A Vacuum Infiltration Inoculation Technique for Detecting *Pseudomonas tomato* in Soil and Plant Tissue

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ABSTRACT

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A vacuum infiltration inoculation technique proved to be highly effective for detecting *Pseudomonas tomato* in extracts from artificially infested field soil and in washings from surfaces of four of six symptomless weed species collected from fields where infected tomato plants had grown the previous year. Populations as low as 10 colony-forming units per gram of soil or per milliliter of test suspension were detected. The procedure consists of immersing the foliage of bare-root Chico III tomato plants (12–15 cm tall) in test suspensions (one drop of Tween 20 added per 100 ml) held in beakers; evacuating to 76 cm Hg for two separate periods (0.5–1.0 and 2 min), each followed by a sudden vacuum release; and placing the transplants at 19–21 C for 14 days to allow lesion development. Isolations were made from individual lesions, and selected laboratory and pathogenicity tests were run to confirm the identity of *P. tomato*. The vacuum infiltration method detects lower levels of *P. tomato* in natural habitats than other methods do and detected *P. tomato* when conventional plating methods with King's medium B failed.

Additional key words: bacterial speck, *Lycopersicon esculentum*, soil assays.

Incidence of bacterial speck of tomato, caused by *Pseudomonas tomato* (Okabe) Altstatt (*P. syringae* pv. *tomato*), has increased in widespread areas of the world in recent years (9). Tomato transplant producers in southern Georgia and tomato growers in the northern United States and Canada who use Georgia transplants have been greatly concerned about the disease since 1978, when more than 160 ha of transplants were rejected for certification by the Georgia Department of Agriculture (1) because of a high incidence of bacterial speck, and other infected transplants were inadvertently shipped to northern areas (12).

Although several recent studies (2,5,7,16–18,21) have contributed to a better understanding of the ecology and epidemiology of *P. tomato,* some important aspects are still poorly understood, especially as they relate to the transplant industry. Little is known about sources of primary inoculum or soil survival of *P. tomato* in southern Georgia. Progress in these deficient areas is hindered by a lack of effective or efficient methods for detecting the organism in its natural habitats. No suitable selective medium for isolating *P. tomato* under a variety of natural conditions has been developed. Methods that have been used to detect the organism include plating on media such as King's medium B (KMB) to allow limited differentiation by fluorescent pigment production (7,16), plating on KMB or other media amended with antimicrobial agents to provide some selectivity (2,5), using a rifampicin-resistant mutant (8), and bioassay procedures involving germinating seed in soil under optimal conditions for disease development (7,12). Plating methods are usually supplemented with pathogenicity tests to confirm the identity of *P. tomato* (5,7,16).

We have used these methods in our work and found that they either lacked selectivity or failed to detect low populations of the pathogen. Detection is most difficult when *P. tomato* is present in natural soil or is associated with symptomless weed hosts where populations are low and are mixed with rapidly growing, saprophytic, fluorescent pseudomonads and other bacteria that are difficult to eliminate with antimicrobial agents. Goto (10) suggested that the vacuum infiltration method originally described by Boosalis (4) could be used to detect low populations of plant pathogens in soil or in association with weeds. We determined the efficiency of the vacuum infiltration technique for detecting *P. tomato* in soil and on suspect symptomless weed hosts.

MATERIALS AND METHODS

We used a highly virulent isolate of *P. tomato* (designated field 3) that originated from tomato transplants growing near Tifton, GA, in 1978. Concentrations of cell suspensions, prepared by washing bacterial growth with sterile, distilled water from plate cultures grown at 25 C for 36–48 hr on KMB (13), were determined with a Bausch & Lomb Spectronic 20 set at 590 nm, and desired concentrations were obtained by appropriate dilution with sterile, distilled water.

Tomato (*Lycopersicon esculentum* Mill. 'Chico III') plants for vacuum infiltration were grown in the greenhouse for 4–5 wk (12–15 cm tall) in pots 10 cm in diameter or 0.35-L plastic cups filled with a methyl bromide-fumigated soil:sand:vermiculite: perlite (6:2:1:1, v/v) mix. Plants, lifted from the soil with minimum

TABLE 1. Vacuum infiltration detection of *Pseudomonas tomato* in pure culture suspensions and in suspensions from soil artificially infested at four levels

	Mean number of lesions per infiltrated plant[b]	
Concentration[a]	Cell suspension	Soil extract[c]
10^1	4.3	0.3
10^2	27.5	3.8
10^3	148.3	12.8
10^4	EN[d]	109.7

[a]Colony-forming units per milliliter of suspension or per gram of soil. Suspensions for direct assay and for soil infestation were prepared from King's medium B plates of *P. tomato* grown for 36 hr at 25 C.
[b]Sixteen plants (four replicates each with four plants) were used for each treatment. Lesions were counted 14 days after vacuum infiltration.
[c]Soil extracts were prepared by homogenizing 50 g of soil and 200 ml of sterile, distilled water in a Waring Blendor for 3 min, mixing vigorously on a wrist-action shaker for 20 min, and centrifuging at 1,400 *g* for 5 min to remove particles.
[d]Extensive necrosis of tissue prevented the counting of individual lesions.

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injury to the root system, were vacuum-infiltrated and replanted into the same pots or cups after treatment.

Vacuum infiltration procedure. Healthy, bare-root tomato plants were inverted to immerse the foliage in approximately 200 ml of each test suspension (amended with two drops of Tween 20) in a 250-ml beaker. Beakers, each containing four to five immersed plants, were placed in a vacuum desiccator 250 mm in diameter. A vacuum of 76 cm Hg was pulled for 0.5–1.0 min, released abruptly, repeated for 2 min, and again released abruptly. Plants were either potted immediately or were held with their roots in water for 5–10 min before repotting. Treated plants were placed in a growth chamber at 19–21 C for 14 days to allow symptom development.

Detection of *P. tomato* in extracts from artificially infested soils. A Dothan loamy sand considered free of *P. tomato* collected near Tifton was artificially infested with *P. tomato* at levels of 10^1, 10^2, 10^3, and 10^4 colony-forming units (cfu) per gram of soil. Cells were incorporated into the soil by atomizing appropriate suspensions into the soil rotating in a cement mixer. Four 50-g samples (replicates) of each infested soil were suspended in 200 ml of sterile, distilled water, homogenized in a Waring Blendor for 3 min, mixed vigorously on a wrist-action shaker for 20 min, and centrifuged at 1,400 *g* for 5 min. The supernatants were collected and used for vacuum infiltration of tomato plants. Samples of each supernatant were also spread on the surface of plates of KMB (0.1 ml per plate). The plates were incubated at 25 C for 48 hr and examined for colonies of *P. tomato* under an ultraviolet lamp.

Detection of *P. tomato* in washings from plant tissue. Seven samples of six weed species possibly harboring epiphytic populations of *P. tomato* were collected in March and April of 1980 from two fields on the University of Georgia Plant Sciences Farm near Athens. High incidence of bacterial speck had occurred on tomato grown in these fields during the summer of 1979. Primrose (*Oenothera* sp.), rye (*Secale cereale* L.), and cudweed (*Gnaphalium* sp.) were collected from field one, and chickweed (*Stellaria media* (L.) Cyr.), mouse-ear cress (*Arabidopsis thaliana* (L.) Heynh.), henbit (*Lamium amplexicaule* L.), and cudweed were collected from field two.

Samples from four separate plants of each species were combined for assay. Only foliage was collected from field one, but foliage and roots of plants from field two were assayed. Foliage and root samples were placed separately in 200 ml of sterile, distilled water in 500-ml flasks and mixed vigorously on a wrist-action shaker for 20 min. The suspending liquid was used directly for vacuum infiltration of tomato plants (foliage samples) or was centrifuged at 1,400 *g* for 5 min to remove excess soil before infiltration (root samples).

The presence of *P. tomato* in the test suspensions was indicated by the appearance of bacterial speck symptoms on the infiltrated plants in 7–10 days. Tests were run on the bacteria isolated from lesions to confirm the identity of *P. tomato*. Lesions from infiltrated plants were comminuted separately in drops of sterile, distilled water and streaked on plates of KMB. Green fluorescent colonies typical of *P. tomato* were picked from the isolation plates and later tested for their oxidase reaction (15), using Taxo differentiation disks (BBL Div. of Becton, Dickinson & Co., Cockeysville, MD 21030); tobacco hypersensitivity (14), by infiltrating suspensions (10^8 cfu/ml) into sections of *Nicotiana tabacum* 'Hicks'; and pathogenicity on tomato. Six-week-old Chico III tomato plants (16–18 cm tall) were inoculated with a cell suspension (10^8 cfu/ml) applied to runoff with a Burgess Model 862 paint sprayer (Vibrocrafters, Inc., Grayslake, IL 60030) held 30–35 cm from the leaf surface to minimize infiltration. Inoculated plants were placed at high humidity (covered with clear polyethylene bags for 36 hr) and were held at 18–21 C for 14 days after inoculation to allow symptom development. A positive pathogenicity test on tomato was considered confirmation of *P. tomato*, because no other known pseudomonad produces symptoms similar to bacterial speck.

Efficacy of the vacuum infiltration method. The efficacy of vacuum infiltration was compared with that of external application (spraying) of bacterial suspensions, and the influence of a humidity period after treatment was tested. Water suspensions containing 10^1, 10^2, 10^3, and 10^4 cfu/ml of *P. tomato* were vacuum-infiltrated into Chico III tomato plants or were sprayed to runoff on the abaxial and adaxial leaf surfaces with a paint sprayer as described above. Twenty plants of each treatment combination were prepared; 10 were placed directly in a growth chamber at 21 C without a high-humidity period, and the other 10 in the same chamber were covered with clear polyethylene bags for the first 36 hr after inoculation. Bacterial speck lesions were recorded 14 days after inoculation.

RESULTS

The vacuum infiltration procedure effectively detected *P. tomato* in extracts from artificially infested field soil (Table 1) and from the foliage and roots of several weed species collected from two naturally infested fields (Table 2). *P. tomato* was detected in soil with population densities as low as 10 cfu/g of soil, and lesion counts on infiltrated tomato plants increased as the soil infestation level increased (Table 1). The bacterium was not recovered from soil supernatants plated directly on plates of KMB, primarily because of high population densities of saprophytes. Portions of the four pure culture suspensions (10^1–10^4 cfu/ml) used to infest the

TABLE 2. Recovery and characterization of green fluorescent pseudomonads from lesions on tomato plants after vacuum infiltration with washings from leaves and roots of six weed species

Weed species[a]	Plant part assayed	Field	Symptoms[b]	Characteristics of isolates: Oxidase reaction	HR on tobacco[c]	Pathogenicity on tomato[d]
Oenothera sp.	Leaves	1	±	+	−	−
Secale cereale	Leaves	1	±	+	−	−
Gnaphalium sp.	Leaves	1	+	−	+	+
	Leaves	2	−			
	Roots	2	−			
Stellaria media	Leaves	2	+	−	+	+
	Roots	2	+	−	+	+
Arabidopsis thaliana	Leaves	2	+	−	+	+
	Roots	2	+	−	+	+
Lamium amplexicaule	Leaves	2	+	−	+	+
	Roots	2	+	−	+	+

[a]Weeds were collected in March and April 1980 from two fields near Athens, GA, which had high incidence of bacterial speck on tomato during the 1979 growing season. Leaf and root samples were suspended in 200 ml of sterile, distilled water in a 500-ml flask and shaken vigorously on a wrist-action shaker for 20 min; the suspending liquid was used for infiltration.

[b]+ = Symptoms of bacterial speck developed on tomato plants vacuum-infiltrated with washings from leaves or roots; − = no symptoms developed; ± = atypical or questionable symptoms developed.

[c]Hypersensitive reaction (HR) determined by infiltrating suspensions (10^8 cfu/ml) into leaf sections of *Nicotiana tabacum* 'Hicks'.

[d]Chico III tomato plants were spray inoculated with a suspension containing 10^8 cfu/ml.

soil were also vacuum-infiltrated directly into tomato plants. Concentrations of 10^4 cfu/ml caused extensive tissue necrosis, and only levels of 10^3 cfu/ml or less produced discrete lesions (Table 1).

Washings from seven of 11 foliage or root samples (four of six weed species) produced typical bacterial speck lesions on tomato plants after vacuum infiltration (Table 2). Some infiltrated plants had 10–50 typical speck lesions. Isolations from these lesions yielded a fluorescent pseudomonad that was oxidase-negative and tobacco-hypersensitive and produced typical bacterial speck symptoms after spray inoculation of Chico III tomato plants in growth chamber tests (Table 2).

Washings from the foliage of two species (*Oenothera* sp. and *S. cereale*) collected from field one produced lesions atypical of bacterial speck (brown spots without halos) on the infiltrated tomato plants. Isolates from these lesions were oxidase-positive and not tobacco-hypersensitive and failed to produce lesions on tomato plants after standard spray inoculation with 10^8 cfu/ml.

Vacuum infiltration proved to be more efficient than standard spray inoculation for detecting low populations of *P. tomato* (Table 3). Population densities as low as 10 cfu/ml in suspension were detected by vacuum infiltration, and lesion counts increased to 10^3 cfu/ml, above which concentration extensive necrosis occurred. Lesions developed on vacuum-infiltrated plants without a high-humidity period after treatment. Spray application of suspensions to tomato plants also allowed detection of *P. tomato* over a wide concentration range, but lesion counts were usually significantly lower than for vacuum infiltration, and a high-humidity period was essential for consistent lesion development (Table 3).

TABLE 3. Lesion counts on leaves of Chico III tomato plants after vacuum infiltration or spray inoculation with four suspensions of *Pseudomonas tomato* with and without a high-humidity period after treatment

Concentration of suspension[a] (cfu/ml)	Application method[b]	Number of bacterial speck lesions per plant[c] No high-humidity period	High-humidity period[d]
10^1	Spray	0.0	0.6
10^1	Vacuum infiltration	1.6*[e]	0.8
10^2	Spray	1.3	2.8
10^2	Vacuum infiltration	11.6*	13.8*
10^3	Spray	0.0	15.0
10^3	Vacuum infiltration	163.5*	100.5*
10^4	Spray	0.0	46.0
10^4	Vacuum infiltration	EN[f]	EN[f]

[a] Suspensions were prepared from King's medium B plates of *P. tomato* grown for 36 hr at 25 C.
[b] Tomato plants (12–15 cm tall) were sprayed to runoff with a paint sprayer or were immersed and infiltrated under a 76-cm Hg vacuum for 0.5–1.0 and 2.0 min, with the vacuum released abruptly after each period.
[c] Lesions were counted on each plant after 14 days at 21 C.
[d] A 36-hr high-humidity period was provided by enclosing each plant in a clear polyethylene bag.
[e] Asterisk indicates that the value is significantly greater than the value for the spray treatment at the same inoculum level, as determined by a *t*-test comparison ($P = 0.05$).
[f] Extensive necrosis of tissue prevented the counting of individual lesions.

DISCUSSION

Although several researchers have used in vitro culture techniques to study the ecology and epidemiology of *P. tomato* (2,5,7,16,18), we have had inconsistent results using these methods under our conditions. When a nonselective medium such as KMB was used to isolate from soil or tissue samples, plates were often overgrown with rapidly growing, nonfluorescent and fluorescent saprophytes that inhibited or masked slower growing colonies of *P. tomato*. Antimicrobial agents added to media (2,5) often fail to eliminate the fluorescent saprophytes. Even when plating techniques are successful, they must be supplemented with laboratory and pathogenicity tests on transfers from individual colonies because *P. tomato* cannot always be distinguished by colony characteristics. Furthermore, *P. tomato* may represent a minor portion of the population of green fluorescent pseudomonads that occur epiphytically on plants. Schneider and Grogan (16), while isolating from suspect symptomless weed species in California, found that only 17 of 1,502 green fluorescent colonies picked from KMB isolation plates were *P. tomato*. Conventional plating techniques may fail to detect low population densities of plant pathogens, especially if they represent a relatively minor portion of the total bacterial population. Goto (10) noted that commonly used isolation procedures allow detection of plant pathogens in plant tissue only when populations are 10^4–10^5 cells per gram or higher. An antibiotic-resistant mutant has been used to study the survival of *P. tomato* in artificially infested field soil (8), but this technique is not useful for detecting populations of the pathogen in nature.

Our results demonstrate that the vacuum infiltration technique is more effective than previously used methods for detecting *P. tomato* under natural conditions. The technique detected low populations in extracts from field soil with high populations of other bacteria and in washings from weed species where the organism apparently survived epiphytically at low levels. *P. tomato* usually was not isolated when washings from leaves and roots of these same weed species were plated directly on KMB during the spring of 1980 (18).

Lesions produced by *P. tomato* on vacuum-infiltrated tomato leaves were identical in appearance to those produced by natural infection or after spray inoculation. Although other fluorescent pseudomonads attacking tomato foliage (6,19,20) could possibly cause lesions after vacuum infiltration, *P. tomato* produces lesions with characteristic yellow halos, whereas other pathogenic species produce indistinct brown lesions without halos. We feel that *P. tomato* can be separated on the basis of symptoms; however, isolations may be required when questionable symptoms appear after infiltration. Fortunately, a few laboratory tests (11) allow separation of *P. tomato* from *P. syringae* pv. *syringae* once isolations are made. We do not know what symptoms would be produced by *Xanthomonas vesicatoria* or *Corynebacterium michiganense* after vacuum infiltration. Although symptomatology was used to distinguish infection by *P. tomato* from that by *X. vesicatoria* and *C. michiganense* on young tomato plants (3), isolation may be necessary if the presence of the latter organisms is suspected.

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