Detoxification of Fusaric Acid by a Fusaric Acid-Resistant Mutant of *Pseudomonas solanacearum* and its Application to Biological Control of Fusarium Wilt of Tomato

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ABSTRACT

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Bacteria capable of detoxifying fusaric acid were isolated and tested for their ability to protect tomato plants from the wilting disease caused by *Fusarium oxysporum* f. sp. *lycopersici* Race 1. The bacteria used were fusaric acid-resistant mutants derived from an avirulent strain of *Pseudomonas solanacearum*. The capability of the bacteria to detoxify fusaric acid was assayed by incubating tomato callus cultures in filtrates of bacteria that had been grown in the presence of fusaric acid. Gas chromatography indicated that the detoxification of fusaric acid by the bacteria could be due to changes in the carboxyl group in the position alpha to the nitrogen of the compound. Fusaric acid detoxification was also demonstrated by protection of tomato leaf cuttings that had been treated with bacteria before treatment with fusaric acid. Intact tomato plants were protected from wilt when they were pretreated with fusaric acid-detoxifying bacteria before inoculation with the pathogenic fungus. These results suggest that fusaric acid is important in symptom development of wilting disease of tomato, and our approach shows promise for biocontrol.

Biological control of plant diseases, especially soilborne diseases, has been the subject of much research in the last two decades. The major conventional methods have been the isolation of bacteria in the spermosphere or rhizosphere of plants and their application to the protection of crops from soil diseases (1,3,19). Rhizobacteria with antifungal activities have been selected and directly mixed with field soil or used for coating plant seeds or tubers (7,8). However, the antagonistic effects of applied bacteria estimated in in vitro pair-culturing were not satisfactorily reproduced under the soil conditions, and the results obtained were not necessarily consistent and sometimes judged noneffective (1). Thus, there is an urgent need to establish a promising strategy for biological control of soilborne plant diseases.

The purpose of the present study is to establish a new strategy for the control of Fusarium wilt of tomato plants by use of fusaric acid-resistant mutants of an avirulent strain of *Pseudomonas solanacearum*. These mutants detoxify fusaric acid, a nonspecific wilt-inducing toxin produced by *Fusarium* species (6).

MATERIALS AND METHODS

Growth of tomato plants. Tomato plants (*Lycopersicon esculentum* Mill. 'Zuiko' and 'Ponderosa,' purchased from Takii Seed Co., Ltd., Kyoto, Japan) were grown in a temperature-controlled greenhouse (25 ± 2 C) for 30 days and used for the following experiments.

Induction and culture of tomato callus tissues. Callus tissues were induced from tomato (Zuiko) leaf-explants according to methods previously described (18) and cultured on solid Murashige-Skoog (MS) (12) medium with 0.5 mg of 2, 4-dichlorophenoxyacetic acid and 0.05 mg of kinetin per liter. The medium was adjusted to pH 5.7 with NaOH and solidified with 0.8% agar. The explants were cultured at 26 C for 20–30 days under a constant illumination of 3,000–4,000 lx. Callus tissues were subcultured at an interval of 20 days for several passages and transferred to liquid MS medium (without agar) (18). The tissues were shaken at 110 strokes/min for 48 hr, and the dissociated callus cells (single cells and 2–10 cell aggregates) were collected on a stainless steel sieve (pore size, 25 μm diameter). These callus cultures were used for examining the toxicity of fusaric acid

remaining in the culture filtrates of fusaric acid-treated bacteria.

Culture conditions of bacteria and separation of an avirulent strain. A virulent strain (U-10) of *Pseudomonas solanacearum* was kindly supplied by Dr. H. Tanaka, Utsunomiya Experimental Station, Japan Tobacco, Inc. The bacteria were grown at 26 C for 48 hr on agar medium containing 10 g of Bacto-peptone, 1 g of casamino acids, and 10 g of glucose in 1 L of water (solid PCG medium), and were stored in sterilized water at room temperature. Avirulent bacteria were separated from the virulent U-10 strain based on morphology of colonies formed on solid PCG medium containing 0.05 mg of 2,3,5-triphenyltetrazolium chloride per liter (9). A small afluidal colony was suspended in sterilized water. The bacterial suspension was diluted to form 10–20 colonies per petri dish. This procedure was repeated three or four times, and finally a small afluidal colony was isolated and designated as U-10A.

Induction and identification of fusaric acid-detoxifying mutant bacteria. Avirulent bacteria (U-10A) exponentially growing in liquid PCG medium were collected by centrifugation and suspended in the same medium containing various concentrations of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) as a mutagen. After a shaking overnight, the bacteria were collected, washed, suspended in water, and spread on solid PCG medium containing various concentrations of fusaric acid (5-*n*-butyl-picolinic acid, purchased from Sigma Chemical Co., St. Louis, MO). After several days of incubation, colonies formed were suspended, diluted, and respread on fusaric acid-containing medium. By repeating these procedures, fusaric acid-resistant mutants were isolated and further screened for the capacity to detoxify fusaric acid. Culture filtrates were obtained from mutants that had been grown in shake-culture for 48 hr in liquid PCG medium containing 100 μg of fusaric acid per milliliter. Toxicity of the filtrates was examined by using tomato callus cultures. Because 10 μg of fusaric acid per milliliter was enough to completely kill tomato callus cells (17), the bacterial culture filtrates were diluted 10-fold with MS medium before estimating the remaining fusaric acid with tomato callus cells. After 24 hr of incubation, the viability of callus cells was examined by staining the cells with fluorescein diacetate (20). The bacteria that effectively lowered the toxicity of fusaric acid were selected as fusaric acid-detoxifying isolates, and one of the most effective was designated as the A-16 strain. Some taxonomic characteristics of strains U-10A and A-16 were examined (5), and the genus and species of both bacteria were confirmed (14). Avirulence of both U-10A and A-16 were confirmed by inoculating these bacteria into tomato plants (Zuiko and Ponderosa), both of which are susceptible to the original strain (U-10).

Gas chromatographic analysis of culture filtrates of A-16. A-16 was precultured for 48 hr in PCG medium containing 100 μg of fusaric acid per milliliter, transferred to mineral medium (7 g of Na_2HPO_4, 3 g of KH_2PO_4, 0.5 g of NaCl, 1 g of NH_4Cl, 11.1 mg of $CaCl_2$, and 246.5 mg of $MgSO_4$, and 100 mg of fusaric acid in 1 L of water) (MI medium) and shake-cultured for 24 hr. After removing bacteria by membrane filtration, the filtrate was lyophilized and extracted with methanol. The extract was treated with *N,O*-bis-(trimethylsilyl)acetamide and trimethylchlorosilane at 80 C and injected into a gas chromatographic column (1 m in length) packed with 1% OV1 (Gas-Chro. Co., Ltd., Tokyo, Japan). The temperature of the column was increased 5 C every 3 min from 80 to 240 C.

Introduction of bacteria into excised tomato branches and fusaric acid treatment. The top two or three branches (each having five compound leaves) were excised from 30-day-old tomato, and the cuttings were dipped in bacterial suspensions (10^6–10^9 cells/ml) of U-10A or A-16 for several hours. After known volumes of bacteria suspensions were absorbed, the cuttings were transferred to water containing fusaric acid. Throughout the experiment, the open ends of the tubes were shielded with Parafilm to reduce water evaporation, and the net amounts of sample solutions absorbed by tomato cuttings were recorded.

Inoculation with bacteria and *Fusarium*. For introduction of bacteria into plants, roots of 30-day-old tomato plants (Ponderosa susceptible to Race 1) were injured with a razor blade, which was plunged into soil (5 cm downward) at positions 2 cm distant from plants. Ten milliliters of bacterial suspensions (10^7–10^9 cells/ml) of A-16 or U-10A was poured directly onto injured roots. One week later, the plants were removed from the soil, and the roots were dipped for 3–5 min in a slurry of *Fusarium oxysporum* f. sp. *lycopersici* Race 1 (density of microconidia, $3–5 \times 10^6$ spores/ml). The inoculated plants were planted in moist soil and incubated for 1 mo in a greenhouse controlled at 28–30 C. The successful introduction of A-16 into tomato was confirmed by spreading leaf and stem homogenates of surface-sterilized plants on solid MIG medium (MI supplemented with 4 g of glucose per liter) containing 300 μg of fusaric acid per milliliter. These plants were harvested 30 days after inoculation with the *Fusarium* pathogen. The efficiency of wilt disease protection of A-16 was represented by disease indices on *Fusarium*-inoculated plants (see the legend of Fig. 5 below).

RESULTS

Isolation and characterization of avirulent fusaric acid-detoxifying bacteria. An avirulent strain was easily isolated from the original, virulent strain (U-10) of *P. solanacearum,* based on the difference in colony morphology. Small afluidal colonies (U-10A) were isolated from fluidal ones, and these were chemically mutagenized for obtaining fusaric acid-resistant mutants. For estimating the effective dosages of MNNG and fusaric acid, U-10A was shake-cultured in liquid PCG media containing various concentrations (1–1000 μg/ml) of these compounds. The highest concentration of MNNG that permitted U-10A to grow was 50 μg/ml, and the minimum level of fusaric acid for preventing growth of bacteria was 100 μg/ml. Therefore, the concentrations of MNNG for mutagenization and fusaric acid for positive screening of mutants were determined to be 50 and 100 μg/ml, respectively. For obtaining fusaric acid-detoxifying bacteria from these mutants, 496 colonies were randomly selected and shake-cultured for 48 hr in liquid PCG medium containing 100 μg of fusaric acid per milliliter. Three isolates (A-7, -16, and -557) detoxified fusaric acid in the media, and allowed tomato callus cells to retain their culture filtrates 42, 88, and 36% viability, respectively. Both U-10A and A-16 were shown to be *Pseudomonas* spp. by a series of morphological and physiological tests (data not shown).

Avirulence of U-10A and A-16 was confirmed by inoculating the bacteria into tomato plants susceptible to the virulent parent strain, U-10. Wilting was observed in all plants inoculated with U-10 by 7–10 days after inoculation. U-10A and A-16 did not cause any wilting in inoculated plants during a 2-mo experimental period. Thus, A-16 was adopted as a fusaric acid-detoxifying, nonpathogenic bacterium and was further characterized in the following experiments.

Growth of fusaric acid-detoxifying strain. Figure 1 gives the growth of A-16 in the presence of various concentrations of fusaric acid. When the bacteria were cultured in liquid PCG medium containing 100 μg of fusaric acid per milliliter, the growth was slightly suppressed at first but gradually increased and finally reached the level of fusaric acid-free, control cultures. The lag periods of bacterial growth were prolonged when cultures contained higher concentrations of fusaric acid and became remarkable as the concentrations increased to 200–400 μg/ml. In the medium containing the highest concentration (500 μg/ml) of fusaric acid, the growth was almost completely suppressed. However, these bacteria had not been killed because they were capable of growing to saturation (10^9 bacteria/ml) when transferred to medium containing lower concentrations of fusaric acid.

Detoxification of fusaric acid by A-16 strain. Figure 2 shows the level of detoxification by A-16 of fusaric acid in culture medium. A-16 was cultured in fusaric acid (100–500 μg/ml) containing PCG media and then the filtrates were harvested at intervals until A-16 attained its stationary phase (8×10^9–10^{10} bacteria per milliliter). With 100 μg of fusaric acid per milliliter, the toxicity of the culture filtrates rapidly decreased, with 85% of tomato callus cells surviving in treatment with 48-hr culture filtrates. In the cultures,

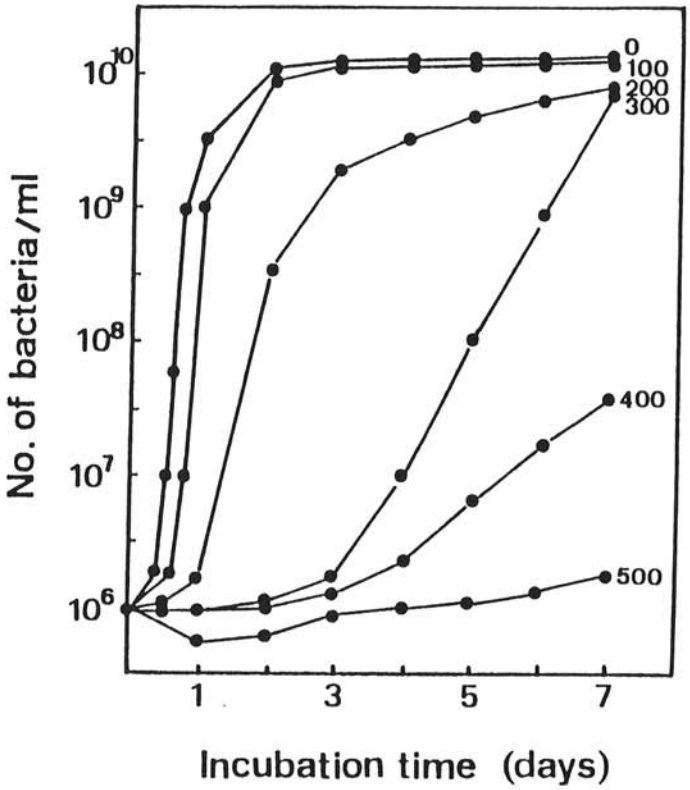

Fig. 1. Growth of fusaric acid-detoxifying mutant A-16 of *Pseudomonas solanacearum* in liquid PCG media containing various concentrations of fusaric acid (indicated on the figure as μg/ml). Bacterial suspensions (10^6 bacteria/ml) were shake-cultured at 110 strokes/min at 26 C. The numbers of bacteria were counted by using a hemacytometer. All values are the average of four separate experiments.

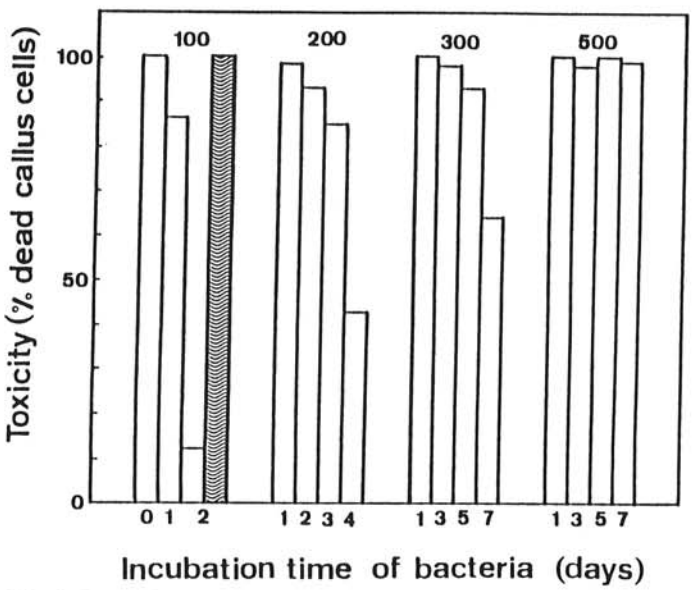

Fig. 2. Detoxification of fusaric acid by A-16 as assessed by toxicity of culture filtrates to tomato callus cells. The fusaric acid-detoxifying isolate A-16 was cultured in PCG media containing various concentrations of fusaric acid, and culture filtrates were harvested at intervals. The filtrates were then 10-fold diluted with MS medium harboring tomato callus cells (10^5–10^6 cells/ml). After 24 hr of incubation, viable tomato cells were counted by vital staining with fluorescein diacetate. Toxicity levels of the culture filtrates were given as lethal rates (%) of tomato cells. The striped column shows 10-fold diluted PCG medium without bacteria. Numbers above the bars represent the concentrations (μg/ml) of fusaric acid. Data are expressed as means of three separate experiments.

containing 200–400 μg of fusaric acid per milliliter, the detoxification was detected only when the bacteria reached the stationary phase. No detoxification was detected in cultures containing 500 μg of fusaric acid per milliliter. The viability of tomato callus cells in 10-fold diluted solution of bacteria-free medium containing 100 μg of fusaric acid per milliliter was practically null, indicating that the control fusaric acid-containing medium was toxic enough to completely kill tomato callus cells. Moreover, it was confirmed that the diluted culture filtrate of A-16 prepared from fusaric acid-free medium was nontoxic.

Gas chromatograms of A-16 culture filtrates (24 hr after inoculation of M1 medium containing 100 μg of fusaric acid per milliliter) are shown in Figure 3. Because fusaric acid was the sole compound silylesterified in this medium, the detection of fusaric acid was simple and accurate. Figure 3 clearly indicates that the peak of authentic fusaric acid added to bacteria-free M1 medium disappeared in the bacterial culture filtrate.

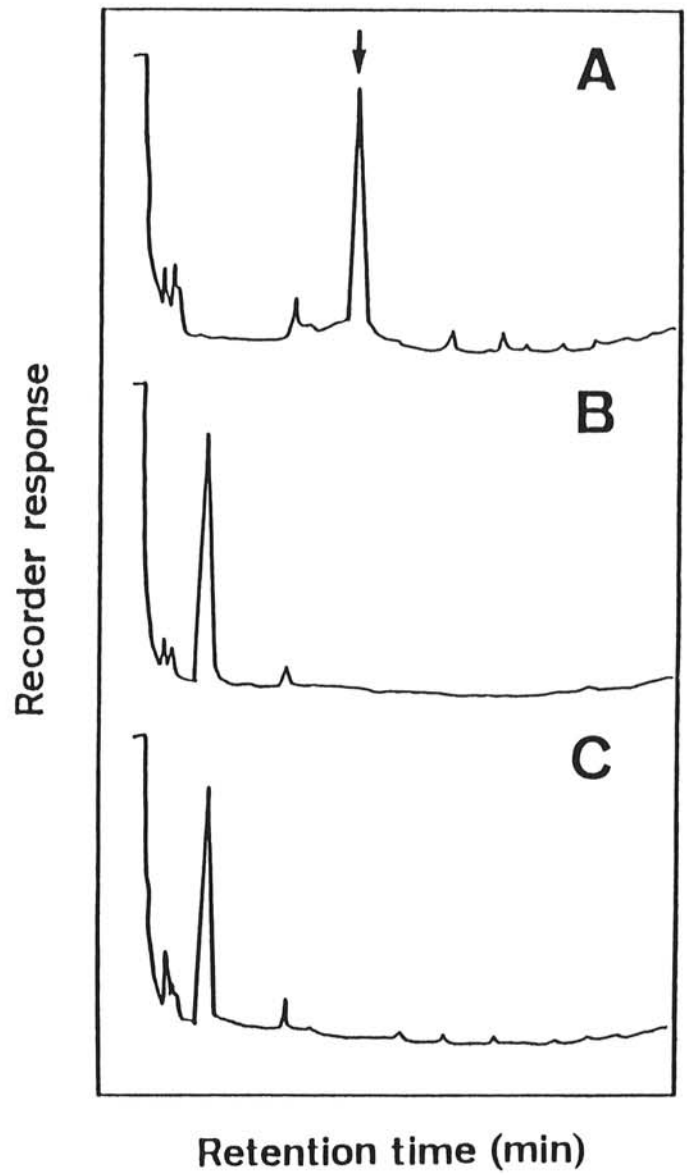

Fig. 3. Gas chromatography of A-16 culture filtrates. The bacteria (10^8 cells/ml) were precultured for 48 hr in liquid PCG medium containing 100 μg of fusaric acid per milliliter, transferred to mineral media containing 100 μg of fusaric acid per milliliter or no fusaric acid, and shake-cultured for 24 hr. The bacteria-free mineral medium with fusaric acid (A), the filtrates of A-16 cultures with fusaric acid (A) and without fusaric acid (B) were lyophilized. After extraction with methanol, the extracts were silylesterified and subjected to gas chromatography. Arrow shows the peak of fusaric acid.

Detoxification of fusaric acid by A-16 as assessed by wilting of excised tomato plants. Because the examination of in situ detoxification of fusaric acid by A-16, the toxicity of fusaric acid to tomato plants was determined by dipping the cuttings into fusaric acid solutions (1–300 μg/ml). Symptoms such as necrosis of the midrib or leaf vein appeared when treated with 100 μg of fusaric acid per milliliter. The cuttings treated with fusaric acid at concentrations higher than 200 μg/ml showed severe midrib necrosis and completely wilted 48 and 24 hr after the treatment with 200 and 300 μg of fusaric acid per milliliter, respectively. In the following experiments, therefore, 300 μg of fusaric acid per milliliter was used because the toxic effects appeared rapidly and clearly. Cuttings were first supplied with 1 ml of bacteria suspensions (10^9 bacteria per milliliter) of U-10A and A-16 or water and then treated with 1 ml of fusaric acid. In plants treated with U-10A and water, severe wilting appeared within 24 hr of treatment with fusaric acid (Fig. 4). On the other hand, no apparent symptoms were observed in plants that had been administered A-16 before fusaric acid treatment (Fig. 4). The relationship between the detoxification and the densities (10^6–10^9 bacteria per milliliter) of bacteria administered was examined. The data indicated that the plants exhibited a remarkable resistance against fusaric acid when 10^8–10^9 A-16 had been introduced into plants. The localization of introduced A-16 was then examined. The bacteria-treated tomato cuttings were treated with fusaric acid or water and cut into some portions. The number of bacteria in each portion was determined by plating the tissue homogenates on MIG media containing 300 μg of fusaric acid per milliliter. The numbers of bacteria were highest in both the basal portions of petioles and the leaves near the cut end regardless of fusaric acid-treatment (10^3–10^4 bacteria per gram fresh weight of tomato). The bacteria were detected in all segments of bacteria-introduced cuttings, but not in water-introduced ones.

Protection of intact plants from Fusarium wilt by pretreatment with A-16. The protective value of A-16 was assessed by inoculating bacteria-treated intact tomato plants with Race 1 of *F. oxysporum* f. sp. *lycopersici* (Fig. 5). The disease indices were scored 30 days after inoculation. In plants treated with U-10A or water, typical severe symptoms of Fusarium wilt were observed, and the plants finally withered within 30 days after inoculation. On the other hand, there were no detectable disease symptoms in tomato plants that had been treated with A-16 at a density of 10^9 cells per milliliter, showing growth similar to noninfected plants. The protective effect of A-16, however, was slightly decreased in the plants treated at 10^8 cells per milliliter and completely lost when 10^7 bacteria were applied. A-16 cells were reisolated from the protected tomato plants that had been administered 10^9 bacteria cells. The numbers of bacteria were 10^3–10^4 and 10^2 bacteria per gram fresh weight of tomato at the time of inoculation and 30 days after inoculation, respectively.

Because the application of A-16 to tomato plants was shown to effectively protect the plants, it was further examined whether these protective effects were due to the antagonistic effects of A-16 against *Fusarium* fungi. When A-16 or U-10A was cocultured with Race 1, essentially no antagonistic activity was observed regardless of culture media (PSA, PDA, Czapek, and Czapek-Dox media) used in the experiment.

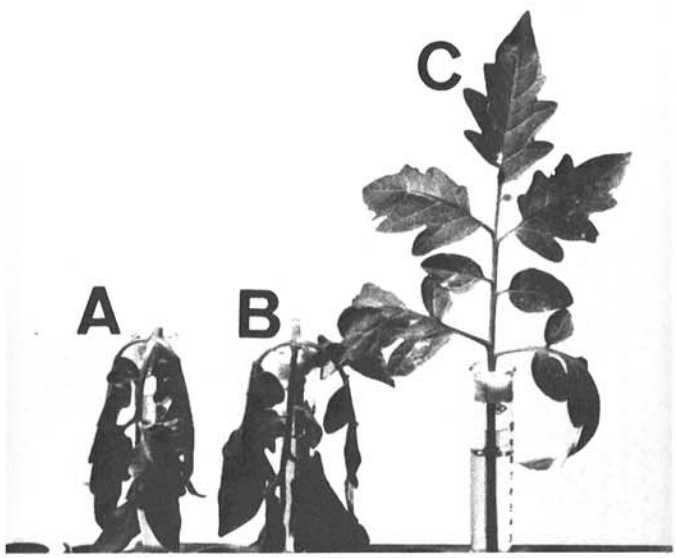

Fig. 4. Detoxification of fusaric acid by A-16 as assessed by protection of tomato leaf cuttings. The cuttings were dipped in water (A) and U-10A (B) or A-16 (C) for 2–3 hr and transferred to water containing fusaric acid (300 μg/ml). Bacterial suspensions contained 10^9 cells/ml.

DISCUSSION

Bacterization and cross-protection have been reevaluated as approaches to biological protection of crop plants against various diseases (1). In some reports dealing with crop protection from Fusarium diseases by microorganisms, avirulent types of *F. oxysporum* were shown to be effective in suppressing the wilt disease caused by other *Fusarium* fungi (4,15). In a few cases, the production of antifungal compounds by rhizobacteria was considered to play an important role in inhibiting the growth of pathogens (7,8,13). In many cases, however, the mechanisms of protection have not been elucidated and remain obscure. The goal of the present study, therefore, was to establish a new strategy for the microbiological control of plant disease.

One of the objectives of this study was to clarify the essential role of fusaric acid in Fusarium wilt by use of fusaric acid-detoxifying mutant bacteria. It would be reasonable to assume that fusaric acid plays some role in wilting, since it has been detected in plants after infection and is present in much higher concentrations in plants infected with virulent strains of *Fusarium* than those inoculated

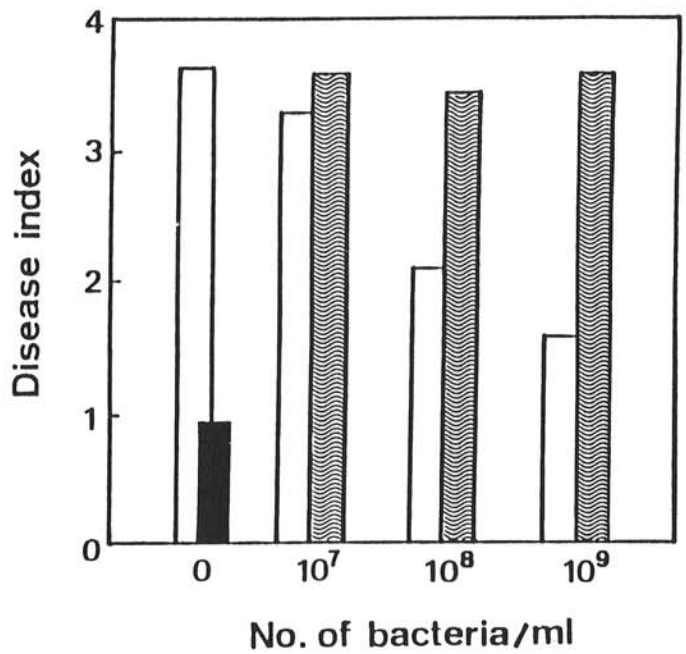

Fig. 5. Evaluation of the protective effect of A-16 against Fusarium wilt. Tomato plants pretreated with A-16 (open column) or U-10A (striped column) were inoculated with Race 1 and incubated for 1 mo. The dark column represents the nontreated and noninfected plants. The protective effects were evaluated according to the following disease index and equation: 0, healthy; 1, slightly retarded growth without any characteristic disease symptom; 2, retarded growth and yellowing of lower leaves; 3, partial wilting; and 4, complete wilting. Evaluation values = $(4a + 3b + 2c + 1d + 0e)/n$, where a–e represent the numbers of plants showing the disease index of 4–0, respectively, and n represents the total number of plants tested. Ten plants were prepared for each treatment for each replicate. Data are expressed as means of three replications of the complete experiment.

with avirulent strains (10,11). However, this toxin could hardly explain host specificity because it is produced by a wide range of formae speciales of *Fusarium* species. Nevertheless, fusaric acid is probably an important virulence factor in pathogenesis in view of its toxicity to plant tissues. This interpretation is supported by recent work of Shahin and Spivey (16), who reported that fusaric acid-resistant regenerants derived from tomato leaf protoplasts were resistant to *F. oxysporum* f. sp. *lycopersici* Race 2. Thus, detoxification of fusaric acid by some means would possibly protect infected plants from this pathogen. The successful protection against Fusarium wilt by use of a fusaric acid-detoxifying mutant not only strongly supports the concept that fusaric acid is involved in wilting activity of *Fusarium* species, but it also contributes to the development of a new protection system. This system could be generally applied to Fusarium diseases of many important crops because wide ranges of *Fusarium* fungi are known to produce this toxin in infected plants.

The principle devised in this study is the first verification of a theoretically constructed procedure for the control of fungal disease by bacteria. At first we determined if an avirulent strain of *P. solanacearum* was effective against Fusarium wilt. An isolated avirulent strain (U-10A) did not effectively suppress the fungal growth either in culture or in treated plants. We then isolated a fusaric acid-detoxifying mutant from the avirulant strain U-10A to determine the significance of fusaric acid detoxification in the wilt symptom and to evaluate its ability to protect plants from the disease. The stable existence of protecting bacteria within plants is a prerequisite for long-term protection. Fusaric acid is a nonspecific and highly toxic compound to various kinds of microorganisms (2,10). In fact, no bacterium was detected on fusaric acid-containing media plated with the homogenates of tomato cuttings which had not been administered A-16. This property of fusaric acid allowed us to easily locate fusaric acid-resistant bacteria in plant materials.

In the present study, a number of fusaric acid-resistant bacteria were induced by mutagenizing U-10A, but only a few mutants (three of about 500 resistant mutants tested) were able to detoxify fusaric acid, indicating that fusaric acid resistance is not always accompanied by fusaric acid detoxification. These mutants, which are fusaric acid-resistant but incapable of detoxifying fusaric acid, did not protect tomato cuttings from the effects of fusaric acid (data not shown).

Although the mechanism of the detoxification of fusaric acid by A-16 has not been fully elucidated in the present study, our gas chromatographic analysis of A-16 culture filtrate indicated the disappearance of a fusaric acid-specific peak. In the present analytical conditions, the detection of fusaric acid depends on the presence of a carboxy group in an alpha-position to the nitrogen because trimethylchlrosilane reacts only with a carboxy group to form trimethylsilylester. Thus, the result suggests that fusaric acid could be cleaved, or the carboxy group might be lost or modified by A-16. A comparative study of toxicity of fusaric acid and synthetic pyridine derivatives indicated that the carboxy group and the aliphatic side chain in the beta-position are essential for toxicity (16). Biochemical analysis of the mechanisms of detoxification is under way.

We expect that the present study will open a new avenue for the biological control of plant diseases. One possible extension of this study is to isolate the gene for fusaric acid detoxification, clone it in *Escherichia coli,* and then transform bacteria that inhabit vascular tissue to obtain promising candidates for biocontrol agents. Our preliminary test indicated that tomato plants treated with fusaric acid-detoxifying transformants of *E. coli* were resistant to fusaric acid (R. Utsumi et al, *unpublished*). Our studies may also provide the basis for molecular cloning of important genes associated with pathogenicity.

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