Reduction of Infection by *Pseudomonas syringae* pv. *tomato* Using a Nonpathogenic, Copper-Resistant Strain Combined with a Copper Bactericide

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ABSTRACT

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A nonpathogenic Tn*5* mutant of *Pseudomonas syringae* pv. *tomato* retained its ability to survive epiphytically on tomato leaves, and coinoculations of the mutant with a pathogenic strain significantly reduced the incidence of bacterial speck in greenhouse tests. When a derivative of the nonpathogen with resistance to high levels of copper was coinoculated with a copper-sensitive pathogenic strain to plants treated with a copper bactericide, significantly greater control was achieved than with either the nonpathogen or the copper treatment alone.

Control of bacterial speck of tomato (*Lycopersicon esculentum* Mill.), caused by *Pseudomonas syringae* pv. *tomato* (Okabe) Young et al, has been reported using copper compounds and antibiotics (6,15,16,22). However, these treatments are not always effective (8,16,19), especially when environmental conditions are highly favorable for disease development (10). Reduced effectiveness of copper compounds has also been associated with copper resistance in *P. s.* pv. *tomato* (Cooksey, *unpublished*), which was widespread in southern California isolates of *P. s.* pv. *tomato* (2,7). Similarly, copper resistance in *Xanthomonas campestris* pv. *vesicatoria* has been associated with reduced effectiveness of copper sprays (1,17).

Several reports have shown that *P. s.* pv. *tomato* can colonize tomato surfaces and survive for extended periods as an epiphyte (5,20,21). The ability to survive epiphytically on resistant as well as susceptible tomato varieties (5) suggests that this ability is independent of pathogenicity. If a nonpathogenic strain could be found that retained its ability to colonize surfaces epiphytically, then it might be possible to use such a strain to compete with the pathogen for colonization of infection sites and achieve a biological control of bacterial speck (12,18). In addition, such a control method could be integrated with conventional copper sprays if the nonpathogen carried copper resistance genes, such as those already cloned and characterized from *P. s.* pv. *tomato* (3). This paper reports the use of a nonpathogenic mutant of *P. s.* pv. *tomato* with copper resistance for biological control of bacterial speck and the compatibility and enhancement of this control method with copper sprays.

MATERIALS AND METHODS

Bacterial strains and inoculum. *P. s.* pv. *tomato* strains PT12 and PT23 were isolated from bacterial speck lesions on plants in southern California tomato fields in 1982 and 1983, respectively. PT12 is a copper-sensitive strain, and PT23 carries the copper-resistance plasmid pPT23D (2,3,7). The minimum inhibitory concentration (MIC) of cupric sulfate as previously defined (2) was 0.6 mM for PT12 and 1.8 mM for PT23. PT23.2 is a spontaneous rifampicin-resistant mutant of PT23 (4). A nonpathogenic mutant of PT23.2, designated PT23.200, was obtained by transposon mutagenesis and screening of mutants on tomato plants as previously described (2,4). A spontaneous mutant of PT23.200 that grew on media containing 3.2 mM cupric sulfate was designated PT23.201.

Inoculum was prepared by growing each strain on mannitol-glutamate (11) agar supplemented with yeast extract at 0.25 g/L (MGY medium) for 2 or 3 days at room temperature, resuspending in distilled water, and diluting to the appropriate cell densities using Klett-Summerson colorimeter readings based on previous dilution plating results.

Survival of the nonpathogenic mutant on tomato leaves. Populations of PT23.2 and PT23.200 were compared over an 8-day period after either spraying onto leaf surfaces or infiltrating into leaf tissues. Tomato seedlings were grown in a greenhouse (three plants/4-in. pot) and inoculated at 4–5 wk after four fully expanded leaves had developed. Greenhouse temperatures ranged from a low of 17–19 C at night to a high of 28–32 C during the day. Plants were grown under natural light during the winter and spring months for these experiments. Pots were irrigated twice daily with an automatic drip system; no attempts were made to maintain wetness of foliage by misting or to alter the humidity of the greenhouse. For spray inoculations, bacteria were suspended in distilled water to about 2×10^7 colony-forming units (cfu) per milliliter and applied with a hand sprayer until leaves were evenly wetted. For infiltrations, bacteria suspended to about 2×10^7 cfu/ml were inoculated with a Hagborg device (9) to produce circular water-soaked areas of 1.5 cm in diameter or greater on each of the three terminal leaflets of the fourth leaf of each plant. The water-soaked area was marked lightly with a pen, and leaf disks were cut from within this area on each of the sample dates. At each sample date for both spray-inoculated and infiltrated plants, a leaf disk was cut with a 1.2-cm cork borer from each of three terminal leaflets on the fourth leaf of each of three plants in a pot. These nine leaf disks were pooled, and this was replicated four times for each sample date. Pooled leaf disks were blended in 20 ml of distilled water and plated in serial dilutions onto MGY agar containing cycloheximide (100 μg/ml) and either rifampicin (40 μg/ml) for PT23.2 or rifampicin and kanamycin (25 μg/ml) for PT23.200.

Biological and chemical control tests. Tomato seedlings were grown individually in 4-in. pots in the greenhouse for 4–5 wk before inoculation. Greenhouse temperatures and irrigation methods were as described above, with no attempts to increase humidity or leaf wetness. Kocide 101 (cupric hydroxide) was sprayed on plants at the label rate (2.4 g/L) with Rohm and Haas B1956 spreader-sticker at 0.3 ml/L 1 day before inoculation. Bacteria were suspended in distilled water and diluted to a final concentration of about 2×10^7 cfu/ml for PT12 and 5×10^8 cfu/ml for PT23.200 and PT23.201. Inoculation of PT12 at a concentration of 2×10^7 cfu/ml had consistently produced 10–200 lesions per leaflet in previous experiments, which closely

approximated the disease severity we had observed in field outbreaks of bacterial speck; lower inoculum levels produced too few lesions to obtain quantitative differences between treatments, and higher inoculum levels produced to many lesions to count accurately. For coinoculations, PT12 and PT23.200 were suspended to twice their final concentrations and mixed in a 1:1 ratio to keep the inoculum dosage of PT12 constant throughout the treatments. Approximately 10 ml of bacterial suspension was sprayed on each plant (to runoff) with a hand sprayer from a distance of 6 in.; the plants were removed from greenhouse benches to a separate area for inoculations to prevent drift of the spray between treatments. Three consecutive experiments were performed with 6–14 replications per experiment arranged in randomized complete blocks on greenhouse benches. Two weeks after inoculation, bacterial speck lesions were counted on the terminal leaflet of the third, fourth, fifth, and sixth leaves of each plant.

RESULTS

Populations of the nonpathogenic mutant PT23.200 of *P. s.* pv. *tomato* initially decreased when sprayed onto tomato leaves until the second to fourth days after inoculation, when populations became more stable at about 1.4×10^3 cfu/cm^2 to 5.5×10^3 cfu/cm^2 (Fig. 1A). When infiltrated into leaf tissues, the mutant survived well and maintained populations of about 2×10^4–2×10^5 cfu/cm^2 of leaf area (Fig. 1B). Populations of the pathogenic parental strain PT23.2 reached much higher levels than the nonpathogenic mutant when sprayed onto leaf surfaces or infiltrated into leaf tissues. This result was expected, since a pathogenic response was observed by the fourth day after infiltrating PT23.2, and numerous lesions were visible by the fourth day after spraying PT23.2 onto leaf surfaces.

The use of the nonpathogenic mutant PT23.200 significantly reduced the incidence of bacterial speck lesions when it was coinoculated with the pathogenic strain PT12 in three greenhouse experiments (Table 1). Coinoculation with the nonpathogenic strain PT23.201, which was selected for higher levels of copper resistance from PT23.200, reduced the incidence of speck lesions significantly more than coinoculations with PT23.200. When PT23.200 was coinoculated with PT12 to plants previously treated with Kocide 101, better reduction was provided than with the coinoculation of PT12 and PT23.200 in the absence of Kocide 101, but in only one of the trials was this reduction significantly greater that the preinoculation treatment with Kocide 101 alone. However, in all three trials, coinoculation of PT23.201 and PT12 following Kocide 101 treatment provided a significantly greater reduction in lesion numbers than either preinoculation treatment with Kocide 101 alone or the coinoculation of PT23.201 and PT12 without Kocide 101.

DISCUSSION

Reduction of bacterial speck of tomato was achieved in greenhouse tests using a nonpathogenic Tn*5* mutant of *P. s.* pv. *tomato*. In addition, when applications of Kocide 101 preceded coinoculations with the nonpathogenic copper-resistant mutant PT23.201 and a copper-sensitive pathogenic strain, a significantly greater reduction was achieved than with either the Kocide 101 preinoculation treatment alone or the coinoculation of PT23.201 and the pathogen without the Kocide 101 treatment. It is not known why PT23.201, with resistance to high levels of copper, provided a greater reduction of speck lesions in the absence of Kocide 101 applications than its moderately copper-resistant parent strain PT23.200; further tests will examine the epiphytic survival of PT23.201 compared with PT23.200 to help clarify this point. Our results suggest that nonpathogenic derivatives of *P. syringae* pathovars might be effective biological control agents for diseases caused by their pathogenic parental strains. Similarly, ice

TABLE 1. Incidence of bacterial speck of tomato caused by copper-sensitive strain PT12 of *Pseudomonas syringae* pv. *tomato* after treatment with Kocide 101 and coinoculation with nonpathogenic copper-resistant strains PT23.200 and PT23.201

Inoculum[x]	Preinoculation treatment with Kocide 101[y]	Lesions per leaflet[z] (no.) Trial 1	Trial 2	Trial 3
PT12	−	115.0 a	23.6 a	5.2 a
	+	10.0 c	5.4 c	2.4 b
PT12 + PT23.200	−	36.0 b	10.7 b	2.2 b
	+	11.6 c	4.3 c	1.0 c
PT12 + PT23.201	−	19.6 c	3.6 c	0.6 c
	+	4.8 d	1.4 d	0.3 d

[x] Inoculum concentrations were approximately 2×10^7 cfu/ml for PT12 and 5×10^8 cfu/ml for PT23.200 and PT23.201.

[y] Kocide 101 was applied at the label rate of 2.4 g/L 1 day before bacterial inoculations.

[z] Mean of four leaflets from 12, 14, and 6 plants in trials 1, 2, and 3, respectively. Data were log-transformed before statistical analysis. The values presented are antilogs of the transformed means. Values followed by the same letter within a column do not differ significantly ($P = 0.05$) according to the Student-Newman-Keuls' test.

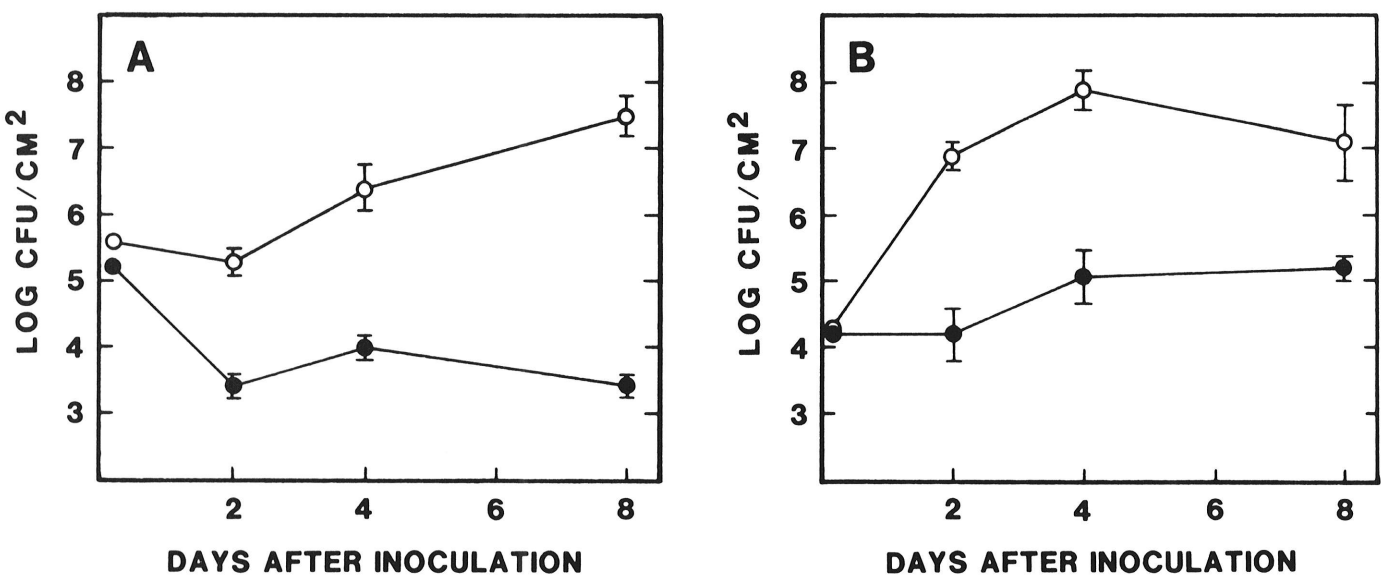

Fig. 1. Populations of *Pseudomonas syringae* pv. *tomato* PT23.2 (o) and the nonpathogenic Tn*5* mutant PT23.200 (•) after **A,** spraying or **B,** infiltrating tomato leaves. Data are means of four replicates, and vertical bars indicate standard deviations.

nucleation-deficient mutants of *P. syringae* and *P. fluorescens* were shown to effectively inhibit ice nucleation by their ice+ counterparts (13,14). Our results suggest that if the nonpathogenic mutants are also copper-resistant, then the application of such agents could be compatible with, and enhanced by, conventional copper sprays. We are presently constructing strains with stable deletions in pathogenicity genes, instead of Tn*5* insertions, and with copper resistance genes stably incorporated into the genome, rather than on a plasmid, for field tests of this control method.

Our greenhouse tests involved only a copper-sensitive pathogenic strain, and since a high proportion of *P. s.* pv. *tomato* strains from southern California were shown to be copper-resistant (2), it is probable that the copper treatment would not be as effective in the field. However, copper applications could enhance the ability of the copper-resistant biological control agent to compete with other epiphytic species and colonize leaf surfaces. Copper applications might also enhance the ability of the native copper-resistant pathogens to compete with other species, and this possibility points to the need for alternatives to copper bactericides.

The nonpathogenic mutant survived epiphytically on tomato leaves over an 8-day period, which further supports the idea that epiphytic survival is independent of pathogenicity. Experiments are planned to determine whether the epiphytic populations of nonpathogenic strains can provide a protection against pathogenic strains inoculated several days after the nonpathogen. In addition, the minimum level of the nonpathogenic strain that will provide disease reduction needs to be determined.

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