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Integrated biological control of bacterial speck and spot of tomato under field conditions using foliar biological control agents and plant growth-promoting rhizobacteria

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Abstract

Integration of foliar bacterial biological control agents and plant growth promoting rhizobacteria (PGPR) was investigated to determine whether biological control of bacterial speck of tomato, caused by *Pseudomonas syringae* pv. *tomato*, and bacterial spot of tomato, caused by *Xanthomonas campestris* pv. *vesicatoria* and *Xanthomonas vesicatoria*, could be improved. Three foliar biological control agents and two selected PGPR strains were employed in pairwise combinations. The foliar biological control agents had previously demonstrated moderate control of bacterial speck or bacterial spot when applied as foliar sprays. The PGPR strains were selected in this study based on their capacity to induce resistance against bacterial speck when applied as seed and soil treatments in the greenhouse. Field trials were conducted in Alabama, Florida, and California for evaluation of the efficacy in control of bacterial speck and in Alabama and Florida for control of bacterial spot. The foliar biological control agent *P. syringae* strain Cit7 was the most effective of the three foliar biological control agents, providing significant suppression of bacterial speck in all field trials and bacterial spot in two out of three field trials. When applied as a seed treatment and soil drench, PGPR strain *Pseudomonas fluorescens* 89B-61 significantly reduced foliar severity of bacterial speck in the field trial in California and in three of six disease ratings in the field trials in Alabama. PGPR strains 89B-61 and *Bacillus pumilus* SE34 both provided significant suppression of bacterial spot in the two field trials conducted in Alabama. Combined use of foliar biological control agent Cit7 and PGPR strain 89B-61 provided significant control of bacterial speck and spot of tomato in each trial. In one field trial, control was enhanced significantly with combined biological control agents compared to single agent inoculations. These results suggest that some PGPR strains may induce plant resistance under field conditions, providing effective suppression of bacterial speck and spot of tomato, and that there may be some benefit to the integration of rhizosphere-applied PGPR and foliar-applied biological control agents.

Keywords: *Bacillus pumilus*; *Pseudomonas fluorescens*; *Pseudomonas putida*; *Pseudomonas syringae*; *Xanthomonas campestris* pv. *vesicatoria*; *Xanthomonas vesicatoria*; Bacterial speck; Bacterial spot; Biological control; Plant growth-promoting rhizobacteria; Induced systemic resistance; Tomato; *Lycopersicon esculentum*

1. Introduction

Bacterial speck of tomato, caused by *Pseudomonas syringae* pv. *tomato*, and bacterial spot of tomato, caused by *Xanthomonas campestris* pv. *vesicatoria* and *Xanthomonas vesicatoria*, are among the most economically important bacterial diseases in many tomato-growing regions of North America and the world (Goode and Sasser, 1980). Lesions occur on leaves and may cause an entire leaflet to turn yellow and drop. As the diseases progress, the lesions may spread to stems, petioles, and flowers. Yield reductions can result from the reduced photosynthetic capacity of infected foliage, leaf defoliation, flower abortion, and from

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lesions on the fruit that render them unsuitable for the fresh market or for processing. Bacterial speck is more severe under cool and humid conditions, whereas bacterial spot is favored by warm and rainy weather. Both diseases may cause significant reductions in tomato yield, especially if the infection appears early in the season (Pohronezny and Volin, 1983; Yunis et al., 1980). The efficacy of current strategies for control of bacterial speck and spot is limited. Cultural practices do not provide sufficient control of the diseases and have not been generally adopted by commercial growers (Conover and Gerhold, 1981; Lawton and MacNeill, 1986). Copper bactericides, applied alone or in combination with ethylenebis-dithiocarbamate (EBDC) fungicides, have been traditionally used to control the diseases (Conlin and McCarter, 1983; Conover and Gerhold, 1981; Jardine and Stephens, 1987; Jones and Jones, 1985; Marco and Stall, 1983). However, ineffective disease suppression due to development of copper resistance in the pathogen populations in many areas (Bender and Cooksey, 1986; Marco and Stall, 1983; Pernezny et al., 1995; Silva and Lopes, 1995) and increased public concern about detrimental effects of pesticide residues have made alternative or complementary methods to control these diseases desirable.

Considerable efforts have been directed to identify genetic resistance to bacterial speck and spot (Jones et al., 1998; Pitblado and MacNeill, 1983; Pitblado et al., 1984; Scott et al., 1997). The *Pto* gene has been demonstrated to confer resistance in tomato to race 0 strains of *P. syringae* pv. *tomato* that express the gene *avrPto* (Martin et al., 1993; Ronald et al., 1992). However, the occurrence of race 1 strains of the pathogen lacking the *avrPto* may hamper the mass release of *Pto* into commercial cultivars (Donner and Barker, 1996; Habazar and Rudolph, 1997; Lawton and MacNeill, 1986). With regard to bacterial spot of tomato, commercial cultivars resistant to the disease are not available, partially because the effectiveness of disease resistance does not appear to persist (Jones et al., 1998; Scott et al., 1997). Another control practice that may be applicable to tomato is chemically induced systemic acquired resistance (SAR). A synthetic compound, acibenzolar-*S*-methyl (Actigard; Bion) has been reported to induce SAR and provide significant suppression of bacterial speck and spot in field trials (Abbasi et al., 2002; Louws et al., 2001; Wilson et al., 2002). Thus, chemically induced SAR may be an effective method for control of bacterial speck and spot, although the application of these chemicals remains to be optimized since negative impact on plant growth or yield have been reported (Csinos et al., 2001; Louws et al., 2001; Romero et al., 2001).

Biological control may provide an additional tool to these chemical approaches for bacterial disease management. Bacterial biological control agents are now commercially available for the control of crown gall, fire blight of pear and several other diseases (Backman et al., 1997; Kloepper, 1993; Lindow et al., 1996; Lindow and Wilson, 1999; Wilson, 1997, 2004; Wilson and Backman, 1999). Selected bacteriophages have been demonstrated to be effective under greenhouse and field conditions for control of bacterial spot of tomato and have been commercialized (Balogh et al., 2003; Flaherty et al., 2000; Obradovic et al., 2004). Although less effort has been directed toward the use of nonpathogenic bacteria for control of bacterial speck and spot, recent studies showed that some biological control agents, especially a foliar bacterial strain *P. syringae* Cit7, consistently suppressed bacterial speck and spot under field conditions at several locations in North America (Byrne et al., 2005; Wilson et al., 2002). It was speculated that the bacterial strain Cit7 provided protection of tomato via mechanisms including induced resistance (Wilson et al., 2002). While induced plant resistance by foliar bacterial biological control agents has not been intensively studied, induced systemic resistance (ISR) by plant growth-promoting rhizobacteria (PGPR) has been the subject of many investigations in recent years (Kloepper et al., 1999, 1992; van Loon et al., 1998; Wei et al., 1996; Zehnder et al., 2001). Treatment of seed or root with PGPR significantly reduced severity of anthracnose, angular leaf spot and cucurbit wilt diseases on cucumber (Raupach and Kloepper, 1998; Raupach and Kloepper, 2000; Wei et al., 1996; Zehnder et al., 2001), and southern blight, bacterial wilt, *Tobacco mosaic virus* (TMV) and *Tomato mottle virus* (ToMoV) in tomato (Anith et al., 2004; Jetiyanon et al., 2003; Murphy et al., 2000; Zehnder et al., 2001). It was hypothesized, therefore, that some PGPR strains might provide systemic protection against bacterial speck and spot of tomato under natural environmental conditions.

The main goal of this study was to determine whether the control of bacterial speck and spot of tomato could be improved through the combined use of foliar biological control agents applied to the leaves and ISR-eliciting PGPR applied to the roots. Three foliar biological control agents were included in the study: *P. syringae* strain Cit7; *Pseudomonas fluorescens* strain A506; and *P. putida* strain B56 (Wilson et al., 2002). While these foliar bacterial strains have been shown to provide protection against both bacterial speck and bacterial spot of tomato, only a relatively moderate level of disease control was achieved (Byrne et al., 2005; Wilson et al., 2002). Hence, a collection of PGPR strains was screened for the capacity to elicit ISR in tomato plants and used to determine whether some combinations of PGPR and foliar biological control agents could improve disease control efficacy.

2. Materials and methods

2.1. Bacterial strains

Bacterial strains *P. fluorescens* A506 (Lindow et al., 1996) and *P. syringae* Cit7 (Lindow, 1985) were provided by S.E. Lindow (University of California, Berkeley, CA). *Pseudomonas putida* strain B56 was isolated from tomato leaves in Florida (Wilson et al., 2002). In a previous study, these bacterial strains significantly reduced foliar severity of bacterial speck of tomato (Wilson et al., 2002). *Pseudomonas syringae* Cit7 and *P. putida* B56 were also moderately

effective in suppression of bacterial spot under both greenhouse and field conditions (Byrne et al., 2005). A collection of fifty PGPR strains, 23 from the USA and 27 from China, was included in initial greenhouse screening as a seed and root treatment. The PGPR strains have either elicited ISR in cucumber, tomato, or tobacco (Liu et al., 1995a,b; Murphy et al., 2000; Raupach and Kloepper, 1998; Raupach and Kloepper, 2000; Wei et al., 1991, 1996; Zehnder et al., 2001) or have been used in China in various crops to enhance plant growth and control diseases (Chen et al., 1996). Two PGPR strains *P. fluorescens* 89B-61 and *Bacillus pumilus* SE34 were selected for evaluation in conjunction with the foliar bacterial strains after the two strains had demonstrated efficacy in greenhouse assays in this study.

Pseudomonas syringae pv. *tomato* (*Pst*) strain PT12 was provided by D.A. Cooksey (University of California, Riverside, CA), and *Pst* strain 95-3 was isolated from tomato in California. *X. campestris* pv. *vesicatoria* (*Xcv*) strain AD17 (race T1) and *X. vesicatoria* 938 (race T3) were isolated from symptomatic tomato foliage collected in Alabama (AL) and Florida (FL), respectively. Bacterial cultures were maintained for long-term storage at −80 °C in tryptic soy broth (TSB) amended with 20% v/v glycerol.

2.2. Greenhouse bioassays

Experiments were conducted in the greenhouse to screen PGPR strains for induction of systemic protection of tomato plants against bacterial speck and to evaluate disease control efficacy of foliar biological control agent/PGPR combinations. The PGPR strains were grown on tryptic soy agar (TSA) at 28 °C for 24–48 h, and single colonies were transferred to TSB and incubated with shaking at 25 °C for 24 h. Bacterial cultures were centrifuged at 5000 rpm for 10 min and the pellet was resuspended in 0.85% sodium chloride solution to reach approximately 10^9 CFU/ml for seed treatment. Tomato seeds (cv. Rutgers, Michael–Leonard, Grant Park, IL) were soaked in the bacterial suspensions for 30 min and then planted in seed trays with $3 \times 3 \times 4$-cm cells containing Promix (Premier Peat, Riviere-du-Loup, Quebec) and incubated in the greenhouse. The plants were fertilized as described previously (Wilson et al., 2002) and watered daily by overhead watering. Two to three weeks later tomato seedlings were transplanted into 10-cm plastic pots containing Promix. Soil drenches were applied at the time of seedling transplanting by pouring 100 ml of bacterial suspensions (10^7 CFU/ml) into the Promix in each pot. Tomato plants were maintained in the greenhouse with temperatures of 20–22 °C (night) and 23–26 °C (day) and 12 h photoperiod.

The pathogen, *Pst* strain PT12, was spray inoculated onto upper and lower leaf surfaces 2 weeks after transplanting. To prepare suspensions of the pathogen, PT12 was grown on King's medium B (KB; King et al., 1954) plates at 28 °C for 24 h. The cells were suspended in sterile phosphate buffer (10 mM, pH 7.0) and adjusted to approximately 10^8 CFU/ml. Tomato plants were incubated in the greenhouse under conditions as described previously (Wilson et al., 2002).

Two foliar biological control agents, Cit7 and A506, were used in combination with two selected PGPR strains to evaluate the efficacy in suppression of bacterial speck in the greenhouse. Application of PGPR strains and inoculation of the pathogen were as described above. Bacterial strains Cit7 and A506 were grown on KB plates at 28 °C for 24 h, and bacterial cells were suspended in sterile phosphate buffer (10 mM, pH 7.0). Bacterial suspensions were adjusted to 10^8 CFU/ml and spray inoculated onto the foliage of tomato 24 h prior to inoculation of the pathogen. The plants were maintained in the greenhouse under disease-conducive conditions (Wilson et al., 2002) from 24 h before application of the foliar biological control agents until symptom development.

2.3. Experimental design and disease assessment in the greenhouse

The experimental design employed in the greenhouse studies was a randomized complete block (RCB) with 4–5 replicates. Ten leaflets were randomly collected from each plant approximately 7 days after inoculation of the pathogen. Lesion numbers on each leaflet were counted and the area of each leaflet was measured by an image analysis system (AgVision Monochrome system, Porta-Trace, Gagne, NY). Disease data were subjected to log transformation and expressed as $\log_{10}$ (lesions + 1)/cm^2. Analysis of variance was performed using the ANOVA or GLM procedures of the Statistical Analysis System (SAS Institute, Cary, NC). Means were compared using Duncan's multiple range test at $P = 0.05$. Biological control effectiveness was quantified as the percentage reduction in lesion numbers per unit leaf area compared to the pathogen-only control. Bacterial strains that provided significant disease suppression were assayed in three experiments under greenhouse conditions.

2.4. Field experiments

Field experiments were conducted in Shorter, AL (spring 1997 and 1998), Bradenton, FL (spring 1997), and Davis, CA (spring 1997) to evaluate the efficacy of combined use of selected PGPR and foliar biological control agents in control of bacterial speck of tomato. Field trials for control of bacterial spot were conducted in Shorter, AL (fall 1997 and summer 1998) and Bradenton, FL (spring 1998). Five- to six-week-old seedlings were transplanted into raised beds covered with polyethylene mulch and fumigated with 98% methyl bromide and 2% chloropicrin at 400 lb/A and placed 0.5 m apart within a row on 1.3 m centers. Experimental plots consisted of a single row which was 5 m long with 10 plants per row. Buffer zones with 2 m spacing without planting of tomato seedlings were maintained between plots. Plants were irrigated through drip irrigation (AL),

seep irrigation (FL), or overhead irrigation (CA). A randomized complete block design was employed with four (CA) or five (AL, FL) replicate rows.

The PGPR strains and foliar biological control agents were either applied separately or in combination. The PGPR strains were applied as seed treatment plus soil drench and foliar biological control agents were sprayed onto tomato foliage. Two PGPR strains, *P. fluorescens* 89B-61 and *B. pumilus* SE34, were used in field tests. Preparation of bacterial suspensions and seed treatments were conducted as in the greenhouse assays. Bacterial suspensions (approximately 10^7 CFU/ml) were applied as a drench, at the rate of 5 ml per transplant cell, to the roots of 5- to 6-week-old seedlings immediately prior to planting in the field. Foliar biological control agents were grown on KB at 28 °C for 24 h, washed from plates and suspended in phosphate buffer (30 mM, pH 7.4). Bacterial suspensions, adjusted to 10^8 CFU/ml using a spectrophotometer, were applied weekly to the tomato plants using a CO_2-powered backpack sprayer for a total of 8 weeks. Chemical treatments [copper hydroxide (Kocide DF) and Mancozeb (Manex) or copper hydroxide plus Mancozeb (Mankocide), Griffin, Valdosta, GA] were applied according to label specifications. Field plots were inoculated 1–3 times with either *Pst* strains PT12 (AL) and 95-3 (CA) or *Xcv* AD17 (AL). Natural infection was usually present in Florida; but to ensure presence of the diseases, field plots were inoculated with a local isolate of *Pst* or with strains T1 and T3 of *Xcv*. Standard insecticides and fungicides were applied onto the foliage weekly for control of other pests.

2.5. Disease assessment of field plots

In field trials in AL, foliar disease was rated by sampling 20 or 30 leaflets per replicate row and counting lesions or was rated visually in the field as a percentage of necrotic foliage (0–100%) per replicate row as described previously (Wilson et al., 2002). In FL, disease was rated using a modified Horsfall–Barratt rating system (Horsfall and Barratt, 1945) for bacterial speck or by counting infected plants and lesions on each sampled leaflet for bacterial spot. In CA, disease was rated on foliage between the 2nd and 4th internode 3 weeks after inoculation with the pathogen using the following scale: 1 = extremely limited lesions, little chlorosis; 2 = limited lesions, moderate chlorosis; 3 = moderate lesions (>15 per compound leaf), moderate chlorosis; 4 = numerous lesions, moderate coalesced lesions, moderate defoliation; 5 = severe defoliation, numerous coalesced lesions, stunting, chlorosis. Area under the disease progress curve (AUDPC) was calculated where applicable using the method of Shaner and Finney (1977). Disease data expressed as percentage were subjected to an arcsine transformation where appropriate. Analysis of variance was performed using the ANOVA or GLM procedures of SAS (SAS Institute, Cary, NC), and means were compared using Duncan's Multiple Range Test at $P = 0.05$.

3. Results

3.1. Greenhouse study

Eight out of fifty PGPR strains tested significantly suppressed bacterial speck in three experiments (Table 1). Among the eight strains, *P. fluorescens* strain 89B-61, *B. pumilus* strain SE34, and *B. pasteurii* strain M-38 provided the greatest disease suppression with a mean reduction in lesion numbers greater than 60%. *Burkholderia gladioli* strain IN26 also reduced disease significantly and was the most consistent (having the lowest standard error of the mean, Table 1). Other strains that provided significant disease suppression in all three trials were *B. cereus* strain 83-6, *Stenotrophomonas maltophilia* strain IN287, *B. cereus* strain M-22, and *B. amyloliquifaciens* strain IN937a (Table 1).

The PGPR strains SE34 and 89B-61 were selected and used in combination with foliar application of the biological control agent *P. syringae* Cit7 to evaluate their efficacy in control of bacterial speck on tomato in the greenhouse. In two independent trials, PGPR strain 89B-61 provided similar levels of disease reduction (76.3 and 52.8%) to the

Table 1
PGPR strains that significantly reduced bacterial speck on tomato leaves in greenhouse assays

Bacterial strain[a]	Reduction in number of lesions/cm^2 leaf (%)[b]				
	Experiment I	Experiment II	Experiment III	Mean	SE[c]
Pseudomonas fluorescens 89B-61	61.53	58.49	71.43	63.8	6.77
Bacillus pumilus SE34	50.00	73.98	65.87	63.3	12.20
Bacillus pasteurii M38	55.26	64.15	67.46	62.3	6.31
Bacillus cereus 83-6	45.16	68.33	62.70	58.7	12.08
Burkholderia gladioli IN26	54.58	55.43	62.70	57.6	4.46
Stenotrophomonas maltophilia IN287	42.58	65.61	56.35	54.9	11.59
Bacillus cereus M-22	46.77	61.54	55.56	54.6	7.43
Bacillus amyloliquifaciens IN937a	47.53	41.51	57.94	49.0	8.31

[a] Bacteria were applied by soaking tomato seeds in bacterial suspensions for 30 min before sowing and by soil drench immediately after transplanting the tomato seedlings.

[b] Samples of 10 leaves per replicate taken approximately 7 days after inoculation of the pathogen. Percentage reduction in lesion numbers was calculated in comparison to the pathogen-only control.

[c] Standard error of the means.

foliar biological control agent Cit7 (72.0 and 56.0%). In one of the two trials, combined use of 89B-61 and Cit7 provided the largest disease reduction (82.2%), which was significantly higher than that of either Cit7 or 89B-61 applied alone (Table 2). Disease was significantly reduced by PGPR strain SE34 in one of the two experiments; however, disease reduction was not significantly greater with combined use of SE34 and Cit7. *P. fluorescens* strain A506 was tested in one experiment, alone or in combination with the two PGPR strains; but A506 did not provide significant disease reduction and did not enhance the efficacy of the PGPR strains (data not shown).

3.2. Control of bacterial speck in field trials

Three foliar biological control agents and two PGPR strains were applied either separately or used in combinations. In the 1997 field trial in AL, bacterial speck was significantly reduced by the PGPR strains 89B-61 and SE34 in two of three disease evaluations. Foliar biological control agents Cit7 and A506 also provided significant disease suppression in two of three disease evaluations (Table 3). In addition, the AUDPC was reduced significantly by *P. syringae* Cit7. Application of the foliar biological control agents in conjunction with the PGPR strains did not enhance disease suppression significantly (Table 3). The combination of copper hydroxide and Mancozeb provided the highest disease suppression in this field trial based on the AUDPC values (Table 3).

An experiment for control of bacterial speck was repeated in AL in spring 1998 with two foliar biological control agents and two PGPR strains. *P. syringae* Cit7 provided significant disease suppression in two of three disease evaluations, compared with the pathogen-only control (Table 4), while *P. fluorescens* strains A506 and 89B-61 resulted in significant disease reduction in one of three disease evaluations (Table 4). Combined use of foliar biological control agents and PGPR strains did not provide higher levels of disease suppression.

In the CA field trial (spring 1997), the PGPR strain 89B-61 applied alone and the foliar biological control agent Cit7 applied alone gave the greatest disease suppres-

Table 2
Comparison of efficacy of biological control agents employed alone or in combination in control of bacterial speck of tomato under greenhouse conditions

Treatment	Application method[a]	Experiment I		Experiment II	
		Number of lesions[b]	% reduction	Number of lesions[b]	% reduction
Control		1.86 a		1.25 a	
B. pumilus SE34	Seed and root	0.42 bcd	77.4	1.09 a	12.8
P. fluorescens 89B-61	Seed and root	0.44 bc	76.3	0.59 c	52.8
P. syringae Cit7	Foliar	0.52 b	72.0	0.55 c	56.0
P. syringae Cit7 + *B. pumilus* SE34	Foliar (Cit7), seed and root (SE34)	0.36 cd	80.6	0.74 bc	40.8
P. syringae Cit7 + *P. fluorescens* 89B-61	Foliar (Cit7), seed and root (89B-61)	0.33 d	82.2	0.58 c	53.6

[a] "Seed and root" indicates seed soaking before sowing plus soil and root drench immediately after transplanting; "foliar" indicates foliar spray.
[b] Samples of 10 leaves per replicate taken 7 days after inoculation of the pathogen. Values represent the number of lesions/cm^2 leaf area. The disease data were log-transformed prior to analysis. Within each column, values followed by the same letter indicates no significant difference according to Duncan's multiple range test at $P = 0.05$. Percentage reduction in lesion numbers was calculated in comparison to the pathogen-only control.

Table 3
Efficacy of biological control agents in control of bacterial speck in a field experiment in Shorter, AL (spring 1997)

Treatment	Application method[a]	Lesion number/leaflet[b,d]			AUDPC[c,d]
		4/30	5/7	5/13	
Control		20.4 a	20.4 ab	19.0 a	329 a
Copper + Mancozeb	Foliar	3.4 g	13.8 c	8.1 c	202 c
B. pumilus SE34	Seed and root	10.2 cd	21.4 ab	10.7 b	298 ab
P. fluorescens 89B-61	Seed and root	10.2 cd	20.4 ab	10.0 bc	278 ab
P. syringae Cit7	Foliar	8.5 de	17.2 bc	10.0 bc	250 bc
P. syringae Cit7 + *B. pumilus* SE34	Foliar (Cit7), seed and root (SE34)	7.3 ef	15.2 c	10.2 bc	243 bc
P. syringae Cit7 + *P. fluorescens* 89B-61	Foliar (Cit7), seed and root (89B-61)	5.9 f	19.4 b	10.7 bc	252 bc
P. fluorescens A506	Foliar	14.1 b	19.9 b	8.5 c	278 ab
P. fluorescens A506 + *B. pumilus* SE34	Foliar (A506), seed and root (SE34)	13.1 bc	24.7 a	9.5 c	329 a
P. fluorescens A506 + *P. fluorescens* 89B-61	Foliar (A506), seed and root (89B-61)	12.8 bc	19.4 b	13.8 b	299 ab

[a] "Seed and root" indicates seed soaking before sowing plus soil and root drench immediately before transplanting; "foliar" indicates foliar spray. Plants were transplanted into the field in mid March and foliar biological control agents were sprayed weekly beginning the last week of March. The pathogen was applied once to the plants in early April.
[b] Samples of 30 leaflets per replicate taken at different dates. Disease data were log-transformed prior to analysis.
[c] AUDPC = area under the disease progress curve based on lesion number per leaflet at the three assessment dates.
[d] Within each column, values followed by the same letter indicates no significant difference according to Duncan's multiple range test at $P = 0.05$.

Table 4
Efficacy of biological control agents in control of bacterial speck in a field experiment conducted in Shorter, AL (spring 1998)

Treatment	Application method[a]	Lesion number/leaflet[b,d]				Disease severity[c,d]	
		5/7	% reduction	5/19	% reduction	5/26	% reduction
Control		32.1 a	—	12.8 a	—	39.10 ab	—
Copper + Mancozeb	Foliar	13.1 ef	59.2	6.6 bc	48.4	23.38 de	40.2
B. pumilus SE34	Seed and root	28.5 ab	11.2	8.5 abc	33.6	41.43 a	0.0
P. fluorescens 89B-61	Seed and root	24.1 abc	24.9	9.7 ab	24.2	26.57 cd	32.0
P. syringae Cit7	Foliar	14.1 ef	56.1	7.1 bc	44.5	30.86 bcd	21.1
P. syringae Cit7 + *B. pumilus* SE34	Foliar (Cit7), seed and root (SE34)	13.8 ef	57.0	8.5 abc	33.6	32.14 abcd	17.8
P. syringae Cit7 + *P. fluorescens* 89B-61	Foliar (Cit7), seed and root (89B-61)	12.2 f	62.0	5.8 c	54.7	29.08 bcd	25.6
P. fluorescens A506	Foliar	16.8 de	47.7	9.5 abc	25.8	36.88 abc	5.7
P. fluorescens A506 + *B. pumilus* SE34	Foliar (A506), seed and root (SE34)	19.4 cd	39.6	6.8 bc	46.9	28.44 cd	27.3
P. fluorescens A506 + *P. fluorescens* 89B-61	Foliar (A506), seed and root (89B-61)	25.3 abc	21.2	9.2 abc	28.1	32.46 abcd	16.9

[a] "Seed and root" indicates seed soaking before sowing plus soil and root drench immediately before transplanting; "foliar" indicates foliar spray. Plants were transplanted into the field in mid March and foliar biological control agents were sprayed weekly beginning the last week of March. The pathogen was applied once to the plants in early April.
[b] Samples of 20 leaflets per replicate taken at different dates. Disease data were log-transformed prior to analysis.
[c] Visual ratings of foliar disease severity (0–100%), numbers are arcsine transformed.
[d] Within each column, values followed by the same letter indicate no significant difference according to Duncan's multiple range test at $P = 0.05$.

Table 5
Efficacy of biological control agents in control of bacterial speck of tomato in a field experiment conducted in Davis, CA (spring 1997)

Treatment	Application method[a]	Foliar disease[b,c]	% reduction
Control		2.90 a	—
Copper hydroxide	Foliar	2.80 a	3.4
B. pumilus SE34	Seed and root	2.80 ab	3.4
P. fluorescens 89-B61	Seed and root	1.40 e	51.7
P. syringae Cit7	Foliar	1.60 de	44.8
P. syringae Cit7 + *B. pumilus* SE34	Foliar (Cit7), seed and root (SE34)	2.30 c	20.7
P. syringae Cit7 + *P. fluorescens* 89B-61	Foliar (Cit7), seed and root (89B-61)	1.80 d	37.9
P. fluorescens A506	Foliar	3.10 a	0.0
P. fluorescens A506 + *B. pumilus* SE34	Foliar (A506), seed and root (SE34)	2.75 ab	5.2
P. fluorescens A506 + *P. fluorescens* 89-B61	Foliar (A506), seed and root (89B-61)	2.50 bc	13.8

[a] "Seed and root" indicates seed soaking before sowing plus soil and root drench immediately before transplanting; "foliar" indicates foliar spray. Plants were transplanted into the field in mid April and foliar biological control agents were sprayed weekly beginning the last week of April. The pathogen was applied once to the plants in early May.
[b] Ratings made on region between 2nd and 4th internode at 3 week after inoculation with *Pseudomonas syringae* pv. *tomato* strain 95-3 on a sampling of eight leaflets per replicate. 1 = extremely limited lesions, little chlorosis; 2 = limited lesions, moderate chlorosis; 3 = moderate lesions (>15 per compound leaf), moderate chlorosis; 4 = numerous lesions, moderate coalesced lesions, moderate defoliation; 5 = severe defoliation, numerous coalesced lesions, stunting, chlorosis.
[c] Within each column, values followed by the same letter indicates no significant difference according to Duncan's multiple range test at $P = 0.05$.

sion, with means of 51.7 and 44.8% disease reduction, respectively. No enhancement in disease suppression was achieved when foliar biological control agents and PGPR strains were combined. The copper bactericide Kocide treatment did not result in significant disease reduction (Table 5).

In the field experiment conducted in FL in 1997, disease suppression provided by combined use of foliar biological control agent Cit7 and PGPR strain 89B-61, based on AUDPC values, was significantly higher than that of the two agents applied separately and not significantly different from the chemical treatment copper hydroxide plus Mancozeb (Table 6). The foliar biological control agent *P. syringae* Cit7 also reduced disease significantly when applied alone. Application of the foliar biological control agent *P. putida* strain B56 with either of the two PGPR strains provided no significant disease reduction (Table 6).

3.3. Control of bacterial spot in field trials

The bacterial strains Cit7 and 89B-61 were tested in a field trial in FL in spring 1998. Combined use of PGPR strain 89B-61 and foliar biological control agent Cit7 provided significant disease suppression, based on AUDPC values (Table 6). Seed and root treatment with 89B-61 or foliar spray with Cit7 alone provided some disease reductions, though these reductions were not statistically different from the untreated control (Table 6).

Field experiments were conducted with two foliar biological control agents Cit7 and B56 and two PGPR strains 89B-61 and SE34 in AL in 1997 and 1998. In the field trial conducted in fall 1997, all four bacterial strains, when applied separately, provided significant protection against the disease (based on AUDPC) compared with the pathogen-only control (Table 7). There was a slight numerical

Table 6
Efficacy of biological control agents in control of bacterial speck and spot of tomato in field trials in Bradenton, FL

Treatment	Application method[a]	AUDPC[b,c]	
		Speck (spring 1997)	Spot (spring 1998)
Control		63 a	1051 a
Copper + Mancozeb	Foliar	41 d	NT
B. pumilus SE34	Seed and root	57 a	NT
P. fluorescens 89B-61	Seed and root	59 a	921 ab
P. syringae Cit7	Foliar	51 b	632 ab
P. syringae Cit7 + *B. pumilus* SE34	Foliar (Cit7), seed and root (SE34)	49 bc	NT
P. syringae Cit7 + *P. fluorescens* 89B-61	Foliar (Cit7), seed and root (89B-61)	45 cd	394 b
P. putida B56	Foliar	59 a	NT
P. putida B56 + *B. pumilus* SE34	Foliar (B56), seed and root (SE34)	59 a	NT
P. putida B56 + *P. fluorescens* 89B-61	Foliar (B56), seed and root (89B-61)	60 a	NT

[a] "Seed and root" indicates seed soaking before sowing plus soil and root drench immediately before transplanting; "foliar" indicates foliar spray. Plants were transplanted into the field in mid March and foliar biological control agents were sprayed weekly beginning the last week of March. The pathogens were applied once to the plants in early April.
[b] AUDPC = area under the disease progress curve based on three disease ratings that were initiated 3 weeks after pathogen inoculation and once every 2 weeks thereafter. Ratings are based on a modified Horsfall–Barratt rating system. NT = not tested.
[c] Within each column, values followed by the same letter indicates no significant difference according to Duncan's multiple range test at $P = 0.05$.

Table 7
Efficacy of biological control agents in control of bacterial spot in a field experiment conducted in Shorter, AL (fall 1997)

Treatment	Application method[a]	Lesion number/leaflet[b,d]				AUDPC[c,d]
		9/29	10/6	10/13	10/23	
Control		4.4 a	4.0 a	6.1 a	7.9 a	232 a
Copper + Mancozeb	Foliar	1.3 cde	2.4 bc	2.1 bcd	3.3 def	111 ef
B. pumilus SE34	Seed and root	1.8 bcd	2.7 bc	2.8 bc	4.5 bcd	148 bcde
P. fluorescens 89B-61	Seed and root	2.3 b	3.3 ab	3.1 b	5.0 bc	158 bc
P. syringae Cit7	Foliar	1.8 bcd	2.8 abc	2.0 bcd	4.1 cdef	129 bcde
P. syringae Cit7 + *B. pumilus* SE34	Foliar (Cit7), seed and root (SE34)	2.0 bc	2.3 bc	1.7 de	3.6 cdef	121 cdef
P. syringae Cit7 + *P. fluorescens* 89B-61	Foliar (Cit7), seed and root (89B-61)	1.8 bcd	2.0 cd	1.7 de	3.5 cdef	121 cdef
P. putida B56	Foliar	1.9 bcd	2.5 bc	2.6 bcd	5.0 bc	152 bcd
P. putida B56 + *B. pumilus* SE34	Foliar (B56), seed and root (SE34)	2.7 b	3.4 ab	2.4 bcd	5.6 b	164 b
P. putida B56 + *P. fluorescens* 89B-61	Foliar (B56), seed and root (89B-61)	2.5 b	2.6 bc	1.9 cde	4.5 bcd	140 bcde

[a] "Seed and root" indicates seed soaking before sowing plus soil and root drench immediately before transplanting; "foliar" indicates foliar spray. Plants were transplanted into the field in mid August and foliar biological control agents were sprayed weekly beginning the last week of August. The pathogen was applied once to the plants in early September.
[b] Samples of 20 leaflets per replicate taken at different dates. Disease data were log-transformed prior to analysis.
[c] AUDPC = area under the disease progress curve based on lesion number per leaflet at the four assessment dates.
[d] Within each column, values followed by the same letter indicate no significant difference according to Duncan's multiple range test at $P = 0.05$.

Table 8
Efficacy of biological control agents in control of bacterial spot in a field experiment in Shorter, AL (summer 1998)

Treatment	Application method[a]	Lesion number/leaflet[b,d]			AUDPC[c,d]
		6/16	6/23	6/30	
Control		41.7 a	41.7 a	23.5 ab	693 a
Copper + Mancozeb	Foliar	13.8 c	16.0 d	17.6 b	411 d
B. pumilus SE34	Seed and root	25.3 b	27.8 b	25.9 a	579 b
P. fluorescens 89B-61	Seed and root	21.4 b	23.5 bc	23.5 ab	489 bcd
P. syringae Cit7	Foliar	20.9 b	19.0 cd	18.1 b	444 cd
P. syringae Cit7 + *B. pumilus* SE34	Foliar (Cit7), seed and root (SE34)	22.4 b	28.5 b	22.4 ab	534 bc
P. syringae Cit7 + *P. fluorescens* 89B-61	Foliar (Cit7), seed and root (89B-61)	20.9 b	29.9 b	20.9 ab	573 b

[a] "Seed and root" indicates seed soaking before sowing plus soil and root drench immediately before transplanting; "foliar" indicates foliar spray. Plants were transplanted into the field in late April and foliar biological control agents were sprayed weekly beginning the first week of May. The pathogen was applied to the plants once a week for 3 weeks beginning in mid May.
[b] Samples of 20 leaflets per replicate taken at different dates. Disease data were log-transformed prior to analysis.
[c] AUDPC = area under the disease progress curve based on lesion number per leaflet at the three assessment dates.
[d] Within each column, values followed by the same letter indicate no significant difference according to Duncan's multiple range test at $P = 0.05$.

decrease in AUDPC when Cit7 and 89B-61 or SE34 were applied jointly, although this decrease was not significant. When used separately, the foliar biological control agent Cit7 and PGPR strain SE34 provided disease reductions which were not significantly different (based on AUDPC) from the copper hydroxide plus Mancozeb treatment (Table 7).

Foliar biological control agent Cit7 and PGPR strain 89B-61 individually provided significant disease reduction (based on AUDPC) in the field trial conducted in AL in fall 1998. AUDPC values in the plots treated by the two strains were not significantly different from that of the chemical control (copper hydroxide plus Mancozeb). The PGPR strain *B. pumilus* SE34 also provided significant disease reduction compared with the pathogen-only control. Combined use of PGPR strains and Cit7 did not result in greater disease suppression (Table 8).

4. Discussion

The limitations of traditional approaches for control of bacterial speck and spot of tomato necessitate the exploration of alternative or complementary control methods such as biological control. However, the limited efficacy of available biological control agents (Byrne et al., 2005; Wilson et al., 2002) indicates that integration of different disease control approaches will be essential to achieve satisfactory disease suppression. In an attempt to develop an integrated biological control approach for bacterial speck and spot of tomato, foliar biological control agents applied as foliar spray were combined with selected PGPR strains applied both as a seed treatment and a soil drench. In the field trial conducted in FL in 1997, combined use of the PGPR strain 89B-61 and the foliar biological control agent Cit7 provided significantly greater suppression of bacterial speck than that provided by the biological control agents applied alone (Table 6). An increase in efficacy of control of bacterial spot was obtained by the combined use of these two bacterial strains in the field trials conducted in AL in fall 1997 and in FL in spring 1998 (Tables 6 and 7). However, combined use of these two strains did not enhance suppression of bacterial spot in AL in 1998 (Table 8); bacterial speck in CA in 1997 (Table 5); or in two of six ratings of bacterial speck in AL in 1997 and 1998 (Tables 3 and 4), indicating that combined use of these two biological control agents is not always synergistic. Nevertheless, the combination of Cit7 and 89-B61 gave numerically the lowest disease severity in at least one rating in 3 out of 4 field experiments with bacterial speck and 2 out of 3 experiments with bacterial spot, strongly suggesting at least some additive effect of these two agents.

Attempts to integrate biological control with chemical control or cultural practices or to use mixtures of biological control agents have been the subject of investigation in control of several plant diseases (Baker, 1990; Jetiyanon et al., 2003; Mazzola et al., 1995; Pierson and Weller, 1994; Raupach and Kloepper, 1998; Wei et al., 1996). However, efforts have not been directed to the integration of foliar-applied and rhizosphere-applied biological control agents. While this study indicates some benefit from the integration of rhizosphere-applied PGPR and foliar-applied biological control agents, the results obtained are not enough to justify the practical application of these combinations to improve the efficacy in control of bacterial speck or spot. Although enhancements in control efficacy were observed, there was no consistent trend toward improved disease suppression by these combinations from one field trial to another. It is unknown why improved efficacy occurred with some strain combinations but not others or occurred in one field trial but not another. Lack of improved efficacy with combined use of the biological control agents is possibly because PGPR-inoculated plants reduced epiphytic colonization or activity of some of the foliar biological control agents. This reduction might result in a level of activity comparable to that of the PGPR strain used alone.

Foliar biological control agent *P. syringae* Cit7 consistently provided significant disease suppression in control of both bacterial speck and spot. This finding was consistent with previous studies that showed foliar application of Cit7 was effective in control of bacterial speck and spot of tomato under field conditions at multiple locations in North America (Byrne et al., 2005; Wilson et al., 2002). The results of this study also provide evidence that the selected PGPR strains may provide significant suppression of bacterial speck and spot on tomato under field conditions. Since the first successful field trials with PGPR in 1996 that reduced bacterial wilt symptoms in cucumber (Wei et al., 1996), several studies have demonstrated PGPR-elicited ISR under field conditions (Jetiyanon et al., 2003; Murphy et al., 2000; Raupach and Kloepper, 1998, 2000; Wei et al., 1996; Zehnder et al., 2001). The PGPR strain *P. fluorescens* 89B-61 significantly reduced severity of angular leaf spot of cucumber caused by *P. syringae* pv. *lachrymans* in field trials conducted in AL (Wei et al., 1996). Induced resistance in tomato plant against *Tobacco mosaic virus* (TMV) and *Tomato mottle virus* (ToMoV) was obtained under field conditions by seed treatment and soil drench with PGPR strains *B. pumilus* SE34 and *B. amyloliquefaciens* IN937a (Murphy et al., 2000; Zehnder et al., 2001). In this study, seed and root treatment with PGPR strains 89B-61 and SE34 provided significant suppression of bacterial spot of tomato in AL field trials. Strain 89B-61 was also the most effective treatment in control of bacterial speck in a field trial conducted in CA in 1997. ISR-elicited by the PGPR strains was speculated to be the mechanism involved in the suppression of the diseases.

The efficacy of the selected PGPR strains and foliar biological control agents in control of bacterial speck of tomato in the field was not as consistent as in greenhouse assays. Strain 89B-61 significantly reduced the disease in all five greenhouse assays, while in the field trials significant suppression of bacterial speck was achieved only in CA in 1997 and in three out of six disease evaluations in AL in 1997 and 1998. Failure to provide significant disease suppression against bacterial speck also occurred in field trials in CA

(1997) and AL (1998) with the foliar biological control agent A506 and in FL (1997) with foliar biological control agent B56. In contrast, the foliar biological control agent Cit7 provided consistent protection in all four field trials. Many factors in the field trials may have contributed to the inconsistency of these bacteria in control of the disease. For example, field infection of tomato plants may involve different strains of the pathogen *P. syringae* pv. *tomato*, in contrast to single strain infection in the greenhouse, and the biological control agents may provide different levels of protection against different pathogen strains. In the case of PGPR strains, seed and root treatments with these strains in the greenhouse were applied 5 and 2 weeks, respectively, prior to inoculation of the pathogen (or 6 and 3 weeks prior to disease assessment). In the field trials, disease assessment was taken 10 or more weeks after seed treatment or more than 5 weeks after root treatment. These extended intervals between PGPR treatment and disease assessment may have had some effect on the efficacy. Furthermore, environmental conditions in the field may have been less favorable to establishment and activity of the biological control agents, resulting in variable disease control efficacy.

Efficacy against both bacterial spot and speck supports previous observations that PGPR provide systemic protection against multiple pathogens. PGPR-elicited ISR has been reported to be effective against bacterial, fungal, and viral pathogens of cucumber (Jetiyanon et al., 2003; Liu et al., 1995a,b; Raupach and Kloepper, 1998; Wei et al., 1996) and tomato (Jetiyanon et al., 2003; Jetiyanon and Kloepper, 2002; Murphy et al., 2000; Zehnder et al., 2001). The study also adds to the list of diseases against which *B. pumilus* SE34 and *P. fluorescens* 89B-61 have provided protection under field conditions. Isolate SE34 has been reported to protect against TMV and ToMoV in tomato and 89B-61 has been reported to protect against angular leaf spot and anthracnose in cucumber under field conditions (Murphy et al., 2000; Wei et al., 1996; Zehnder et al., 2001). It is speculated that other PGPR strains that showed promising results against bacterial speck in the greenhouse assays in this study may also be able to provide protection against diseases other than bacterial speck on tomato because of the potential of PGPR strains to induce plant resistance to several pathogens.

In this study, we demonstrated that foliar application of some biological control agents in conjunction with PGPR seed and root treatment may improve suppression of bacterial spot and speck of tomato. Practical application of these combinations remains to be justified since no particular combination of PGPR and foliar biological control bacteria in the field trials showed consistently elevated disease suppression compared with PGPR or foliar biological control agents applied alone. The results do support further study of the combined use of these foliar and rhizosphere biological control agents; for example, the impact of PGPR root application on population dynamics of the foliar biological control agents in the phyllosphere, the colonization and population dynamics of the PGPR strains in the rhizosphere under different field conditions, and the impact of combined use of PGPR and foliar biological control agents on phyllosphere populations of the pathogens. Such studies may allow the effective deployment of integrated foliage-applied and seed/root-applied biological control agents against bacterial speck and spot of tomato in the future.

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