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Protection of Tomato Seedlings against Infection by *Pseudomonas syringae* pv. Tomato by Using the Plant Growth-Promoting Bacterium *Azospirillum brasilense*†

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***Pseudomonas syringae* pv. tomato, the causal agent of bacterial speck of tomato, and the plant growth-promoting bacterium *Azospirillum brasilense* were inoculated onto tomato plants, either alone, as a mixed culture, or consecutively. The population dynamics in the rhizosphere and foliage, the development of bacterial speck disease, and their effects on plant growth were monitored. When inoculated onto separate plants, the *A. brasilense* population in the rhizosphere of tomato plants was 2 orders of magnitude greater than the population of *P. syringae* pv. tomato (10^7 versus 10^5 CFU/g [dry weight] of root). Under mist chamber conditions, the leaf population of *P. syringae* pv. tomato was 1 order of magnitude greater than that of *A. brasilense* (10^7 versus 10^6 CFU/g [dry weight] of leaf). Inoculation of seeds with a mixed culture of the two bacterial strains resulted in a reduction of the pathogen population in the rhizosphere, an increase in the *A. brasilense* population, the prevention of bacterial speck disease development, and improved plant growth. Inoculation of leaves with the mixed bacterial culture under mist conditions significantly reduced the *P. syringae* pv. tomato population and significantly decreased disease severity. Challenge with *P. syringae* pv. tomato after *A. brasilense* was established in the leaves further reduced both the population of *P. syringae* pv. tomato and disease severity and significantly enhanced plant development. Both bacteria maintained a large population in the rhizosphere for 45 days when each was inoculated separately onto tomato seeds (10^5 to 10^6 CFU/g [dry weight] of root). However, *P. syringae* pv. tomato did not survive in the rhizosphere in the presence of *A. brasilense*. Foliar inoculation of *A. brasilense* after *P. syringae* pv. tomato was established on the leaves did not alleviate bacterial speck disease, and *A. brasilense* did not survive well in the phyllosphere under these conditions, even in a mist chamber. Several applications of a low concentration of buffered malic acid significantly enhanced the leaf population of *A. brasilense* (>10^8 CFU/g [dry weight] of leaf), decreased the population of *P. syringae* pv. tomato to almost undetectable levels, almost eliminated disease development, and improved plant growth to the level of uninoculated healthy control plants. Based on our results, we propose that *A. brasilense* be used in prevention programs to combat the foliar bacterial speck disease caused by *P. syringae* pv. tomato.**

Tomato plants are hosts to *Azospirillum brasilense*, a plant growth-promoting bacterium (PGPB) (7, 8), and *Pseudomonas syringae* pv. tomato, the causal agent of bacterial speck disease (35, 47). This disease is of moderate economic importance to tomato production under greenhouse and field conditions (24). Whereas *A. brasilense* increases the growth and yield of tomato plants (14), *P. syringae* pv. tomato decreases production (51). Although *A. brasilense* is known as a rhizosphere bacterium (7), some strains are ephiphytic (7, 10), and *P. syringae* pv. tomato is largely an epiphytic foliar bacterium (15, 36). Nevertheless, both bacterial species are capable of colonizing seeds, leaves, and the rhizosphere. Plant foliage is heavily colonized by both bacterial species only under mist chamber conditions (4) that favor bacterial speck infections (12). The presence of low levels of these bacterial species under dry leaf conditions is known as well (4, 36).

Despite progress in our understanding of the molecular mechanisms of *P. syringae* pv. tomato pathogenicity (20), including the sources of genetic resistance to the pathogen (6, 18, 33, 34, 39, 50; V. F. Lawson and W. L. Summers, abstr., HortScience **17:**503) and the genes which confer resistance to the disease (30, 41), this knowledge has not yet translated into an efficient strategy to control this minor, but occasionally devastating, foliar disease. Disease control is still based on traditional chemical and physical methods (3), and despite some significant successes, achieved mainly by the induction of systemic resistance in plants (1, 27, 45, 52) and the displacement of a pathogen by nonvirulent strains of the same pathogen or by ecologically similar antagonistic strains (28, 43, 44, 48, 49), biological control of foliar bacterial pathogens is still largely at the experimental stage.

The aim of this study was to measure the fluctuations in the populations of the two bacterial species, belonging to different genera, on the foliage and in the rhizospheres of tomato plants inoculated with one or both species. The effect of the relative sizes of the bacterial populations on the development of bacterial leaf speck disease in tomato plants and on plant growth was monitored.

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† This study is dedicated to the memory of the late Avner Bashan and Uzi Bashan from Israel.

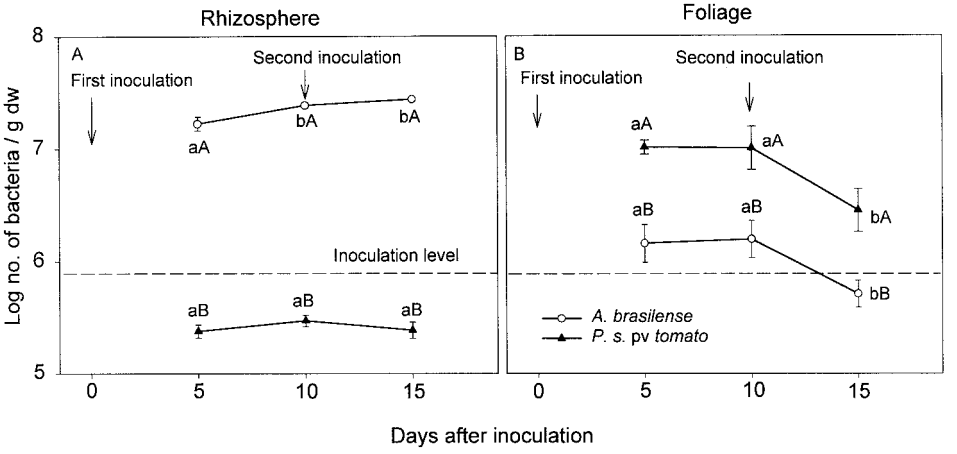

FIG. 1. Sizes of *P. syringae* pv. tomato and *A. brasilense* populations in the rhizospheres and on the foliage of tomato plants when the bacteria were inoculated on separate plants. Points located on the same line and denoted by different lowercase letters differ significantly at a P of ≤ 0.05 by one-way ANOVA. Points which share the same time position and are denoted by different capital letters differ significantly at a P of ≤ 0.05 by Student's t test. Bars represent standard errors (SE); missing bars indicate that the SE is smaller than the point.

MATERIALS AND METHODS

Organisms and growth conditions. *A. brasilense* Cd (DSM 1843) and a naturally triple-antibiotic-resistant mutant (oxytetracycline, rifampin, and kanamycin, 200 μg of each per liter) of *P. syringae* pv. tomato (WT-1-ORK) from our laboratory culture collection were used in this study and grown as described previously (4). *P. syringae* pv. tomato, strain WT-1 (12), was originally isolated from infected tomato plants growing in a greenhouse during an epidemic in 1975. In preliminary greenhouse and growth chamber tests, there was no difference in virulence between *P. syringae* pv. tomato WT-1-ORK used in this study and wild-type *P. syringae* pv. tomato WT-1 (unpublished data).

Tomato plants (*Lycopersicon esculentum* Mill) of the susceptible fresh-market cultivar Pik Red (Joseph Harris Co., Rochester, N.Y.) (24) were grown in black, 500-ml pots containing a sterile (tyndallized) commercial potting substrate (Sunshine Mix 3, special fine; Fisons Horticulture, Mississauga, Ontario, Canada) in a greenhouse as previously described (16). The commercial greenhouse cultivation of tomato plants in steamed, nonsoil substrates, where plants are subjected to a high relative humidity and the condensation of free water drops on leaf surfaces, is commonplace (12).

Inoculation and detection techniques. Seeds and leaves were inoculated with *A. brasilense* Cd and *P. syringae* pv. tomato, as described previously, after the plants were preconditioned under mist conditions (4, 12). Leaves were inoculated at the three- to five-true-leaf stage with a handheld pneumatic sprayer from a height of 25 to 35 cm above the plant. Plants were sprayed until runoff occurred. Tomato seeds or leaves were inoculated with monocultures of each bacterium separately or with both species together at the intervals mentioned for each experiment. In each case, the total inoculation level was 10^6 CFU/ml. These concentrations are optimal for plant growth promotion by *Azospirillum* sp. (14), for avoiding the growth inhibition known to be induced by high cell concentrations (2, 25), and for preventing atypical symptom formation caused by high concentrations of *P. syringae* pv. tomato (12). Plants were incubated under mist chamber conditions in the greenhouse with a temperature regime of 28°C during the day and 22°C at night and with natural illumination. Mist diffuser jets applied mist every 30 min for 5 s, creating permanently wet leaves with minimal dripping of liquids from the surfaces. To increase the *A. brasilense* Cd population on the leaves, diluted malic acid was sprayed onto the leaves prior to inoculation; before application, the malic acid was adjusted to pH 6.5 with 0.01 g of NaOH per liter and then dissolved in 0.01 M phosphate-buffered saline, pH 6.5, to achieve a final concentration of 0.02 g/liter.

After the leaves were homogenized as previously described (38), bacteria were specifically detected and enumerated by an enzyme-linked, immunosorbent assay for *A. brasilense* Cd (11, 26) and by the plate count method for *P. syringae* pv. tomato on nutrient agar plates supplemented with an antibiotic package (200 μg of each antibiotic [oxytetracycline, rifampin, and kanamycin] per liter of medium). Rhizosphere populations were sampled as previously described (4, 11, 14). The total number of cultured bacteria in the phyllospheres and in the rhizospheres of plants growing under dry ambient temperature conditions were enumerated by the plate count method on nutrient agar (Difco, Detroit, Mich.) supplemented with cycloheximide (250 mg/liter; Sigma) after 72 h of incubation at 28 ± 2°C.

Evaluation of disease severity and bacteriocin activity. Disease severity was evaluated visually and scored using a disease index with a range of 0 to 3 (0 signifies a healthy-looking plant; 1 signifies 2 to 5 specks together or spread over each leaf; 2 signifies 6 to 10 specks; and 3 signifies more than 10 specks), as previously described (50).

Experimental design and statistical analysis. The plants subjected to the various treatments were randomly placed in a growth chamber or a mist chamber. Each treatment was replicated five times, and three pots served as a single replicate; each pot contained two plants. Data from the three pots were combined, and the entire experiment was analyzed by analysis of variance (ANOVA) or by Student's t test at a P of ≤ 0.05. This was done because tomato seedlings are small in the initial stages of growth yet need space to grow. Six individual seedlings supplied enough dry matter to avoid the inaccuracies that occur when dry weight is determined for individual, very small plants. All experiments were repeated two or three times. The detection intervals of both bacterial species varied among the experiments, ranging from the time of inoculation to 15 days postinoculation in short-term experiments and up to 60 days postinoculation in longer experiments. Nevertheless, both *A. brasilense* Cd and *P. syringae* pv. tomato organisms were enumerated at the same intervals during each experiment.

RESULTS

Sizes of *P. syringae* pv. tomato and *A. brasilense* populations in the rhizospheres and on the foliage of tomato plants when each bacterium was inoculated individually. Five or 10 days after seed inoculation, the *A. brasilense* populations in the rhizospheres of tomato plants (over 10^7 CFU/g [dry weight] of root) were 2 orders of magnitude greater than the *P. syringae* pv. tomato populations (over 10^5 CFU/g [dry weight] of root) in the rhizospheres of separate tomato plants. No further increases in the population sizes were detected later (Fig. 1A). On the same plants, under mist chamber conditions, the leaf populations of *P. syringae* pv. tomato were 1 order of magnitude greater than the *A. brasilense* populations 5 days after inoculation. The populations did not increase further with time, and they later decreased (Fig. 1B). All of these changes occurred against the background of a natural microbial population of 100 to 1,000 CFU of culturable phyllosphere bacteria per g (dry weight) of leaf. The total cultured rhizosphere population was constant before and immediately after inoculation,

TABLE 1. Total cultured bacterial populations on leaves and in the rhizospheres of tomato plants prior to competition experiments

Location of populations	Mean bacterial population (CFU/g [dry wt]) ± SE		
	Plants growing under dry ambient conditions	Plants preconditioned with 24-h misting	Plants immediately after inoculation[a]
Phyllosphere	$2.3 \times 10^2 \pm 9 \times 10^1$	$4.4 \times 10^3 \pm 1.4 \times 10^3$	$2.1 \times 10^3 \pm 6 \times 10^2$
Rhizosphere	$1.7 \times 10^5 \pm 3 \times 10^4$	$2.1 \times 10^5 \pm 4 \times 10^4$	$1.9 \times 10^5 \pm 2 \times 10^4$

[a] Excluding populations of *P. syringae* pv. tomato and *A. brasilense*.

regardless of the preconditioning of the leaves with mist, and it was lower than the population of inoculated bacteria (Table 1). A second inoculation of *P. syringae* pv. tomato or *A. brasilense* into the rhizosphere, 10 days after the initial inoculation, did not increase the *P. syringae* pv. tomato and *A. brasilense* populations there or in the plant foliage (Fig. 1).

Sizes of *P. syringae* pv. tomato and *A. brasilense* populations in the rhizospheres of tomato plants and their effects on the dry weights of tomato seedlings when the bacteria were inoculated as a mixed culture on seeds. The inoculation of seeds with a mixed culture (*A. brasilense* and *P. syringae* pv. tomato) in which the populations of the bacteria were initially similar (10^6 CFU/ml) resulted in a reduction of the pathogen population in the rhizosphere and a temporary (at 10 days) increase in the *A. brasilense* population (Fig. 2A). In addition, when plants were grown from seeds inoculated with a mixture of both bacterial species, bacterial speck disease did not develop, even after plants were transferred to mist chamber conditions that favor disease development (data not shown). Compared to inoculation with the pathogen alone, mixed inoculation increased plant biomass, although to a lesser extent than inoculation with *A. brasilense* alone (Fig. 2B).

Sizes of *P. syringae* pv. tomato and *A. brasilense* populations and the development of bacterial leaf speck disease on tomato leaves inoculated with a mixed bacterial culture. Compared to inoculation with *P. syringae* pv. tomato alone, leaf inoculation with a mixed bacterial culture under mist conditions significantly reduced the *P. syringae* pv. tomato population and significantly decreased disease severity (Fig. 3A and B). Challenging plants with *P. syringae* pv. tomato 4 days after *A. brasilense* was established on the leaves reduced the population of *P. syringae* pv. tomato and disease severity even further (Fig. 3A and B). The presence of *A. brasilense* on the plant, whether from inoculation in a mixed culture with *P. syringae* pv. tomato or from inoculation alone before challenge with the pathogen, significantly enhanced plant development (Fig. 3C).

Both bacteria maintained large populations in the rhizosphere for 45 days when each was inoculated separately onto tomato seeds. However, *P. syringae* pv. tomato did not survive in the rhizosphere in the presence of *A. brasilense* (Table 2).

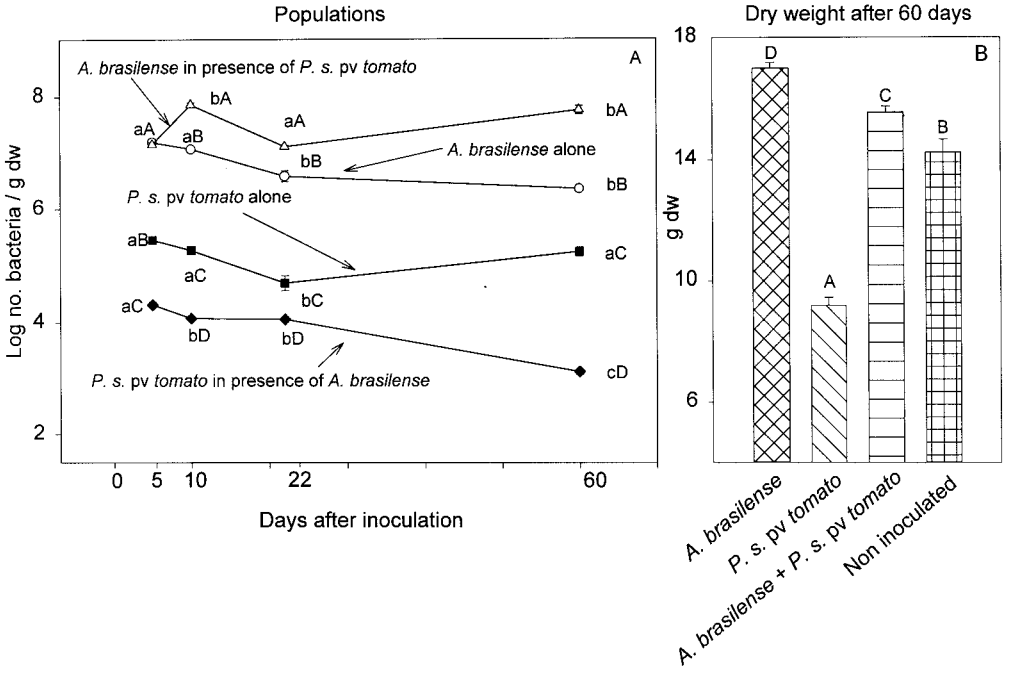

FIG. 2. Sizes of *P. syringae* pv. tomato and *A. brasilense* populations in the rhizospheres of tomato plants (A) and their effects on the dry weights (dw) of tomato seedlings (B) when the bacteria were inoculated alone and together onto seeds. (A) Points located on the same line and denoted by different lowercase letters differ significantly at a P of ≤ 0.05 by one-way ANOVA. Points which share the same time position and are denoted by different capital letters differ significantly at a P of ≤ 0.05 by one-way ANOVA. (B) Columns denoted by different capital letters differ significantly at a P of ≤ 0.05 by one-way ANOVA. Bars represent SE; missing bars indicate that the SE is smaller than the point.

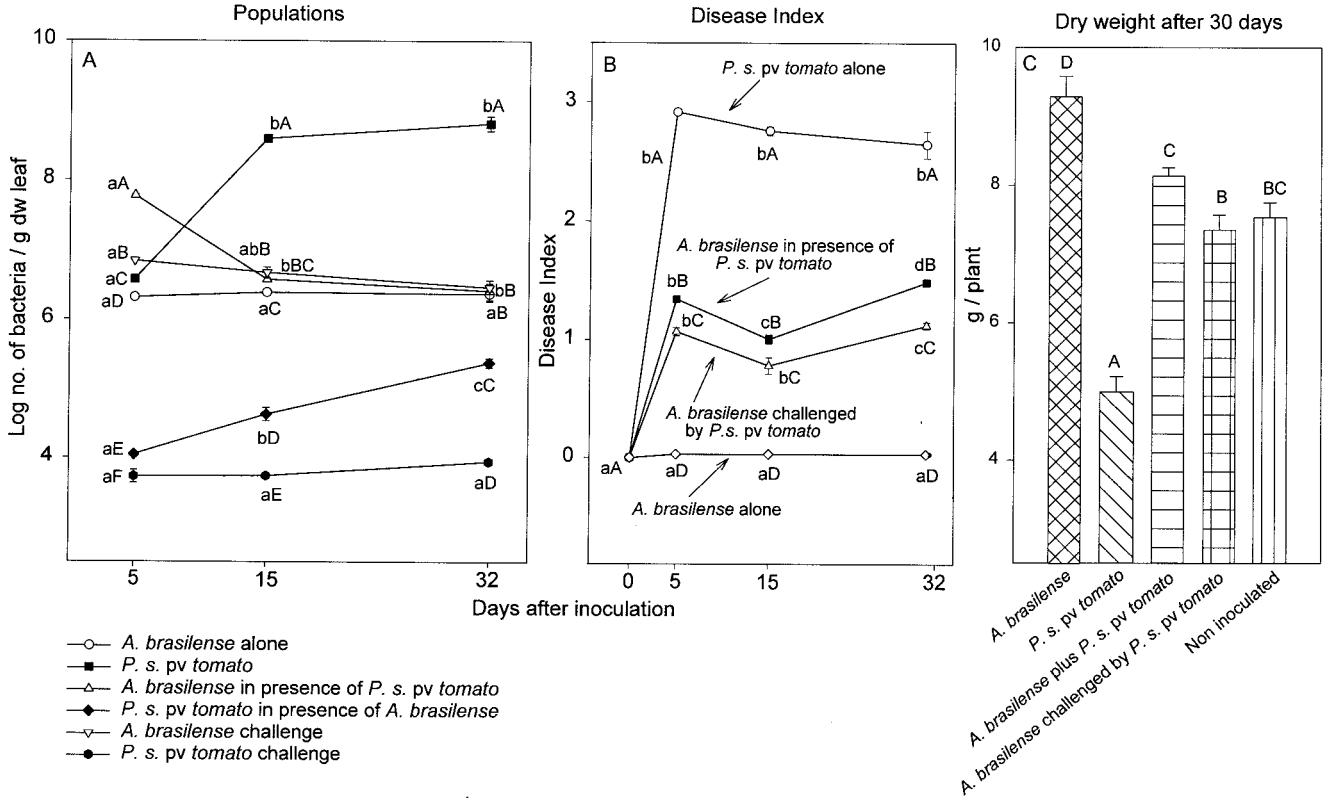

FIG. 3. Sizes of *P. syringae* pv. tomato and *A. brasilense* populations (A), the development of bacterial leaf speck disease in tomato leaves (B), and the effect on dry weight of the plants (C) after inoculation of the bacteria onto leaves. (A and B) Points located on the same line and denoted by different lowercase letters differ significantly at a P of ≤ 0.05 by one-way ANOVA. Points which share the same time position and are denoted by different capital letters differ significantly at a P of ≤ 0.05 by one-way ANOVA. (C) Columns denoted by different capital letters differ significantly at a P of ≤ 0.05 by one-way ANOVA. Bars represent SE; missing bars indicate that the SE is smaller than the point.

Foliar inoculation with *A. brasilense* after *P. syringae* pv. tomato was allowed to establish itself on leaves for 2 days did not alleviate bacterial speck disease (Table 3). Under these conditions, *A. brasilense* did not survive well in the phyllosphere, even under mist conditions.

Development of bacterial speck disease on tomato leaves after leaf inoculation with *A. brasilense* and malic acid. Several applications of a low concentration of buffered malic acid significantly enhanced the size of the *A. brasilense* leaf population, decreased the population of *P. syringae* pv. tomato to an almost undetectable level (Fig. 4A), nearly eliminated disease development (Fig. 4B), and improved the growth of the inoculated plants to the level of the uninoculated, healthy control plants (Fig. 4C). Long-term treatment of tomato leaves with malic acid, however, resulted in a pale chlorosis of the leaves after the third and subsequent applications (data not shown).

DISCUSSION

Most biological control strategies use biocontrol agents that suppress plant pathogens either by producing inhibitory substances (19) or by displacing (outcompeting) the pathogen (17, 23, 32, 40, 43, 49). *Azospirillum* spp. are not known as typical biocontrol PGPBs; *A. brasilense* lacks the capacity to produce significant amounts of antibacterial substances (apart from some bacteriocins and siderophores) (31, 37, 42). This bacterium is also unable to induce systemic resistance in plants (5). However, *A. brasilense* is considerably rhizo-competent (10)

TABLE 2. Long-term survival of *P. syringae* pv. tomato and *A. brasilense* in the rhizospheres of tomato plants whose seeds were inoculated with one or both species

Bacterial species inoculated	Mean bacterial population level in roots (CFU/g [dry weight]) ± SE after:			
	5 days		45 days	
	A. brasilense	*P. syringae* pv. tomato	*A. brasilense*	*P. syringae* pv. tomato
A. brasilense alone	$2.5 \times 10^7 \pm 4 \times 10^6$		$3.4 \times 10^6 \pm 7 \times 10^5$	
P. syringae pv. tomato alone		$3.3 \times 10^5 \pm 3 \times 10^4$		$1.8 \times 10^5 \pm 2 \times 10^4$
A. brasilense + *P. syringae* pv. tomato	$5.5 \times 10^7 \pm 8 \times 10^6$	$1.3 \times 10^4 \pm 5 \times 10^3$	$4.4 \times 10^6 \pm 6 \times 10^5$	Undetectable

TABLE 3. Development of bacterial leaf speck disease of tomato after leaf inoculation with *P. syringae* pv. tomato followed by inoculation with *A. brasilense* 3 days later[a]

Bacterial species	Bacterial population level in plants after 10 days (CFU/g [dry wt])		Disease severity index[b]	Plant dry wt after 15 days (mg)
	A. brasilense	*P. syringae* pv. tomato		
A. brasilense alone	7.1×10^6 A		0	88 B
P. syringae pv. tomato alone		7.3×10^8 A	2.87 A	63 A
P. syringae pv. tomato + *A. brasilense*	4.6×10^4 B	5.9×10^8 A	2.79 A	66 A

[a] Values followed by a different letter within each column indicate results that differ significantly at a P of ≤ 0.05 by one-way ANOVA.
[b] Disease severity was scored from 0 to 3 as described in Materials and Methods.

and is able to multiply to form large populations on leaves under wet conditions (4) that are also favorable to many leaf pathogens. These capacities led us to ask whether *A. brasilense* can displace a leaf pathogen (*P. syringae* pv. tomato) and in the process reduce disease severity and improve plant growth.

In this study, competition between two different bacterial species, one pathogenic and the other a PGPB hosted by the same plant, was probably a mechanism of biocontrol of this leaf disease. The displacement of *P. syringae* pv. tomato cells by *A. brasilense* was demonstrated by the reduced colonization of *P. syringae* pv. tomato in the rhizosphere and on leaf surfaces in the presence of *A. brasilense*. A similar phenomenon occurred when *A. brasilense* was mixed with the mangrove rhizosphere bacterium *Staphylococcus* sp. (22). The mechanism for the displacement is not known, but it is likely that *A. brasilense* is better able to obtain nutrients or to colonize plant surfaces. Earlier studies showed that both bacteria are mainly epiphytic on tomato leaves and not endophytic (4, 15).

Alternatively, the protective mechanism of *A. brasilense* might be indirectly explained by the plant growth promotion effect. That is, due to the positive influence of *A. brasilense*, a more robust plant might be able to fend off the *P. syringae* pv.

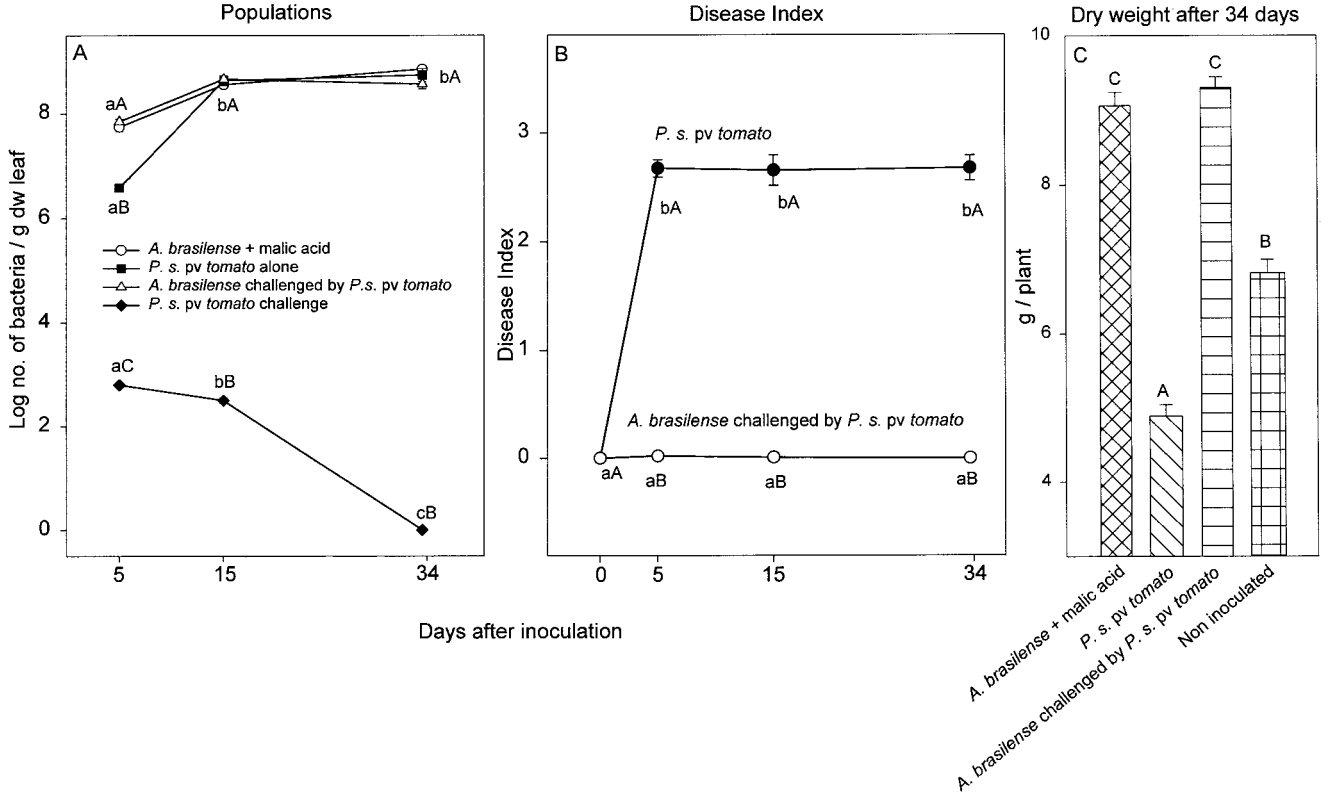

FIG. 4. Sizes of *P. syringae* pv. tomato and *A. brasilense* populations (A), development of bacterial speck disease in tomato leaves (B), and the effect on dry leaves of the plants (C) after leaf inoculations. (A and B) Points located on the same line and denoted by different lowercase letters differ significantly at a P of ≤ 0.05 by one-way ANOVA. Points which share the same time position and are denoted by different capital letters differ significantly at a P of ≤ 0.05 by one-way ANOVA (A) and by Student's t test (B). (C) Columns denoted by different capital letters differ significantly at a P of ≤ 0.05 by one-way ANOVA. Bars represent SE; missing bars indicate that the SE is smaller than the point. For clarity, values for *A. brasilense* inoculated alone onto leaves are not presented, as similar information is available in other graphs; values for *P. syringae* pv. tomato supplemented with malic acid are also not presented, as these were almost identical to those from inoculation with *P. syringae* pv. tomato alone. For clarity, some letters indicating statistical significance in panel A were also eliminated.

tomato infection more readily. This possibility is consistent with the observation that prior colonization of the plant by the pathogen abolished any protective effects.

Azospirillum prefers malic acid and several other organic acids, like lactate, fumarate, and succinate, as carbon growth substances (29), while the metabolism of organic acids in *P. syringae* pv. tomato is not efficient. *P. syringae* pv. tomato prefers sugars, like galactose, glucose, sorbitol, and sucrose (13), which *A. brasilense* cannot metabolize (21). These affinities were exploited by *A. brasilense* to preferentially enhance its population on leaves; malic acid has no effect on the proliferation of *P. syringae* pv. tomato, as it probably cannot metabolize it. Organic acids metabolized by *A. brasilense* by the Embden-Meyerhof-Parnas hexose phosphate pathway via the enzymes malate dehydrogenase and lactate dehydrogenase (21, 46) are the basis of most *Azospirillum* culture media (9, 29). The increase in the population of *A. brasilense*, a consequence of malic acid application, probably helped *A. brasilense* to displace *P. syringae* pv. tomato more extensively than it did in the absence of malic acid. Despite the minor yellowing of leaves following malic acid treatment, this approach of enhancing phyllosphere competition by supplying a PGPB with specific nutrients warrants further exploration. Industrial-grade organic acids are available in inexpensive bulk quantities, as they are used regularly by the food industry. Perhaps the yellowish color is a symptom of metabolic inhibition in the host plant caused by the high levels of *A. brasilense* on the leaves; *A. brasilense* is known to inhibit the growth of roots at very high concentrations (2, 25). Alternatively, this application perhaps induced a minor chlorosis, a common symptom of nutrient stress in plants.

We propose that our data support the notion that PGPBs, such as *Azospirillum* sp. and probably other phyllosphere PGPBs (17, 28, 43, 44, 48, 49), can be used in programs to combat foliar bacterial diseases. For the protection of plants, the displacement of a pathogen by a competing species and the competitor's superior adaptation to an ecological niche may prove to be as effective as direct pathogen inhibition or the induction of systemic resistance. This is especially valid when a suitable biocontrol PGPB that inhibits pathogens by secreting antimicrobial compounds has not been identified, as is the case for some foliar pathogens of crops.

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