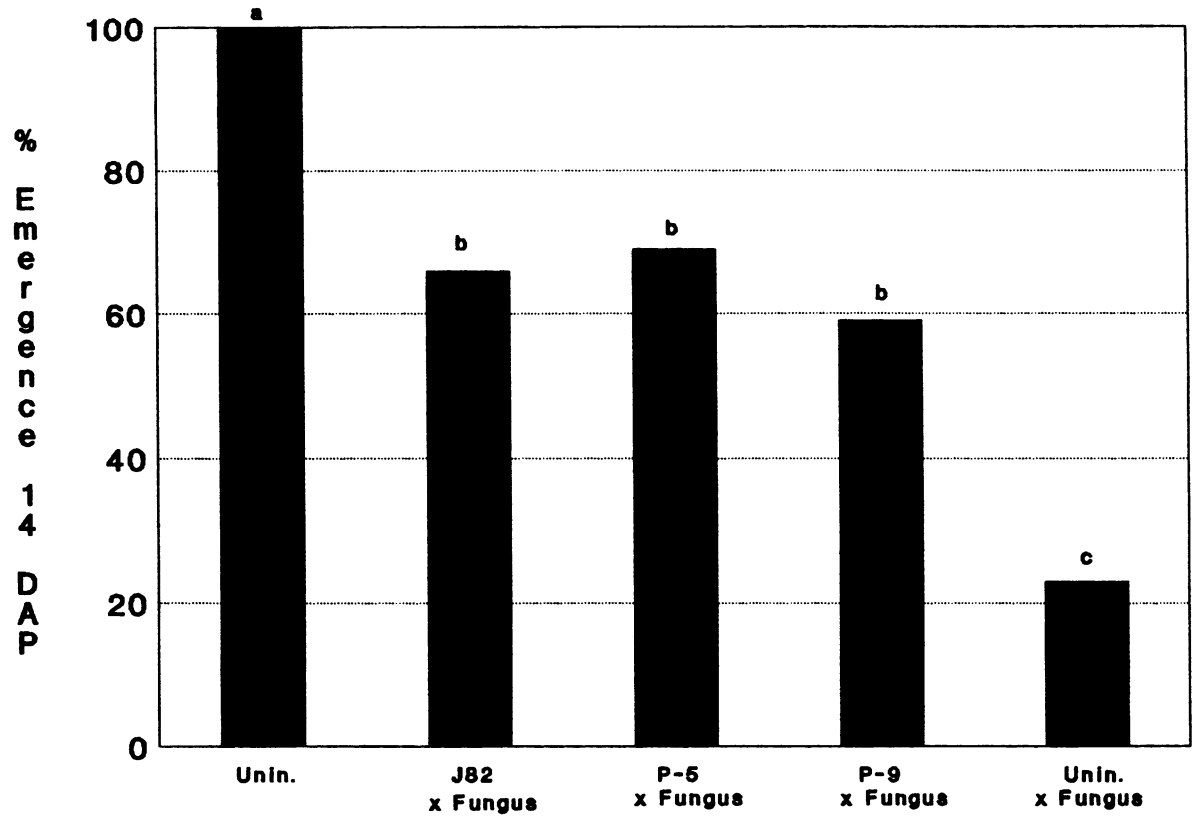

FIG. 2. Effect of antibiosis-negative mutants P-5 and P-9 on sunflower emergence in the presence of *S. sclerotiorum* in a growth chamber experiment. Abbreviations: Unin., uninoculated control; DAP, days after planting. Means designated with the same letter are not significantly different ($P = 0.05$) according to Duncan's multiple-range test.

Experimentally derived molecular weights for the three compounds were found to be 257, 227, and 193, respectively (data not shown). The HPLC gradient program was able to reproducibly elute the three antibiotic compounds between 12 and 18 min. Two major bands were evident by TLC of J82 culture supernatant. The first had an R_f value of 0.33 and a positive ninhydrin reaction, corresponding to the synthetic aminopyrrolnitrin standard. The second had an R_f value of 0.5 and a negative ninhydrin reaction, corresponding to the synthetic pyrrolnitrin standard. We were unable to confirm isolation of the monochloroaminopyrrolnitrin analog by using TLC, although a very small zone of inhibition (<1 mm) was observed at the point of sample application for J82. J82 cells grown in PNP produced higher concentrations of pyrrolnitrin than cells grown in KMB or NB (data not shown).

Four mutants which showed no antibiosis against *S. sclerotiorum* in vitro were selected after *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine mutagenesis at a frequency of 0.03%. When two of the mutants were inoculated onto sunflower seeds and challenged with the fungus in growth chamber experiments, there was no significant difference between the mutants and parent wild-type strain in protecting the seeds against the fungus (Fig. 2). Rhizosphere colonization counts on sunflower roots of the two antibiosis-negative mutants P-5 and P-9 measured 6.4 and 6.3 log CFU g of root^{-1}, respectively, compared to 6.4 log CFU g of root^{-1} for J82. The other two mutants gave similar levels of protection (data not shown). The two mutants P-5 and P-9 were unable to synthesize any of the three compounds produced by the parent wild-type strain in the three media tested (data not shown).

Pyrrolnitrin was originally reported by Arima et al. (2) to be from *P. pyrrocinia*. Roitman et al. (10) reported the isolation of the same three compounds from a strain of *P. cepacia* isolated from apple leaves, and Lambert et al. (7) showed that four *P. cepacia* isolates from the rhizosphere of maize produced pyrrolnitrin and another unknown antifungal compound. Homma et al. (5) isolated pyrrolnitrin and two pseudane derivatives from a *P. cepacia* strain isolated from a lettuce root and suggested that the chemicals might be involved in disease suppression.

It is possible that these mutants were able to synthesize small quantities in the rhizosphere in response to plant exudates. Conceivably, it would take small amounts of pyrrolnitrin to control *S. sclerotiorum* in the rhizosphere since the MIC is 0.01 μg ml^{-1} in vitro (data not shown). Thomashow et al. (13) showed that *P. fluorescens* 2-79 produced 27 to 43 ng of phenazine g of root^{-1}, which gave good biological control of the take-all fungus (*Gaeumannomyces graminis* var. *tritici*) in the rhizosphere. Our results suggest that antibiotic production by *P. cepacia* J82 is not the main mechanism for controlling *S. sclerotiorum*. Other mechanisms of control may be competition for nutrients or siderophore production.

The inoculum level used in this field experiment may be impractical and uneconomical. We have shown (8) that inoculum levels as low as 10^5 cells seed^{-1} on sunflowers will

give colonization counts of 3×10^6 cells g of root^{-1} measured 3 weeks after planting in growth chamber experiments. However, we have not determined if this inoculum level is adequate to protect the seeds against *S. sclerotiorum*. Parke (9) reported that to effectively control *Pythium ultimum* on pea seed, an initial population of 10^8 cells seed^{-1} is required.

Although questions remain as to the efficacy of *P. cepacia* J82rif and J51rif under field conditions, these isolates may have some potential as biological agents for controlling *S. sclerotiorum* on sunflowers and other crop species.

We thank Fujisawa Co. for the pyrrolnitrin, N. Mahoney for the aminopyrrolnitrin, D. Rotenberg and J. Kimpell for critical review of the manuscript, D. Rotenberg for preparing the figures, Eli Lilly Corp. for *N. crassa* (M45-846), and S. Alt and C. Hopka for help with the field experiments.

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