Biological Control of Bacterial Speck of Tomato Under Field Conditions at Several Locations in North America

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ABSTRACT

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Bacterial speck of tomato, caused by *Pseudomonas syringae* pv. *tomato*, continues to be a problem for tomato growers worldwide. A collection of nonpathogenic bacteria from tomato leaves plus *P. syringae* strains TLP2 and Cit7, *P. fluorescens* strain A506, and *P. syringae* pv. *tomato* DC3000 *hrp* mutants were examined in a greenhouse bioassay for the ability to reduce foliar bacterial speck disease severity. While several of these strains significantly reduced disease severity, *P. syringae* Cit7 was the most effective, providing a mean level of disease reduction of 78% under greenhouse conditions. The *P. syringae* pv. *tomato* DC3000 *hrpA*, *hrpH*, and *hrpS* mutants also significantly reduced speck severity

under greenhouse conditions. The strains with the greatest efficacy under greenhouse conditions were tested for the ability to reduce bacterial speck under field conditions at locations in Alabama, Florida, and Ontario, Canada. *P. syringae* Cit7 was the most effective strain, providing a mean level of disease reduction of 28% over 10 different field experiments. *P. fluorescens* A506, which is commercially available as Blight-Ban A506, provided a mean level of disease reduction of 18% over nine different field experiments. While neither *P. syringae* Cit7 nor *P. fluorescens* A506 can be integrated with copper bactericides due to their copper sensitivity, there exist some potential for integrating these biological control agents with "plant activators", including Actigard. Of the *P. syringae* pv. *tomato* DC3000 *hrp* mutants tested, only the *hrpS* mutant reduced speck severity significantly under field conditions.

Bacterial speck disease of tomato, caused by *Pseudomonas syringae* pv. *tomato*, occurs worldwide (9,11-14,19,23,31,48,51-53,55,56). In North America, bacterial speck is most prevalent in processing tomatoes in the Midwestern United States and Ontario, Canada, and in fresh-market tomatoes in the mountain areas of North Carolina. The disease occurs occasionally in processing tomatoes in California when the spring is particularly cool and wet, and is similarly sporadic in fresh-market tomatoes in Florida and Virginia. Yield reductions result from the reduced photosynthetic capacity of infected foliage and from lesions on the fruit that render them unsuitable for the fresh market.

While copper-based bactericides (13,16,57,73) have been used to control bacterial speck, the rapid development of plasmid-mediated copper-tolerance has rendered these compounds relatively ineffective (1,5,48,56). Combining these compounds with the ethylene bisdithiocarbamate (EBDC) fungicides, maneb or mancozeb, has provided improved levels of control even with copper-tolerant populations (16). However, these copper-EBDC combinations are only moderately effective and are possibly carcinogenic. The antibiotic streptomycin also has been used to control bacterial speck (13,16), but resistance is common in pathogen populations (55) and thus, the antibiotic is rarely used. The recently introduced "plant activators" appear promising for control of bacterial diseases of fruits and vegetables. One such plant activator, acibenzolar-S-methyl (CGA-245704 or Actigard [Syngenta, Greensboro, NC]), provides good control of both speck and spot of tomato (42,53). These first generation plant activators are not the final

solution, however, since they may also reduce fruit yield when applied at recommended application rates and frequencies and suppress defense pathways effective against insect pests (58).

Resistance to race 0 of *P. syringae* pv. *tomato*, conferred by the gene *Pto* (47,49,50), has been introduced into several processing tomato varieties but relatively few fresh-market tomato varieties. Unfortunately, hybrid varieties heterozygous for *Pto* are not completely resistant to speck (25,46). Furthermore, the *Pto* gene normally does not confer resistance to race 1 of the pathogen (9,24,31) and will not confer disease resistance where this race is present (19). Transgenic plants overexpressing either *Pto* (59) or *Prf* (46) appear to be resistant to this race, however, it remains to be seen whether such transgenic lines will be commercialized.

In recent years, there has been substantial interest in the biological control of plant pathogens (3,41,67-70,72). While biological control agents have been introduced successfully into commercial horticulture for control of crown gall (10), caused by *Agrobacterium tumefaciens*, and fire blight of pear (39), caused by *Erwinia amylovora*, commercial biological control of diseases caused by pathovars of *P. syringae* has not yet been implemented. However, frost injury caused by ice nucleation active (Ice^+) *P. syringae* strains can be reduced substantially by applying non-pathogenic non-ice nucleation active (Ice^-) *P. syringae* strains (33-37) or other Ice^- organisms such as *P. fluorescens* A506 (BlightBan A506) (39). Biological control of frost injury is effective because the incidence of frost injury is proportional to the logarithm of the population size of Ice^+ *P. syringae* (38), and prior colonization of a leaf or blossom by an Ice^- organism reduces the attainable population size of an immigrant Ice^+ *P. syringae* (33, 36). In theory, it should be possible to use this process called preemptive competitive exclusion to control any bacterial disease in which incidence and/or severity are related to the population

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size of the pathogen on the leaf or blossom surface. Such a relationship has been demonstrated for *P. syringae* pv. *syringae* (32), but not for *P. syringae* pv. *tomato* though this pathogen has been reported to colonize tomato leaf surfaces “epiphytically” (2,6,24, 44,54,57,66). Although small-scale biological control experiments have been performed for several *P. syringae* pathovars including *P. syringae* pv. *glycinea* on soybean (43,63–65), *P. syringae* pv. *savastanoi* on olive (62), *P. syringae* pv. *syringae* on barley (7,8) and bean (40), and *P. syringae* pv. *tomato* (4,12–15,17), no large-scale, multilocation field trials, such as the one described in this study, have been reported.

In this study, a greenhouse assay was used to evaluate the ability of nonpathogenic leaf-associated bacteria to reduce bacterial speck disease severity, and those strains with the most potential as biological control agents were tested under field conditions at multiple locations in North America. In addition to nonpathogenic bacteria isolated from tomato foliage, we tested the following: *P. fluorescens* A506, which previously had been shown to preemptively exclude Ice⁺ *P. syringae* strains and control frost injury (39); *P. syringae* strains TLP2 and Cit7 (35,36) because of the assumption that these closely related bacteria may be effective in preemptive exclusion of *P. syringae* pv. *tomato*; and *hrp* mutants of *P. syringae* pv. *tomato* because studies with undefined avirulent mutants (40,60–62) and *hrp* mutants (20–22,45) of other bacterial phytopathogens have shown that these strains are potential biological control agents. Our field studies also included Actigard and combinations of Actigard with either *P. syringae* Cit7 or *P. fluorescens* A506.

MATERIALS AND METHODS

Bacterial strains. Nonpathogenic bacteria were isolated from symptomless tomato leaves in Alabama (AL) and Florida (FL) on tryptic soy agar (TSA, Difco Laboratories, Detroit, MI) containing 100 µg/ml of cycloheximide to suppress fungi. Bacteria were tentatively identified using the Sherlock GC-FAME system (MIDI Inc., Newark, DE) and stored at –80°C in tryptic soy broth (TSB, Difco Laboratories) containing sterile 20% (vol/vol) glycerol. Bacterial strains *P. fluorescens* A506 (39), *P. syringae* TLP2, and *P. syringae* Cit7 (35) were provided by S. E. Lindow (University of California, Berkeley). BlightBan A506 was provided by S. Kelly (Plant Health Technologies, Boise, ID). *P. syringae* pv. *tomato* strain PT12 was isolated from tomatoes in California and was provided by D. A. Cooksey (University of California, Riverside). *P. syringae* pv. *tomato* strain DCT6D1 was isolated in Leamington, Ontario, Canada (18). *P. syringae* pv. *tomato* strain DC3000 was isolated in the United Kingdom (18). The *hrp* mutants (*hrpA*, *hrpH*, and *hrpS*) of *P. syringae* pv. *tomato* DC3000 were provided by S. Y. He (Michigan State University, East Lansing).

Greenhouse experiments. Tomato (cv. Agriset 761) seeds were sown in 3 × 3 × 4-cm cell packs containing Promix (Premier, Red Hill, PA). After 2 to 3 weeks, seedlings were transplanted in 10.2-cm square plastic pots. Plants were maintained with weekly fertilization alternating 15-16-17 complete fertilizer with 15-5-15 N-P-K. Four- to five-week-old tomato plants (approximately 15 to 20 cm high) were placed under intermittent mist on a greenhouse bench for 24 h prior to inoculation with potential biological control agents. All bacterial strains were cultured on TSA at 28°C for 24 h, except A506, TLP2, Cit7, and DC3000 *hrp* mutants, which were cultured on King’s medium B (29) amended with rifampicin at 100 µg/ml. Bacteria were suspended in sterile phosphate buffer (0.01 M, pH 7.0), and suspensions were adjusted to 1 × 10⁸ CFU/ml using a spectrophotometer. Suspensions were inoculated on the abaxial and adaxial surfaces of tomato leaves until runoff using a hand-held sprayer, and plants were incubated under intermittent mist for 24 h. Treatments were arranged in a randomized complete block (RCB) design with four replications (where each replicate

consisted of a single plant). Plants were subsequently challenged with a suspension of the pathogen *P. syringae* pv. *tomato* strain PT12, except for experiments with *hrp* mutants, which were challenged with the wild-type parent DC3000, at a concentration of 1 × 10⁸ CFU/ml. Plants were incubated under intermittent mist until symptom appearance, at which time the misting was terminated.

Ten leaflets were randomly sampled from each of the four plants per treatment on a single occasion between 7 and 10 days after inoculation with the pathogen. Lesions were counted, and leaf area determined for each leaflet using an image analysis system (Agvvision, Decagon Devices, Inc., Pullman, WA). Disease severity data [$\log_{10}$ ((lesions + 1)/cm²)] were analyzed by analysis of variance (ANOVA) or GLM procedures of SAS (SAS Institute, Cary, NC). Means were compared using Duncan’s Multiple Range (DMR) test at *P* = 0.05. Bacterial strains that provided the highest level of disease reduction (compared to the pathogen-only control) were selected for field experiments.

Field experiments. Field experiments were conducted in Shorter, AL (spring 1996, 1997, and 1998), Bradenton, FL (spring 1996 and 1997), and London, ON, Canada (ON) (spring 1996 and 1997). Five- to six-week-old transplants were planted in raised beds covered with polyethylene mulch, which had been fumigated with 98% methyl bromide at 400 lb/A (450 kg/ha) (AL and FL), or bare ground (ON) and placed 0.5 m apart on 1.2-m centers. Plants were irrigated through drip irrigation (AL) or sip irrigation (FL), and fertilizer was applied at recommended rates through the same system. Field plots were arranged either in an RCB design with five (AL and FL) or four (ON-1997) replications or in a Latin square design with four replications (ON-1996). Bacteria were grown on TSA or King’s medium B (AL and FL) for 24 h at 28°C prior to inoculation, washed from plates, and suspended in phosphate buffer (0.03 M, pH 7.4). Suspensions were adjusted to a concentration of 1 × 10⁸ CFU/ml using a spectrophotometer. The suspensions were applied weekly to tomato foliage using a CO₂-powered backpack sprayer until leaves were visibly wet. In ON, bacteria were grown in nutrient broth (18) at 25°C overnight on a shaker (150 rpm) to 2 to 4 × 10⁹ CFU/ml, pelleted by centrifugation, and resuspended in sterile distilled water to 3 to 6 × 10⁸ CFU/ml. The suspensions were applied weekly to tomato foliage using an RL-FLO-Master (Root-Lowell Mfg. Co., Lowell, MI) sprayer until leaves were visibly wet. The chemical treatments, copper alone, copper plus an EBDC, or Actigard were applied according to label specifications. Biological and chemical treatments were applied 8 to 10 times during the season. Field plots were inoculated with a suspension of *P. syringae* pv. *tomato* strain PT12 (AL) or DCT6D1 (ON). Natural infection was present in FL, but where no disease was seen, plants were inoculated once with *P. syringae* pv. *tomato* strain PT12 (≈10⁸ CFU/ml). Field experiments in ON were conducted under permit (Health Canada Research Permit No. 58-RP-96). Field experiments in AL with *P. syringae* pv. *tomato* strain DC3000 *hrp* mutants were conducted under permit (APHIS 97-023-01r).

In the AL field trials, foliar disease severity was rated either visually as a percentage of necrotic foliage (0 to 100%) per replicate row (1996) or by sampling 20 to 50 leaflets per replicate row and counting lesions (1996, 1997, and 1998). Incidence was based on the presence or absence of lesions on sampled leaflets (1996). In ON, foliar disease incidence was expressed as the number of leaflets out of 50 showing speck-like lesions (24 50-leaflet samples per treatment), and disease severity was expressed as the number of speck-like lesions on 20 randomly sampled leaflets (16 20-leaflet samples per treatment). In FL, foliar severity was based on a Horsfall-Barratt scale where: 1 = 0%; 2 = 0 to 3%; 3 = 3 to 6%; 4 = 6 to 12%; 5 = 12 to 25%; 6 = 25 to 50%; and 12 = 100% defoliation.

Determination of bacterial population sizes. In AL, *P. fluorescens* A506, *P. syringae* TLP2, and *P. syringae* Cit7 population dynamics were determined to assess the ability of these

biological control agents to survive on tomato foliage under field conditions. As plants were inoculated weekly with the biological control agents, leaflets were sampled immediately prior to inoculation to assess survival since the last inoculation. Five leaflets per replicate row per treatment (a total of 25 leaves per treatment) were sampled randomly, placed individually in sterile plastic bags, and transported back to the laboratory on ice. Twenty milliliters of sterile phosphate buffer (0.01M, pH 7.0) was added to each bag. Leaflets were sonicated for 10 min to dislodge bacterial cells and agitated briefly to suspend the bacterial cells. The washwater was diluted by an amount appropriate to the sample and plated on King's medium B amended with rifampicin at 100 µg/ml and cycloheximide at 100 µg/ml using a spiral plater (Autoplate 3000, Spiral Biotech, Bethesda, MD). Following incubation for 72 h at 28°C, colonies were enumerated using a laser counter (Spiral Biotech).

In ON, two 10-g leaf samples were randomly collected from the 40 plants of each replicate of each treatment at selected times (as indicated in the Figures) throughout the growing season. Each 10-g sample was assayed for *P. syringae* pv. *tomato* populations as described previously (18). The *P. fluorescens* A506 populations were determined by plating the various sample dilutions on King's medium B amended with rifampicin at 50 µg/ml, streptomycin at 10 µg/ml, and cycloheximide at 75 µg/ml.

RESULTS

Greenhouse experiments. *P. fluorescens* A506, *P. syringae* TLP2, and *P. syringae* Cit7 provided moderate to high levels of disease control in all greenhouse experiments. Over the course of 14 to 17 experiments in the greenhouse, Cit7 provided a mean level of control of 78.4% (standard deviation (SD) = 8.9%), A506 provided a mean level of control of 61.2% (SD = 16.4%), and TLP2 provided a mean level of control of 59.8% (SD = 15.5%) (data not shown). Not only did Cit7 provide a significantly ($P < 0.0001$) higher mean level of disease control than TLP2 or A506, but the variability in the level of control as measured by standard deviation was also considerably less. During these experiments, the mean level of disease severity in the pathogen-only control varied between approximately 0.3 and 2.5 lesions per cm² perhaps due to fluctuating conditions in the greenhouse. There was, however, no significant correlation between the level of control provided by these three biological control agents and the disease severity in the pathogen-only control (data not shown).

Approximately 70 nonpathogenic bacterial strains isolated from tomato foliage were tested in the greenhouse assay for the ability to reduce bacterial speck disease severity. From this first round of

screening of the 70 strains, four were selected for field studies in AL and FL during that year (1996) based upon the level of disease reduction being equal to or greater than that provided by Cit7, TLP2, and A506: Cu9 (69.4% reduction), Cu14 (71.0% reduction), B52 (73.3% reduction), and B41 (79.5% reduction) (data for other strains not shown). A subset of 36 of these 70 strains, including Cu9, Cu14, B52, and B41, were tested in an additional two experiments and the mean level of disease reduction across three experiments was calculated: Cu14 (mean 28.6% reduction), Cu9 (mean 48.2% reduction), B52 (mean 56.2% reduction), and B41 (mean 65% reduction) (data for other strains not shown).

Under greenhouse conditions, the *P. syringae* pv. *tomato* strain DC3000 *hrp* mutants provided significant ($P = 0.05$) reductions in bacterial speck severity, however, none of the *hrp* mutants were as effective as Cit7, TLP2, or A506 (data not shown). Since the *hrp* mutants provided significant reductions in severity, they were employed in a field experiment in 1997.

Field experiments: 1996. The four nonpathogenic bacterial strains, Cu9, Cu14, B52, and B41 that were selected from the first round of screening, and strains Cit7, TLP2, and A506 were tested for efficacy against bacterial speck under field conditions in AL and FL (Tables 1 and 2). Due to regulatory constraints, only Cit7, TLP2, and A506 were included in the field experiments in ON (Table 3). Copper-based bactericides, alone or in combination with mancozeb, were included for comparison. Since the mean level of reduction in disease provided by Cu9, Cu14, B52, and B41 in greenhouse experiments was much lower than the initial level determined during the single round of screening, and since the levels of control in the field (Tables 1 and 2) were, in general, not superior to those provided by the well characterized strains Cit7, TLP2, and A506, it was decided not to include these strains in subsequent field experiments.

Field experiments: 1997. In 1997, experiments were conducted successfully in only AL and ON (Tables 4 and 5). The experiment in AL incorporated the *P. syringae* pv. *tomato* DC3000 *hrp* mutants in addition to Cit7, TLP2, and A506 (Table 4). Additionally, *P. putida* B56 that provided a mean level of control in the greenhouse of 61% (mean of three experiments), and was also effective against the bacterial spot pathogen (M. Wilson, J. M. Byrne, H. L. Campbell, A. C. Dianese, J. B. Jones, and D. A. Cuppels, unpublished data) was incorporated into this experiment (Table 4). Of the *P. syringae* pv. *tomato* DC3000 *hrp* mutants, only the *hrpS* mutant significantly ($P = 0.05$) reduced disease severity and then only in the first disease assessment. Since the *hrp* mutants did not show the potential to provide higher levels of control of speck than the well-characterized strains Cit7, TLP2, and A506, they were not included in subsequent field experiments.

TABLE 1. Efficacy of biological control agents in the control of bacterial speck (foliar incidence, foliar severity, and fruit incidence) on tomato (cv. Agriset 761) in a field experiment conducted in Alabama (spring 1996)

	Foliar incidence ^t	Foliar severity ^u	Foliar incidence ^v	Foliar severity ^w	Fruit incidence ^x
Control	71.1 a ^y	18.3 a ^y	60.1 a ^y	28.8 a ^y	1.7 a ^y
Kocide DF	29.2 c (71.2) ^z	3.3 c (81.9) ^z	37.9 d (49.1) ^z	15.0 cd (70.2) ^z	0.5 a (56.1) ^z
BlightBan A506	46.5 b (37.5)	11.7 abc (36.0)	47.7 c (26.7)	23.3 ab (34.7)	1.3 a (32.9)
<i>Pseudomonas fluorescens</i> A506	49.9 b (30.7)	15.8 ab (13.6)	54.0 abc (12.4)	26.7 ab (14.0)	0.9 a (34.8)
<i>P. syringae</i> TLP2	50.8 b (28.6)	15.8 ab (13.6)	51.9 abc (16.9)	25.4 ab (20.6)	0.8 a (51.7)
<i>P. syringae</i> Cit 7	46.5 b (37.5)	8.2 bc (55.2)	49.7 bc (22.2)	20.9 abcd (45.0)	0.3 a (80.4)
B41	49.3 b (31.6)	13.3 ab (27.3)	52.4 abc (16.1)	23.3 ab (34.7)	1.6 a (0)
B52	50.4 b (29.6)	15.5 ab (15.3)	59.8 a (1.0)	19.8 bcd (48.3)	0.5 a (52.0)
Cu9	51.4 b (27.7)	13.3 ab (27.3)	59.4 ab (1.0)	20.0 bcd (41.3)	0.4 a (67.6)
Cu14	52.5 b (25.6)	14.2 ab (22.4)	51.6 abc (17.8)	23.6 ab (27.7)	0.4 a (71.6)

^t Sample of 20 leaflets per replicate taken 4 June 1996.

^u Visual ratings of foliar disease severity (0 to 100%) made 6 June 1996.

^v Sample of 50 leaves per replicate taken 6 June 1996.

^w Visual ratings of foliar disease severity (0 to 100%) made 12 June 1996.

^x Percent infected fruit based on a complete harvest made 13 June 1996.

^y Means in a column followed by the same letter are not significantly different at $P = 0.05$ according to Duncan's multiple range test.

^z Percent disease reduction compared to pathogen-only control.

The experiment in ON incorporated *Paenibacillus* sp. BC87, which provided moderate levels of control in separate greenhouse experiments in ON (data not shown), in addition to Cit7 and A506 (Table 5). This was also the first experiment to attempt to integrate *P. fluorescens* A506 and Actigard. In the first disease assessment, the level of disease reduction achieved by this combination was significantly ($P = 0.05$) higher than that provided by Actigard alone, warranting inclusion of such combination treatments in the 1998 field experiments.

Field experiments: 1998. In 1998, two experiments were conducted successfully in AL (Tables 6 and 7). These experiments incorporated only Cit7, TLP2, A506, or those strains in combination with Actigard (applied at recommended rates). Only in one disease assessment was the level of disease reduction achieved by a biological control agent in combination with Actigard significantly ($P = 0.05$) higher than that provided by Actigard alone (i.e., Cit7 plus Actigard [Table 7]).

Field experiments: Summary. Across all the field experiments, under different climatic conditions and horticultural practices, and with different application methods and users, *P. syringae* strain Cit7 was the most effective biological control agent providing a mean reduction in bacterial speck severity of 28.3%, compared to 18.0 and 14.9% for A506 and TLP2, respectively (Table 8). In contrast to the greenhouse studies, however, the variability was not considerably lower for Cit7 than for the other two strains (Table 8).

Population dynamics of biological control agents. The population dynamics of the biological control agents were determined

to assess the survival of these organisms under different environmental conditions and horticultural practices. In AL, the biological control agents were applied on a weekly basis; leaflet samples to assess population sizes were taken just prior to that application (i.e., population size on the leaves represents that which persisted from the inoculation 7 days prior to the sampling). *P. syringae* strain TLP2 reached a slightly higher population size on tomato foliage than did *P. fluorescens* A506 in 1995 (Fig. 1A), and TLP2 and Cit7 reached slightly higher population sizes than A506 in 1996 (Fig. 1B), however, these differences were not statistically significant. In 1995, TLP2 maintained a peak population size of approximately 1×10^4 CFU/leaflet while A506 maintained a peak population size of approximately 2×10^3 CFU/leaflet (Fig. 1A). In 1996, Cit7 and TLP2 maintained a peak population size of approximately 3.2×10^4 CFU/leaflet, while A506 increased throughout the season reaching approximately 1.6×10^4 CFU/leaflet (Fig. 1B).

In ON, the population size of *P. fluorescens* A506 increased slowly throughout the season and reached approximately 1×10^5 CFU/g fresh weight in 1996 (Fig. 2) and approximately 3.2×10^3 CFU/g fresh weight 1997 (Fig. 3A) (Cit7 and TLP2 population sizes were not measured in ON).

Population dynamics of the pathogen. In ON, the population dynamics of *P. syringae* pv. *tomato* were determined on the foliage of plants treated with different biological control agents in order to provide another measure of efficacy (1996–Fig. 2; 1997–Fig. 3B). The only treatment that significantly reduced epiphytic pathogen population sizes was the Actigard/A506 combination used in 1997 (Fig. 3B).

DISCUSSION

The data presented in this study suggest that while there is potential for biological control of bacterial speck of tomato due to the relatively moderate levels of disease control achieved, any future use of foliar biological control agents is likely to be just one component of an integrated disease management program. The most effective biological control agent, bacterial strain *P. syringae* Cit7, achieved 15 to 50% reduction in foliar disease severity with a mean of 28% reduction when used in different tomato-growing regions of North America (AL, FL, and ON), under different climatic conditions, on different cultivars with different horticultural practices (including fungicide regimes), and different users. *P. syringae* Cit7 maintained an effective population size on the tomato leaflets when applied only once a week; a regime comparable to current chemical application frequencies. Unfortunately, Cit7 did not reduce the incidence of bacterial speck lesions on the fruit in either of the experiments in which this parameter was measured. Neither did Cit7 significantly reduce epiphytic population sizes of *P. syringae* pv. *tomato* in two different experiments in ON (Figs. 2 and 3B). This might suggest that incidence of speck on the fruit is correlated with epiphytic population size of *P. syringae* pv. *tomato* and not with foliar lesion number; hence,

TABLE 2. Efficacy of biological control agents in the control of bacterial speck (foliar incidence and severity) on tomato (cv. Agriset) in a field experiment conducted in Florida (spring 1996)

	Foliar severity ^{u,v}	Foliar severity ^{u,w}	Lesions/leaflet ^{u,x}
Control	4.9 a ^y	3.9	10.2 a ^y
BlightBan A506	4.9 a	4.0	7.7 ab (24.5) ^z
<i>Pseudomonas syringae</i> TLP2	4.9 a	3.9	7.3 ab (28.4)
<i>P. syringae</i> Cit7	4.8 a	4.0	7.7 ab (24.5)
<i>P. fluorescens</i> A506	4.9 a	3.9	7.8 ab (23.5)
B41	4.7 a	3.8	8.5 ab (16.7)
B52	4.8 a	3.6	8.9 ab (12.7)
Cu9	4.7 a	3.9	6.0 ab (41.2)
Cu14	4.8 a	3.7	5.5 ab (46.1)
Kocide 101	4.6 a	3.8	6.2 b (39.2)

^u Rating is based on Horsfall-Barratt scale where: 1 = 0%; 2 = 0 to 3%; 3 = 3 to 6%; 4 = 6 to 12%; 5 = 12 to 25%; 6 = 25 to 50%; 12 = 100% defoliation.

^v Rating made on 9 April 1996.

^w Rating made on 26 April 1996.

^x Ten leaflets from each plot were selected, and lesions were counted on each leaflet. The data were log-transformed prior to analysis.

^y Means in a column followed by the same letter are not significantly different at $P = 0.05$ according to a Duncan's multiple range test.

^z Percent disease reduction compared to pathogen-only control.

TABLE 3. Efficacy of biological control agents in the control of bacterial speck (foliar and fruit incidence) on tomato (cv. H9478) in a field experiment conducted in London, Ontario, Canada (summer 1996)

	Foliar incidence ^v	Foliar severity ^w	Foliar incidence ^x	Fruit yield (kg/10 plants)
Control	40.5 a ^y	182.0 a ^y	28.4 a ^y	44.3 a ^y
BlightBan A506	33.3 c (17.8) ^z	128.4 b (29.5) ^z	21.2 a (25.4) ^z	46.9 a
<i>Pseudomonas syringae</i> TLP2	37.4 b (7.7)	172.8 a (5.1)	22.6 a (20.4)	45.6 a
<i>P. syringae</i> Cit7	36.2 b (10.6)	133.3 b (26.8)	20.8 a (26.8)	51.6 a

^v Samples of 50 leaves per replicate taken 6 August 1996 (56 days after transplanting). Each number represents the mean number of leaves out of 50 showing bacterial speck-like symptoms.

^w Samples of 20 leaves per replicate taken 6 August 1996. Each number represents the mean number of bacterial speck-like lesions present in each 20-leaf sample.

^x Tomatoes were harvested on 12 September 1996 (101 days after transplanting), assessed for bacterial speck-like lesions, and weighed.

^y Means in a column followed by the same letter are not significantly different at $P = 0.05$ according to Duncan's multiple range test.

^z Percent disease reduction compared to pathogen-only control.

although Cit7 reduced foliar disease severity, there was no impact on incidence of disease on the fruit.

The data presented here justify further investigation of the effect of Cit7 on the marketable tomato yield. It should be noted that while by most standards *P. syringae* Cit7 could be considered a successful biological control agent, particularly since it has also been shown to reduce bacterial spot severity (M. Wilson, J. M. Byrne, H. L. Campbell, A. C. Dianese, J. B. Jones, and D. A. Cuppels, *unpublished data*), unless marketable yield is increased, this strain likely has no potential for commercial development. Marketable yield is determined by total yield and the incidence of speck on the fruit. Even if Cit7 did not reduce speck incidence on the fruit (which remains to be confirmed), reduction of foliar disease could increase the total yield (despite the fact that tomatoes can tolerate a certain degree of defoliation) or reduce the number of fruit lost to sun-scald due to the reduction in defoliation. Of course, the fact that Cit7 is an Ice⁺ strain means that any commercial biological control agent developed from the strain could be utilized only after any threat of frost has passed.

As the most effective biological control agent of bacterial speck (this study) and bacterial spot (M. Wilson, J. M. Byrne, H. L. Campbell, A. C. Dianese, J. B. Jones, and D. A. Cuppels, *unpublished data*) to date, *P. syringae* Cit7 is now the subject of investigations to improve overall efficacy through various approaches, including combined application of Cit7 and salicylic acid (27), which induces systemic acquired resistance (SAR) in tomato (11); enhanced colonization of Cit7 through the use of an engineered salicylic acid-catabolizing Cit7 strain and exogenous salicylic acid (27), or an engineered opine-catabolizing Cit7 strain and transgenic opine-producing plants (72); and through the foliar applica-

tion of Cit7 in conjunction with root-application of rhizobacteria which induce systemic resistance (26; P. Ji, H. L. Campbell, J. W. Kloepper, J. B. Jones, T. Suslow, and M. Wilson, *unpublished data*).

The bacterial strain *P. syringae* TLP2 was less effective than Cit7 under both greenhouse and field conditions. This is despite the fact that the Ice⁻ derivatives of the two strains exhibited similar levels of efficacy against Ice⁺ *P. syringae* strains (35,36). These observations, as well as the fact that Cit7 is effective against bacterial spot under greenhouse conditions, while TLP2 is not (M. Wilson, J. M. Byrne, H. L. Campbell, A. C. Dianese, J. B. Jones, and D. A. Cuppels, *unpublished data*), suggest that Cit7 may function via a different mechanism than TLP2.

The bacterial strain *P. fluorescens* A506, either derived from agar culture or from the formulated commercial product Blight-Ban A506, provided 0 to 40% reduction in foliar disease severity with a mean reduction of 18%. *P. fluorescens* A506 also showed some evidence of ability to reduce incidence of bacterial speck on fruit. However, *P. fluorescens* A506 was not as effective as *P. syringae* Cit7 and lacks efficacy against bacterial spot (M. Wilson, J. M. Byrne, H. L. Campbell, A. C. Dianese, J. B. Jones, and D. A. Cuppels, *unpublished data*). Despite these limitations, the fact that *P. fluorescens* A506 is Ice⁻ would make it effective in preemptive exclusion of Ice⁺ bacteria on the foliage of young transplants, protecting them from frost injury. Plus, it is already commercially available as BlightBan A506, which is registered for use on tomatoes; hence, it could be readily integrated into a disease/pest control program on tomato.

It would be desirable to utilize the most successful biological control agents in an integrated pest management (IPM) program

TABLE 4. Efficacy of biological control agents in the control of bacterial speck (foliar severity) on tomato (cv. Agriset 761) in a field experiment conducted in Alabama (spring 1997)

	Foliar severity ^w	Foliar severity ^x
Control	1.29 a ^y	1.44 ab ^y
<i>Pseudomonas putida</i> B56	0.98 b (24.0) ^z	1.49 ab (0) ^z
BlightBan A506	1.07 b (17.0)	1.41 ab (2.1)
<i>P. syringae</i> TLP2	0.99 b (23.2)	1.35 b (6.2)
<i>P. syringae</i> Cit7	1.03 b (20.2)	1.30 b (9.7)
DC3000 <i>hrpS</i>	1.10 b (14.7)	1.35 b (6.3)
DC3000 <i>hrpH</i>	1.31 a (0)	1.38 ab (4.2)
DC3000 <i>hrpA</i>	1.28 a (0)	1.43 ab (0)
Kocide + Manex	0.69 c (46.5)	0.94 c (34.7)
Actigard	0.46 d (64.3)	0.80 d (43.8)

^w Samples of 30 leaflets per replicate row taken 30 April 1997. Means are the log of the number of lesions per leaflet.

^x Samples of 30 leaflets per replicate row taken 7 May 1997. Means are the log of the number of lesions per leaflet.

^y Means in a column followed by the same letter are not significantly different at *P* = 0.05 according to Duncan's multiple range test.

^z Percent disease reduction compared to pathogen-only control.

TABLE 6. Efficacy of biological control agents in the control of bacterial speck (foliar severity) on tomato (cv. Agriset 761) in a field experiment conducted in Alabama (spring 1998)

	Foliar severity ^v	Foliar severity ^w	Foliar severity ^x
Control	1.70 a ^y	0.84 ab ^y	44.31 a ^y
Mankocide	1.40 c (17.6) ^z	0.85 ab (0) ^z	26.06 d (41.2) ^z
<i>Pseudomonas syringae</i> Cit7	1.53 bc (10.0)	0.87 a (0)	27.77 cd (37.3)
<i>P. syringae</i> TLP2	1.39 c (18.2)	0.95 a (0)	38.39 ab (13.4)
<i>P. fluorescens</i> A506	1.54 bc (9.4)	0.99 a (0)	38.20 ab (13.8)

^v Sample of 20 leaflets per replicate taken 7 May 1998. Means are the log of the number of lesions per leaflet.

^w Sample of 20 leaflets per replicate taken 19 May 1998. Means are the log of the number of lesions per leaflet.

^x Visual ratings of foliar disease severity (0 to 100%) taken 26 May 1998. Numbers are arcsine transformed.

^y Means in a column followed by the same letter are not significantly different at *P* = 0.05 according to Duncan's multiple range test.

^z Percent disease reduction compared to pathogen-only control.

TABLE 5. Efficacy of biological control agents in the control of bacterial speck (foliar and fruit incidence) on tomato (cv. H9478) in a field experiment conducted in London, Ontario, Canada (summer 1997)

	Foliar incidence ^v	Foliar severity ^w	Fruit incidence ^x	Fruit yield (kg/10 plants)
Control	22.5 a ^y	45.0 a ^y	22.6 a ^y	47.4 a ^y
BlightBan A506	17.2 b (23.6) ^z	27.0 b (40.0) ^z	17.8 abc (21.2) ^z	48.9 a
<i>Paenibacillus</i> BC87	15.5 b (31.1)	33.5 ab (25.6)	21.7 abc (4.0)	44.3 a
<i>Pseudomonas syringae</i> Cit7	14.7 b (34.7)	26.0 b (42.2)	24.8 a (0)	46.8 a
Actigard	8.3 c (63.1)	10.5 c (76.7)	16.1 bc (28.8)	44.9 a
A506 + Actigard	4.2 d (81.3)	4.8 c (89.3)	14.6 c (35.4)	46.8 a

^v Samples of 50 leaves per replicate taken 25 July 1997 (57 days after planting). Each number represents the mean number of leaves out of 50 showing bacterial speck-like symptoms.

^w Samples of 20 leaves per replicate taken 25 July 1997. Each number represents the mean number of bacterial speck-like lesions present in each 20-leaf sample.

^x Tomatoes were harvested on 12 September 1997 (106 days after planting), assessed for bacterial speck-like lesions, and weighed.

^y Means in a column followed by the same letter are not significantly different at *P* = 0.05 according to Duncan's multiple range test.

^z Percent disease reduction compared to pathogen-only control.

with currently available chemicals or those that are likely to be available in the near future. Such integration has been successfully achieved with BlightBan A506 in the control of fire blight of pear, where the streptomycin-resistant biological control agent is applied in conjunction with the antibiotic (39). Unfortunately, none of the successful biological control agents employed in this study are naturally resistant to copper so that they could be integrated with the current standard chemical control regime of copper plus EBDC. There is potential, however, for the integration of the non-bactericidal plant activators with biological control agents. Under greenhouse conditions, the combined use of Cit7 and salicylic acid, which induces SAR in tomato (11), reproducibly provided significantly greater reductions in disease severity than either agent alone (27). Cit7 (Table 7) and *P. fluorescens* A506 (Table 5) exhibited potential for additive activity with Actigard in control of foliar disease severity. Most notably, the combination of A506 and Actigard was the only treatment that significantly reduced epiphytic *P. syringae* pv. *tomato* populations sizes. Despite these

TABLE 7. Efficacy of biological control agents in the control of bacterial speck (foliar severity) on tomato (cv. Agriset 761) in a field experiment conducted in Alabama (spring 1998)

	Foliar severity ^v	Foliar severity ^w	Foliar severity ^x
Control	1.77 a ^y	0.73 a ^y	35.32 a ^y
Actigard	1.16 b (34.5) ^z	0.59 ab (19.2) ^z	22.59 b (36.0) ^z
<i>Pseudomonas syringae</i>			
Cit7 + Actigard	0.92 c (48.0)	0.51 b (30.1)	20.34 b (0.4)
<i>P. syringae</i> TLP2 + Actigard	1.19 b (32.8)	0.70 ab (4.1)	26.81 b (24.1)
<i>P. fluorescens</i> A506 + Actigard	1.14 b (35.6)	0.63 ab (13.7)	22.40 b (36.6)

^v Sample of 20 leaflets per replicate taken 7 May 1998. Means are the log of the number of lesions per leaflet.
^w Sample of 20 leaflets per replicate taken 19 May 1998. Means are the log of the number of lesions per leaflet.
^x Visual ratings of foliar disease severity (0 to 100%) taken 26 May 1998. Numbers are arcsine transformed.
^y Means in a column followed by the same letter are not significantly different at *P* = 0.05 according to a Duncan's multiple range test.
^z Percent disease reduction compared to pathogen-only control.

TABLE 8. Summary of efficacy of biological control agents *Pseudomonas syringae* Cit7, *P. syringae* TLP2, and *P. fluorescens* A506 against bacterial speck of tomato in several locations in North America in 1996, 1997, and 1998

Experiment (location and date)	Mean reduction in disease severity (%)		
	<i>P. syringae</i> Cit7 ^w	<i>P. syringae</i> TLP2 ^w	<i>P. fluorescens</i> A506 ^x
Alabama (AL), spring 1996 (Table 1)	50.1	17.1	24.6
Florida (FL) spring 1996 (Table 2)	24.5	28.4	24.0
London, Ontario, Canada, (ON) summer 1996 (Table 3)	26.8	5.1	29.5
AL, spring 1997 (Table 4)	15.0	14.7	9.6
AL, spring 1997 ^y	24.0	ND ^z	15.5
FL, spring 1997 ^y	18.9	ND	ND
California, spring 1997 ^y	44.8	ND	0.0
ON, summer 1997 (Table 5)	42.2	ND	40.0
AL, spring 1998 (Table 6)	15.8	10.5	7.7
AL, spring 1998 ^y	21.2	13.4	11.3
Mean	28.3	14.9	18.0

^w Mean reduction in disease severity (%) calculated across available severity ratings in specified field experiment.
^x Mean of reduction in disease severity (%) for *P. fluorescens* A506 and formulated BlightBan A506 combined across available severity ratings in specified field experiment.
^y P. Ji, H. L. Campbell, J. W. Kloepper, J. B. Jones, T. Suslow, and M. Wilson, unpublished data.
^z ND = strain not included in this trial.

promising results, many more trials will be required to determine whether there is a consistent benefit to using biological control agents in conjunction with Actigard.

Nonpathogenic *hrp* mutants have been found to significantly reduce disease severity in the case of several different pathogens, including *Xanthomonas campestris* pv. *vesicatoria* (45), *Erwinia amylovora* (21), and *Ralstonia solanacearum* (20,22). In this study, the *P. syringae* pv. *tomato* DC3000 *hrpA*, *hrpH*, and *hrpS* mutants all significantly reduced disease severity caused by wild-type DC3000 under greenhouse conditions, however, only the *hrpS* mutant significantly reduced disease severity under field conditions where disease was caused by indigenous *P. syringae* pv. *tomato* strains and/or PT12. In *P. syringae*, the *hrpS*-gene product plays a regulatory role and hence, in the *hrpS* mutant the entire *hrp* regulon, including *hrp*-associated (*hpa*) genes, is probably not induced in planta. It is interesting to note that in two other pathogens, *X. campestris* pv. *vesicatoria* (45) and *E. amylovora* (21), the regulatory mutants also have provided the highest

Fig. 1. Population dynamics of biological control agents *Pseudomonas fluorescens* A506 (●), *P. syringae* TLP2 (■), and *P. syringae* Cit7 (▲) on tomato (cv. Agriset 761) under field conditions in Alabama and rainfall during experimental period of A, 1995 and B, 1996.

levels of disease reduction (compared to strains with mutations in nonregulatory or structural genes) perhaps suggesting something about the mechanism of action in these mutants.

Despite the efficacy of the *P. syringae* pv. *tomato* *hrpS* mutant, *hrp* mutants were not employed in subsequent speck field trials due to lack of superiority to nonengineered naturally occurring strains (e.g., Cit7), the regulatory bureaucracy involved in field-releases, and the low probability that *hrp* mutants will be developed commercially in the United States in the near to mid-term future. This contrasts somewhat with the bacterial spot situation in which the *X. campestris* pv. *vesicatoria* *hrpG* mutant is more effective than any of the nonpathogenic strains and has warranted extensive examination under field conditions at different locations (45; W. P. Moss, J. B. Jones, and M. Wilson, unpublished data).

The greenhouse assays, particularly when replicated multiple times, successfully predicted the superior efficacy of *P. syringae* Cit7 over *P. syringae* TLP2 and *P. fluorescens* A506. However, the variability in efficacy observed suggests that a minimum of three repetitions are necessary to get a true picture of the potential of an

individual strain as evidenced by the fact that Cu9 and Cu14 gave relatively high levels of control in the first greenhouse assay, but the mean level of control after three replicate assays was fairly low. The need to screen such large numbers of bacterial strains in replicate greenhouse assays in order to select an organism with potential for field use makes this empirical approach impractical in most instances, underscoring the need to understand the attributes of an effective biological control agent for the targeted pathogen and the need to develop more targeted selection strategies.

Based upon our knowledge of the characteristics of *P. syringae* strains TLP2 and Cit7 (35), *P. fluorescens* A506 (39,68), and the relative efficacy of these strains, we can speculate on some of the important characteristics of a successful biological control agent of bacterial speck of tomato. While *P. syringae* strains Cit7 and TLP2 and *P. fluorescens* strain A506 are not known to produce antibiotics effective against *P. syringae* pv. *tomato*, *P. syringae* strains have been reported to produce various bacteriocins (30,65), and *P. fluorescens* A506 has been reported to produce an iron-inducible antibiotic; hence, a role for an antimicrobial metabolite cannot be ruled out. It has been demonstrated that similarity in in vitro carbon source utilization profiles between the biological control agent and *P. syringae* pv. *tomato* is an important attribute of a successful biological control agent (28), however, TLP2 and Cit7 both have carbon source utilization profiles with a high degree of similarity to *P. syringae* pv. *tomato*; hence, differences in this area are unlikely to account for the superiority of Cit7 over TLP2. Neither were there significant differences in leaf surface colonization between TLP2 and Cit7 in AL experiments. One major difference between the two strains is that Cit7 was also effective against bacterial spot, while TLP2 was not (M. Wilson, J. M. Byrne, H. L. Campbell, A. C. Dianese, J. B. Jones, and D. A. Cuppels, unpublished data). In the case of bacterial spot, preemptive competitive exclusion of epiphytic *X. campestris* pv. *vesicatoria* populations appears less important than it is in *P. syringae* pv. *tomato* (45). It is possible that the effect of Cit7 against spot relates to the ability of Cit7 to partially invade the interior of the leaf in the manner of a pathogen without causing disease symptoms. Following vacuum infiltration into bean (*Phaseolus vulgaris*) leaves, Cit7 was able to multiply, though to a lower population size than pathogenic strains, while TLP2 was not (71). Further, the ability to multiply internally following vacuum infiltration was correlated with the ability to access internal sites following mist inoculation (71). The unique ability of Cit7 to invade certain internal sites without causing disease may lead to pre-

Fig. 2. Population dynamics of biological control agent and pathogen on tomato (cv. H9478) under field conditions in London, Ontario, Canada during the 1996 experiment. Population size of *Pseudomonas fluorescens* A506 (□); population size of *P. syringae* pv. *tomato* strain DCT6D1 on non-treated tomato plants (○); and population size of *P. syringae* pv. *tomato* on tomato plants treated with biological control agent *P. fluorescens* A506 (■), *P. syringae* TLP2 (●), and *P. syringae* Cit7 (▲).

Fig. 3. **A.** Seasonal progression of the biological control agent *Pseudomonas fluorescens* A506 on bacterial speck-infected tomato (cv. H9478) plants grown under field conditions in London, Ontario, Canada during 1997: A506 on plants infected with *P. syringae* pv. *tomato* DCT6D1 (■); and A506 on infected plants sprayed every 10 days with Actigard (62 mg/liter) (□). **B.** Effect of Actigard and various biological control agents on populations of *P. syringae* pv. *tomato* strain DCT6D1 sprayed on field tomato plants (cv. H9478) grown in London, Ontario, Canada in 1997: Control (○); *P. fluorescens* A506 (●); *Paenibacillus* sp. BC87 (■); *P. syringae* Cit7 (▲); Actigard (◆); and *P. fluorescens* A506 plus Actigard (▼).

emptive exclusion of the pathogen from these internal sites or may induce host defense responses that contribute to the disease suppressive ability of this strain. In other words, in addition to the preemptive competitive exclusion of surface *P. syringae* pv. *tomato* populations, to which TLP2 and A506 are limited, Cit7 may have the ability to exclude internal populations or maybe to induce protective host defense responses in tomato. One might speculate, therefore, that the superior biological control agent of speck is one that has the ability to preemptively exclude both surface and internal *P. syringae* pv. *tomato* populations (71) or to additionally induce host defense responses.

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