Herbaceous Weeds Are Not Ecologically Important Reservoirs of *Erwinia tracheiphila*

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ABSTRACT

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The potential of herbaceous weeds commonly growing in or adjacent to cucurbit crops to serve as alternate hosts and overwintering reservoirs of *Erwinia tracheiphila*, a causal agent of cucurbit wilt, was investigated. Methods for isolation, maintenance, long-term storage, and detection of *E. tracheiphila* from infected plants were developed. *E. tracheiphila* was consistently detected by enzyme-linked immunosorbent assay (ELISA) and reisolated from infected, susceptible, cucurbit species. When six common herbaceous weed species were inoculated, *E. tracheiphila* was detected in 49% (combined species) of the plants by ELISA 3 weeks after inoculation. However, we were unable to reisolate *E. tracheiphila* from these plants by standard techniques. Immunoaffinity isolation with a sensitivity of 2 CFU per sample also failed to recover *E. tracheiphila* from weed species. Comparisons of cucumber and goldenrod inoculated with live or formaldehyde-killed *E. tracheiphila* indicated that immunoassays could detect nonviable *E. tracheiphila* systemically spread in plants 3 weeks post-inoculation. In these tests, the pathogen was reisolated only from cucumber plants inoculated with live *E. tracheiphila*. Although we could reproduce serological evidence of *E. tracheiphila* antigen in the weeds investigated, our results do not support the hypothesis that *E. tracheiphila* can infect, survive in, or overwinter in the weed species tested.

Additional keywords: bacterial wilt, *Cucumis sativus*, immunoaffinity isolation, immunogold labeling, *Solidago altissima*

Erwinia tracheiphila induces bacterial wilt of cucurbits, which is especially severe in cucumber (*Cucumis sativus* L.) and cantaloupe (*Cucumis melo* L.) in the eastern United States. Severe wilting of the leaves and eventual plant collapse is believed to result from bacterial occlusion of the vascular elements by the multiplying bacteria (17). Field losses due to bacterial wilt vary annually, but can be as high as 75% of the crop (29).

E. tracheiphila is transmitted by Diabroticite beetles, *Acalymma vittata* (Fabricius) and *Diabrotica undecimpunctata howardi* (Barber), the striped and spotted cucumber beetles (23,26). The proportion of infected plants in a cucurbit crop is correlated to the density of beetle vectors (36), and the rate of disease progression within a plant is directly influenced by the inoculum dose (16). Current protection programs for cucurbit crops rely primarily on insecticides and allow little tolerance

for Diabroticite beetles due to their ability to transmit *E. tracheiphila*. This management strategy is maintained even though the mechanism of transmission, transmission efficiency, and the potential host range of *E. tracheiphila* are not well documented.

The host range of *E. tracheiphila* includes many species of Cucurbitaceae, and overwintering is presumed to be dependent on *A. vittata* (1,18,22,26,28). Contributing to this presumption is the similarity of *E. tracheiphila* to *E. stewartii*, a maize pathogen that overwinters in the gut of a flea beetle (10). However, Harrison et al. (11) state that "a more reasonable explanation is that the bacterium overwinters in the plants or debris that the beetles feed upon in early spring." This sentiment was supported by Leach (14) who points out that "These plants [family Rosaceae] should be given more consideration as possible sources of primary inoculum." More recently, Bassi (2) obtained serological evidence suggesting that the common herbaceous weedy perennials *Solidago altissima* L. (goldenrod) and *Sorghum halepense* L. Pers. (johnsongrass) might serve as hosts for *E. tracheiphila*. Unfortunately, he was unable to isolate *E. tracheiphila* from these weeds. Bassi speculated that his inability to isolate *E. tracheiphila* was due to interference

from other, faster-growing microorganisms. Blua et al. (4) confirmed the occurrence of positive serological responses to *E. tracheiphila* in several herbaceous weed species. These reports suggested that unknown plant sources could be important to overwintering and pathogen spread in cucurbit agroecosystems.

Slow growth of *E. tracheiphila* in culture, as well as differences in viability and virulence among isolates, have impeded progress in understanding the biology and host range of *E. tracheiphila* (7,26). Therefore, our first set of objectives were to develop methods for routine isolation of *E. tracheiphila*, to maintain pathogenic isolates in culture, and establish methods for long term storage. We also surveyed the carbon source utilization of the strains that we obtained and determined a growth curve for the type isolate. Our second set of objectives were to develop specific antisera and to optimize various serological tests, allowing *E. tracheiphila* detection in various plant species and rapid identification of bacterial cultures. With these tools we addressed our primary objective, which was to examine the potential role of herbaceous weedy plant hosts to serve as ecologically significant reservoirs of *E. tracheiphila*. We addressed this objective with serological assays of field-collected weeds, greenhouse studies of mechanically inoculated weeds, immunoaffinity isolation, light and electron microscopy, and experiments to determine the serological response of nonviable bacteria within weeds.

MATERIALS AND METHODS

Bacterial isolation, culture media, long-term storage, growth rate, and carbon source utilization. Three *E. tracheiphila* isolates (33245, 27002, and 27005) were purchased from the American Type Culture Collection (ATCC), Rockville, MD. In the summer of 1993, seven additional isolates were obtained from *Cucumis sativus* (cucumber) and *Cucumis melo* (cantaloupe) vines showing bacterial wilt symptoms in Delaware and Maryland (the Delmarva Peninsula), central Pennsylvania (Pennsylvania Furnace, Centre County), and New Jersey (Somerset, Somerset County). A sterile needle was used to transfer bacteria oozing from vascular bundles of cut, surface-sterilized stems to Difco nutrient agar supplemented with extra agar and peptone

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(NAP) (23 g of Difco nutrient agar, 5 g of Difco agar, and 5 g of Difco Bacto-peptone per liter of water). Alternatively, 2-cm stem sections were surface sterilized in 10% Clorox bleach for 10 s, 70% ethanol for 10 s, and then rinsed in sterile water before being cut transversely, and incubated 5 days in nutrient broth supplemented with extra peptone (NBP) (10 g of Difco nutrient broth and 5 g of Difco Bacto-peptone per liter of water). The NBP cultures were then streaked on NAP plates. Resulting single colonies of the correct morphology and color (7,15) were subcultured onto new plates. New, pure colonies, tentatively identified as *E. tracheiphila*, were allowed to grow for 7 days. Bacteria from these cultures were needle inoculated into 10-day-old cucumber (cv. SMR 18, third-true-leaf stage) stems immediately below the cotyledons by picking up a small but visible amount of *E. tracheiphila* with a sterile dissecting needle and inserting it 1 to 2 mm deep into the stems. Seedlings were examined daily for wilt symptoms for 21 days. Bacteria were reisolated by the isolation methods described above, or by blotting surface-sterilized cut stems, cut transversely, directly onto NAP plates (stem blot isolation). Each isolate was also identified with double antibody sandwich-enzyme-linked immunosorbent assay (DAS-ELISA), as described below.

The following media were compared for routine culture and isolation of *E. tracheiphila*: glucose nutrient agar (GNA) (26) consisting of 23 g of Difco nutrient agar and 5 g of glucose per liter water; yeast extract dextrose calcium carbonate agar (YDC) consisting of 10 g of yeast extract, 20 g of dextrose, 20 g of calcium carbonate, and 15 g of agar per liter of water; and nutrient agar supplemented with

extra agar and peptone (NAP) described above. Growth on each medium was evaluated by measuring the number and size of widely spaced *E. tracheiphila*-ATCC 33245 colonies after a 3-day incubation period at room temperature. Pathogenicity was evaluated by needle inoculation of the resulting colonies into cucumber seedlings.

Strains were maintained at room temperature on NAP and subcultured weekly. Seven-day-old NBP cultures of *E. tracheiphila* were diluted with glycerol (1:1) and placed at -80°C for long-term storage. Because of slow growth, 7-day-old plate or slant cultures were used in inoculation experiments in order to acquire sufficient inoculum. Such cultures maintained pathogenicity for several weeks.

The growth rate of *E. tracheiphila* was determined in triplicate NBP cultures. Three flasks containing 150 ml of NBP were equilibrated on a shaking table (120 rpm; Clinical rotator, Fisher Scientific, Pittsburg, PA) in an environmental chamber at 23°C and then inoculated with 133 CFU (determined by dilution plating of the inoculum) of the *E. tracheiphila* type strain (ATCC 33245). At 4-h intervals, 1-ml samples of the broth were removed from each flask and absorbance (480 nm) was determined with a Beckman DB UV/VIS Spectrophotometer (Beckman, Fullerton, CA). A second aliquot was diluted 10-fold with sterile water and spread onto NAP plates in triplicate. Plates were incubated at 28°C for 3 days before the colonies were counted, and correlated with the mean of the absorbancies at the time of sample removal.

E. tracheiphila isolates were examined for their ability to oxidize carbon sources with Biolog GN Microplates (Biolog,

Hayward, CA). Methods used were those recommended by Biolog (19), except that *E. tracheiphila* was grown on NAP instead of tryptic soy agar (TSA; Difco, Detroit, MI), because no growth occurred on TSA. To distinguish among groups of *E. tracheiphila* isolates that are similar in carbon source use, the matrix of 10 isolates by 34 carbon sources was analyzed with Phylogenetic Analysis Using Parsonomy (PAUP) software (Smithsonian Institution, Washington, DC).

Preparation of antisera and serological assays. Antisera were produced against the *E. tracheiphila* type strain (ATCC 33245) and MD1, a field-collected strain (Table 1). Broth cultures grown in Difco nutrient broth with extra peptone (5 g/liter) (NBP) were washed twice in phosphate-buffered saline (PBS) and resuspended in 2 ml of PBS. Cells of each isolate were fixed with 2% glutaraldehyde and then washed by pelleting and resuspending three times in PBS.

Antisera to ATCC 33245 and MD1 were produced in female New Zealand white rabbits. A 0.5-ml cell sample (10^8 CFU) in PBS was emulsified in 0.5 ml of incomplete Freund's adjuvant and injected subdermally in 10 0.1-ml aliquots into each rabbit. A total of three injections were made at 2- to 3-week intervals and serum was collected approximately 10 weeks after the first injection. Whole serum was stored at -80°C . The IgG component was purified by CM Affi-Gel Blue affinity chromatography according to the manufacturer's directions (Bio-Rad Laboratories, Richmond, CA). The final concentration of IgG was approximately 12 mg/ml for each treatment.

DAS-ELISAs were conducted according to Clark and Bar-Joseph (8) and McLaughlin and Chen (20) with the trapping and conjugated antibodies at a dilution of 1:400. Results of the ELISAs were determined by measuring absorbance of each well at 405 nm with a plate reader (Dynatec, Chantilly, VA) when the *E. tracheiphila* (ATCC 33245) bacterial culture (diluted to approximately 10^6 CFU) control absorbance reached $1.0 A_{405}$. The two antisera were compared by testing with DAS-ELISA the original *E. tracheiphila* isolates and similar bacteria (*E. amylovora*, *E. carotovora* pv. *carotovora*, *E. herbicola* [= *Pantoea agglomerans*], and *E. stewartii* [= *Pantoea stewartii* subsp. *stewartii*]) (21) maintained at Pennsylvania State University. All bacteria were diluted to approximately 10^6 CFU. Antisera produced for this study were also compared with antisera produced against an Arkansas strain of *E. tracheiphila* (H11) by Bassi (2).

Direct tissue blots of cross sections of plant tissues were performed as described by Hsu and Lawson (13). Plant stem segments were cut transversely with a clean razor blade and immediately pressed onto a 1×10 cm strip of nitrocellulose paper

Table 1. Identification of *Erwinia tracheiphila* strains based on enzyme immunoassays (enzyme-linked immunosorbent assay; ELISA) and pathogenicity on cucumber

Strain ^a	Area of origin	Date	Original host	ELISA Abs.405 ^b		Pathogenicity on cucumber ^c
				MD1	33245	
33245	USA	?	Cantaloupe	1.45	1.44	+
27002	USA	?	Cucumber	1.44	1.44	+
27005	Wisconsin	1970	Cucumber	1.45	1.44	+
MD1	Maryland	1993	Cucumber	1.42	1.45	+
MD2	Maryland	1993	Cucumber	1.40	1.44	+
MD3	Maryland	1993	Cucumber	1.36	1.44	+
PA1	Pennsylvania	1993	Cantaloupe	1.21	1.44	+
PA2	Pennsylvania	1993	Cantaloupe	1.13	1.44	+
NJ1	New Jersey	1993	Cucumber	0.97	1.44	+
NJ2	New Jersey	1993	Cucumber	1.10	1.44	+

^a Strains 33245, 27002, and 27005 purchased from ATCC. Other strains isolated from field-infected plants as described in Materials and Methods.

^b Numbers are average absorbance values ($n = 4$) from double antibody sandwich-ELISAs of 7-day-old pure cultures diluted to approximately 10^5 CFU and tested against polyclonal antiserum made against the MD1 or 33245 strains as described in Materials and Methods. Differences among values are not significant. The average absorbance value of healthy cucumber controls was 0.02.

^c Seven-day-old slant cultures from purified single-colony isolates of each strain grown on Difco nutrient agar supplemented with extra agar and peptone (NAP) were stab inoculated to 10 14-day-old cucumber seedlings immediately below the point of cotyledon attachment or in the cotyledon petiole. Seedlings were maintained in the greenhouse and observed for symptoms for 3 weeks. The newly produced leaves were then ELISA-tested to verify infection. Bacteria were reisolated from sterilized stem sections to verify infection and ELISA-tested to verify identity.

(Bio-Rad). A 1- μ l aliquot of *E. tracheiphila* suspended in PBS was spotted at the bottom of each strip as a positive control and a healthy plant was used as a negative control.

Field-collected plants. Serological responses to *E. tracheiphila* antibodies from field-collected herbaceous biannual weeds present in a cucumber (cv. Speedway) field in fall of 1994, and a cantaloupe (cv. Cordell) field in spring of 1995, at the Russell E. Larson Research Farm (Rock Springs, PA) were evaluated. In the fall of 1994, the cucumber field had a high weed density, and approximately 25% of the cucumber plants had died from bacterial wilt during the summer. The weeds collected were *Erigeron strigosus* Muhl. ex Willd. (rough flea bane), *Capsella bursa-pastoris* (L.) Medik. (shepherd's purse), *Rumex obtusifolius* L. (broadleaf dock), *Plantago rugelii* Decne. (blackseed plantain), and *Plantago major* L. (broadleaf plantain). The following spring (June 1995), prior to the planting of cucurbits, the same weed species were collected from the same field with the exception of broadleaf dock and broadleaf plantain, which were not present. For a second fall field survey, weeds were collected in October 1995 in a nearby cantaloupe field (cv. Cordell). This cantaloupe field was less weedy than the cucumber field sampled in the fall of 1994, but had a higher rate of bacterial wilt (35%). Blackseed plantain, broadleaf plantain, and broadleaf dock were collected in the fall of 1995. Healthy tissue negative controls and positive controls were included in each ELISA plate and a positive threshold was set at five times the highest absorbance value for the healthy cucumber control plant in each ELISA plate.

Greenhouse studies. To test six herbaceous weed species (*Capsella bursa-pastoris*, *Plantago lanceolata* (L.), *Rumex crispus* (L.), *Solidago altissima*, *Solidago gigantea* Aiton, and *Sorghum halepense*) and three cucurbit species (*Cucumis melo*, *Cucumis sativus*, and *Citrullis vulgaris*) for their ability to be infected by *E. tracheiphila*, greenhouse-grown seedlings were inoculated with 7-day-old NAP plate cultures of *E. tracheiphila*. The plants were observed for symptoms for 3 weeks and then reisolation and serological tests were conducted. The *S. altissima* and *S. gigantea* plants were grown from rhizomes collected in New Hampshire and Vermont and maintained in the greenhouse for several years. The other seven species tested were grown from seed. All *Solidago* spp. were tested before inoculation and were negative for *E. tracheiphila* by DAS-ELISA. Plants were inoculated with 7-day-old cultures of ATCC 33425 as follows. Inoculum was transferred from plate cultures with a sterile inoculation needle by insertion into the stem immediately below the third to fifth fully expanded leaf of each test plant or at the base of the rosette, depending on the

species. For every 10 inoculated plants of each species, three plants of the same species were designated as mock-inoculated, wounded controls and were stabbed with a sterile inoculating needle in the same location. Three cucumber plants (cv. Discover) were inoculated with the same culture of *E. tracheiphila* to serve as controls. All plants were maintained in the greenhouse for 3 weeks and observed for symptoms of wilt or abnormal growth. After 3 weeks, the plants were harvested, and reisolation attempts and serological tests were conducted. Plant stem segments were surface sterilized, cut transversely, and blotted onto NAP plates or incubated in NBP for 7 days, and then streaked onto NAP plates. Resulting colonies with *E. tracheiphila* colony morphology were tested with DAS-ELISA and reinoculated into cucumber seedlings to verify identity. Following the 3-week period, leaf and stem tissues from each of the original test plants were also cut into small pieces and mixed, and random 0.5-g samples (fresh weight) were tested with ELISA to detect *E. tracheiphila* in the inoculated tissues. Samples were considered positive for *E. tracheiphila* when absorbance values (405 nm) were greater than the mean plus three standard deviations of the noninoculated controls of that species. This threshold was selected to ensure that absorbance values of 99.72% of all plants that were not infected would fall under the threshold, assuming a normal distribution of values from noninfected control plants.

Immunoaffinity isolation. Because of the possibility that the techniques we successfully used to isolate *E. tracheiphila* from infected cucurbits were not adequate to isolate this pathogen from herbaceous weed species, we developed a variation of immunoaffinity isolation (12,27,34).

Thirteen *Plantago lanceolata* (buckhorn plantain) and 13 *Rumex crispus* (curly dock) plants were grown from seed in 0.5-liter pots. Nine plants of both species were needle inoculated at the base of one leaf in each rosette with a 5-day-old culture of *E. tracheiphila* by the same needle inoculation technique described previously. Four plants of each species were wounded at the same location with a sterile needle as negative controls. Three weeks after inoculation, leaves and stems were harvested, surface sterilized, and ground in 2 ml of ELISA sample buffer in mortar and pestles. Two hundred microliters of each of the ground, inoculated, plant samples was immediately pipetted into eight ELISA plate wells coated overnight at 4°C with a 1:100 dilution of anti-*E. tracheiphila* antibodies (one ELISA plate for each of the two species). Each of the four homogenized control samples of both species was pipetted into duplicate wells in the appropriate ELISA plate. A culture of *E. tracheiphila* grown on NAP and suspended in 10 ml of sample buffer was pipetted into

eight wells of each plate as positive controls. Eight wells were filled with buffer as negative controls. The last eight wells of each plate were filled with equal parts of the suspended *E. tracheiphila* culture and the homogenized control plants of the appropriate plant species. This was to ensure that the curly dock and buckhorn plantain plant homogenates did not kill the bacteria during isolation. The samples were incubated in the antibody-coated ELISA plates for 1 h at room temperature and then rinsed three times with PBS. All of the wells were then filled with 200 μ l of NBP and incubated at room temperature for 5 days. The contents of each well were streaked for isolation on NAP. After 5 days of incubation at room temperature the resulting colonies with colony morphology resembling that of *E. tracheiphila* were tested with DAS-ELISA and needle inoculated into cucumber seedlings to verify pathogenicity.

Microscopy. Light microscopy, coupled with scanning (SEM) and transmission (TEM) electron microscopy, was used to study bacteria-host plant tissue interactions and to confirm immunological identification. Cucumbers (cv. Discover) were grown to the three-leaf stage in the greenhouse and stab inoculated with *E. tracheiphila*. Petioles of *E. tracheiphila*-infected cucumbers were fixed 2 weeks after inoculation. For Nomarski interference contrast light microscopy, petioles were fixed in 1% formaldehyde and 2% glutaraldehyde in 0.01 M phosphate buffer (pH 7), postfixed in 1% osmium tetroxide in 0.01 M phosphate buffer (pH 7), dehydrated in an ethanol series, and then embedded in LR White resin (32).

For immunolabeling, specimens were fixed in 2% formaldehyde and 0.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7), rinsed in 0.01 M glycine to inactivate aldehydes, dehydrated to 75% ethanol, and embedded in LR White resin. Protocols for immunogold labeling were variants of those previously described to optimize specificity of labeling (3). Specimens were viewed and photographed on a JOEL 1200 II TEM at the Electron Microscope Facility for the Life Sciences in the Biotechnology Institute at Pennsylvania State University.

To study bacterial wilt disease progress over time in individual plants, 3-week-old cucumber seedlings were stab inoculated with *E. tracheiphila* grown in the greenhouse under ambient temperature (20 to 30°C) and a 15-h photoperiod. Every 24 h following inoculation, two seedlings were harvested and dissected with a razor blade into six sample regions: the cotyledon near the inoculation site, the cotyledon-hypocotyl junction, the hypocotyl, the petiole-leaf junction, and the first-true-leaf blade. Samples were fixed for 2 h in 1.5% glutaraldehyde, 2.5% formaldehyde, and 4% sucrose in 0.1 M phosphate buffer (pH

7.4). Tissues were then dehydrated in ethanol, cryofractured, critical point dried in a Bio-Rad E300 Parr pressure bomb, mounted on stainless steel stubs, and sputter coated with 2.1 nm of gold-palladium.

To examine the interaction of *E. tracheiphila* with a potential weed host, 10 *S. altissima* plants approximately 15 cm tall were wound inoculated in the stem immediately below the fifth fully expanded leaf from the tip. Three weeks later, each plant stem was equally divided into four sections and a 3-cm segment of stem was excised from each section. From each 3-cm segment, 1 cm was used for ELISA, 1 cm for reisolation of bacteria, and 1 cm was fixed and cryofractured as described above and examined for bacteria in vascular bundles. Both cucumber and *S. altissima* specimens were viewed on a JOEL JSM-5400 at the Electron Microscope Facility for the Life Sciences in the Biotechnology Institute at Pennsylvania State University.

Detection of nonviable bacteria. To determine whether our serological tests could detect nonviable *E. tracheiphila* in plant tissues, 10 cucumbers (cv. Discover) at the three-leaf stage were assigned to each of six treatments: (i) uninoculated controls; (ii) plants mock inoculated (wounded) between the remnants of the cotyledons; (iii) stab inoculated with viable, virulent, *E. tracheiphila* (ATCC 33245); (iv) inoculated with viable *E. carotovora* var. *carotovora*; (v) inoculated with a culture of *E. tracheiphila* that was fixed with 2% formaldehyde, 10.2% glutaraldehyde, and 0.05% azide diluted in 0.05 M phosphate buffer and rinsed by pelleting and resuspending three times in PBS; and (vi) inoculated with *E. carotovora* var. *carotovora* fixed with the same procedure. The fixed *E. tracheiphila* and *E. carotovora* var. *carotovora* were also streaked on NAP to confirm that all of the cells were killed. Three weeks after inoculation, all of the cucumber plants were tested with direct tissue blotting immuno-

assays (13) in seven locations: at the inoculation point; 1, 2, and 3 cm above the inoculation point; and 1, 2, and 3 cm below the inoculation point. DAS-ELISA was also used to assay the cucumbers from treatments five and six.

This same experiment was repeated with *S. altissima* and cantaloupe (cv. Superstar) with only *E. tracheiphila* for inoculations. In this second trial, there were five uninoculated, five wounded, five inoculated with fixed *E. tracheiphila*, and 10 goldenrod plants inoculated with live *E. tracheiphila*. There were also wounded, fixed, and live-bacteria-inoculated cantaloupe plants, six of each, as controls. The inoculation point was just below the fifth fully expanded leaf of each goldenrod plant and between the remnants of the cotyledons on the cantaloupe. Three weeks after inoculation the goldenrod plants were sampled by removing 4-cm tissue samples from five parts of the plants: the first stem segment was removed from the top of the plant; the second stem segment extended from 2 cm above to 2 cm below the inoculation point; the third stem segment was removed from just above the soil level; the fourth sample was rhizome from just below the soil surface; and the fifth sample was removed from the distal ends of the roots. Each of the stem and rhizome segments (top, above and below the inoculation point, base and rhizome) was then surface sterilized as described previously, and cut transversely with a sterile razor blade. We blotted one cross section onto a NAP and the other cross section onto a nitrocellulose strip (1 × 10 cm) for direct tissue blotting. We did not try to isolate bacteria or direct tissue blot the root samples. The NAP plates were incubated for 5 days at room temperature and examined for any *E. tracheiphila* colonies. The direct tissue blots were developed as described previously and examined for any blue color change that would indicate the serological detection of *E. tracheiphila*. All of the samples

(including the roots) were ground in 5 ml of ELISA sample buffer and assayed with DAS-ELISA.

RESULTS

Bacterial isolation, culture, long-term storage, growth rate, and carbon source utilization. All seven isolates of *E. tracheiphila* from field-collected cucurbit vines and all three ATCC strains caused bacterial wilt symptoms when inoculated into 14-day-old cucurbit seedlings (Table 1). Needle-inoculated seedlings (third-true-leaf stage) generally showed symptoms of infection in 6 to 8 days. The earliest symptoms of infection ranged from mild, in which local areas of individual leaves became flaccid, to severe, in which two or more whole leaves rapidly became flaccid and then dried within 2 to 3 days. All leaves on infected seedlings completely dried after approximately 2 weeks. All isolates were successfully reisolated from inoculated seedlings by the stem blot technique and produced strong serological responses when subjected to DAS-ELISA. The field-isolated strain MD1 and ATCC strains 33245, 27002, and 27005 were subcultured weekly on NAP for over 4 years without loss of pathogenicity. In addition, after 5 years storage at -80°C in 50% glycerol, all isolates were recovered on NAP and induced wilt symptoms in cucumbers.

Our isolates grew slowly on nutrient agar as previously reported (30), but grew better when extra peptone was added to the agar. We chose to use NAP to isolate and maintain our bacterial cultures, because *E. tracheiphila* colonies were difficult to see on the YDC plates, and *E. tracheiphila* isolates grown on GNA plates varied in virulence and in one case failed to induce wilt. When NBP was used to determine the growth rate of *E. tracheiphila* at 28°C, we calculated a doubling time of approximately 3.5 h.

E. tracheiphila isolates were variable in their ability to metabolize different carbon sources in the Biolog tests (19). Isolates metabolized between 15 and 26 of the 95 different carbon sources tested. However, no two isolates had the same carbon source utilization profiles. All isolates combined metabolized 34 carbon sources. Of these carbon sources, 9 (D-fructose, α-D-glucose, D-mannitol, β-methyl-D-glucoside, sucrose, glucose-1-phosphate, glucose-6-phosphate, D-galactose, and glycogen) were used by all isolates. Cluster analysis with the variation in carbon source oxidation among strains indicated that those from Pennsylvania and New Jersey could be distinguished from the others by their metabolism of succinic acid. Within this grouping, isolates from New Jersey could be distinguished from the Pennsylvania isolates by their metabolism of L-glutamic acid. Some tested strains differed from those described in Bergey's Manual of Determinative Bacteriology (15) and were able to me-

Table 2. Comparison of three antisera made to *Erwinia tracheiphila* for ability to detect a range of *Erwinia* spp. and *Xanthomonas campestris* pv. *pelargonii*

Bacterial strain ^a	Strain antiserum ^b		
	33245	MD1	HI1
<i>E. tracheiphila</i> (ATCC 33245)	1.44 ^c	1.45	1.31
<i>E. amylovora</i>	0.02	0.03	NT
<i>E. carotovora</i> pv. <i>carotovora</i>	0.01	0.03	0.02
<i>E. herbicola</i> (ATCC 13460)	0.04	0.06	0.09
<i>E. stewartii</i>	0.01	0.03	0.00
<i>X. campestris</i> pv. <i>pelargonii</i>	NT	NT	0.05
Buffer control	0.00	0.01	0.00
Healthy cucumber tissue	0.02	0.03	0.01

^a Samples were prepared by diluting 7-day-old slant cultures grown on Difco nutrient agar supplemented with extra agar and peptone (NAP) in enzyme-linked immunosorbent assay (ELISA) sample buffer to give approximately 10⁵ CFU per well. *E. herbicola* and *E. stewartii* are subjective synonyms for *Pantoea agglomerans* and *P. stewartii* subsp. *stewartii*, respectively (21).

^b Antisera were made at Pennsylvania State University against *E. tracheiphila* type-strain 33245, and a strain isolated from an infected cucumber from Maryland (MD1). Antiserum to HI1 was produced by Bassi (2).

^c Numbers are average absorbance values (n = 4) from double antibody sandwich-ELISAs. NT = not tested.

tabolize dextrin, maltose, glycerol, and m-inositol as carbon sources.

Preparation of antisera and serological assays. All *E. tracheiphila* strains tested were positive by DAS-ELISA with polyclonal antibodies produced against ATCC 33245 or MD1 (Table 1). Other bacterial species, including several *Erwinia* species, did not react with these antisera (Table 2). In DAS-ELISAs with detection limits of 10^2 CFU per well of *E. tracheiphila*, reactivity with 10^6 CFU of *E. amylovora*, *E. carotovora* var. *carotovora*, *E. herbicola*, and *E. stewartii* was below the detection limits. Antisera to 33245, MD1, and the previously produced HI1 did not differ in specificity in comparative tests. None of the three antisera gave significant background reactions to healthy cucumber plant sap or to healthy plant sap from any of seven other herbaceous weeds and three other cucurbit species tested with DAS-ELISA.

Field-collected plants. Four of five field-collected weed species indicated positive serological responses to *E. tracheiphila* antibodies in DAS-ELISAs. The most prominent was blackseed plantain collected in the fall of 1994, for which 39 of 47 (83%) of plants tested were ELISA positive. Serological responses to *E. tracheiphila* in DAS-ELISAs were also recorded in three of 22 (13%) broadleaf dock and four of 48 (8%) shepherd's purse. Serological responses in broadleaf plantain and rough fleabane were negative. Attempts to isolate *E. tracheiphila* with stem blots on NAP were unsuccessful. The ELISA values obtained with specific plants indicated the presence of *E. tracheiphila* based on appropriate positive and negative controls and conservatively set positive thresholds. Plants collected in fall 1994 were from a cucumber field with 25% incidence of bacterial wilt and a heavy infestation of *A. vittata*. Diabroticite beetles were observed feeding on the weed species that we surveyed, and some of the plants collected for testing had feeding damage.

When plants from the same cucumber field were tested the following spring (1995), four of 112 of the blackseed plantain were ELISA positive, but none of the 50 shepherd's purse or 50 rough fleabane plants tested positive. Broadleaf plantain and broadleaf dock were not present in the spring of 1995. Beetles were not observed in high numbers when these samples were collected and there was no evidence of feeding on these weeds.

Of 50 blackseed and 50 broadleaf plantain plants collected in fall of 1995, only 2 broadleaf plantain were ELISA positive. These plants were collected from a cantaloupe field with 35% wilt incidence, and although there was a high beetle population, the herbaceous weeds did not show obvious feeding damage. We were unable to isolate *E. tracheiphila* from any of these field-collected plant species.

Greenhouse studies. Six herbaceous weed species and three cucurbit species were tested for their potential to be infected by *E. tracheiphila* (Table 3). Needle inoculation of *E. tracheiphila* into the stems of 10 plants of each of the three cucurbit species tested resulted in bacterial wilt symptoms in all of the cucumbers and cantaloupe, but not the watermelon seedlings. *E. tracheiphila* was reisolated from all cucurbits that tested positive for *E. tracheiphila* with DAS-ELISA. The wounded control cucurbits did not develop symptoms and tested negative with DAS-ELISA. In contrast, none of the *E. tracheiphila*-inoculated herbaceous weed species developed wilt symptoms. Although weeds did not express disease symptoms, five of the six species (*C. bursa-pastoris*, *P. lanceolata*, *R. crispus*, *S. altissima*, *S. gigantea*, and *S. halepense*) were ELISA positive, suggesting infection by *E. tracheiphila*. Attempts to reisolate *E. tracheiphila* from at least 10 plants of all six herbaceous weed species with the stem blot technique onto NAP were consistently unsuccessful. Reisolation of *E. tracheiphila* with immersion of 2-cm stem segments in NBP from several of these weed species was also unsuccessful (Table 3). Therefore, infection of these weedy herbaceous species could not be verified by reisolation of the pathogen.

Immunoaffinity isolation. With immunoaffinity isolations, *E. tracheiphila* was reisolated from all sample wells loaded with *E. tracheiphila* diluted in homogenates of healthy stem and leaf tissue from curly dock or buckhorn plantain. All of the isolates recovered from these immunoisolations were determined to be *E. tra-*

cheiphila based on colony morphology, DAS-ELISA of the isolates, and wilt symptoms that developed when reisolated bacteria were inoculated into cucumbers. No *E. tracheiphila* were isolated from any of the four curly dock or buckhorn plantain mock-inoculated healthy controls. This immunoaffinity technique also failed to isolate *E. tracheiphila* from any of the nine curly dock or buckhorn plantain plants inoculated 3 weeks earlier with the same 5-day-old culture of *E. tracheiphila* used in the dilution test described above. There were few bacterial or fungal contaminants isolated from any of the sample wells and those few contaminants that were isolated did not cause bacterial wilt symptoms when inoculated into cucumbers and were not positive with DAS-ELISA. Failure to recover *E. tracheiphila* utilizing the very sensitive immunoaffinity isolation method with a detection level of two cells per sample was consistent with our earlier failures to reisolate from inoculated herbaceous weeds species in the greenhouse inoculation studies (Table 3).

Microscopy. Light and electron microscopy verified *E. tracheiphila* host tissue specificity (Fig. 1A, B), confirmed serological identity (Fig. 1C, D), and verified negative results of the reisolation tests from *S. altissima*. Three stages of cucumber wilting were identified following inoculation of 3-week-old cucumber seedlings. Wilt stage one occurred 1 to 3 days post-inoculation (dpi). During this time, bacteria were observed only in the cotyledon vascular tissues and no visible symptoms occurred. During wilt stage two, 3 to 5 dpi, one or both cotyledons showed loss of turgor. SEM observations indicated that

Table 3. Comparison of potential plant host species for symptom expression, enzyme-linked immunosorbent assay (ELISA) detection, and viable pathogen recovery 3 weeks post-inoculation (wpi) with *Erwinia tracheiphila*^a

Plant species tested (common name)	Latin name	Number positive / Number tested			
		Symptoms ^b	ELISA ^c	Abs. ^d	Recovery ^e
Cantaloupe	<i>Cucumis melo</i>	10 / 10	10 / 10	1.150	10 / 10
Cucumber	<i>Cucumis sativus</i>	10 / 10	10 / 10	1.117	10 / 10
Curley dock	<i>Rumex crispus</i>	0 / 42	27 / 42	0.185	0 / 17
Goldenrod	<i>Solidago altissima</i>	0 / 37	21 / 37	0.718	0 / 10
Giant goldenrod	<i>Solidago gigantea</i>	0 / 38	16 / 38	0.509	0 / 10
Johnsongrass	<i>Sorghum halepense</i>	0 / 20	0 / 20	0.001	0 / 10
Buckhorn plantain	<i>Plantago lanceolata</i>	0 / 30	12 / 30	0.122	0 / 10
Shepard's purse	<i>Capsella bursa-pastoris</i>	0 / 35	20 / 30	0.352	0 / 10

^a All plants were from direct seeded seedlings, except for goldenrods grown from field-collected rhizomes. All healthy plants of each species tested negative for *E. tracheiphila* by ELISA prior to inoculation. Plants were inoculated by stem wounding with 7-day-old cultures of *E. tracheiphila* (ATCC 33452) grown on Difco nutrient agar supplemented with extra agar and peptone (NAP).

^b Number of plants showing symptoms of wilt or abnormal growth 3 wpi / total number of plants tested.

^c Number of plants with positive ELISA values / total number of plants tested 3 wpi. Threshold ELISA values for positives equaled the mean of noninoculated healthy controls plus three standard deviations for each species. Based on these criteria, the positive threshold absorbance value for all species was about 0.05.

^d Average ELISA values (absorbance 405 nm) for the plants of each species tested for recovery of *E. tracheiphila* 3 wpi.

^e Number of plants from which viable *E. tracheiphila* were recovered from surface-sterilized stem sections incubated in Difco nutrient broth supplemented with extra peptone (NBP) medium or by stem tissue blots onto nutrient agar. Identification of resulting isolates was verified by ELISA.

bacteria had spread to 75% of the hypocotyl-cotyledon junction vascular bundles (Fig. 1B) and 33% of the hypocotyl vascular bundles. At wilt stage three, 5 to 8 dpi, severe wilting occurred and both cotyledons were shriveled and dried. The first true leaf showed loss of turgor and severe wilt. Typically, within 24 h of initiating leaf wilt the entire plant wilted and collapsed. At wilt stage three, bacteria were observed to have spread from the cotyledon, up the stem, and into the petiole of the first leaf. SEM and light microscopy observations indicated that 100% of the petiole vascular bundles contained bacteria (Fig. 1A, B). Even though plants showed severe wilt and bacteria were observed in most xylem vessel elements during late infection, bacteria were never observed completely plugging or occluding vessel

elements. Typically, bacteria were observed only around the periphery of the cell adhering to the cell wall. In some samples, aggregates of bacteria were observed filling much of the lumen of some vessel elements. The morphology and tissue localization of bacterial cells were consistent with descriptions of *E. stewartii* (5) and *E. tracheiphila* (17). However, no vascular deterioration was observed associated with the bacteria and bacterial cells were never seen associated with any other cell types in infected cucumber plants, even at wilt stage three. Identity of bacteria in cucumber vascular tissues as *E. tracheiphila* was verified by immunogold labeling of bacterial cells in situ (Fig. 1C, D). Colloidal gold was concentrated around the periphery of all bacterial cells within a vessel and did not label cytoplasmic components

of the host cells or of the bacterial cells (Fig. 1D). The antibodies were specific for cell wall-associated epitopes unique to *E. tracheiphila*. Bacteria in infected tissues treated with preimmune serum or for which *E. tracheiphila* antibody was deleted were not labeled. No bacterial cells were observed in tissues of two healthy cucumber plants examined as controls.

Only 11 of 40 stem segments from inoculated *S. altissima* plants were ELISA positive for *E. tracheiphila* 3 weeks post-inoculation (wpi). Eight of these 11 ELISA positive stems originated from stem segments 1 cm away from the inoculation point. However, *E. tracheiphila* could not be reisolated from any of these plants with stem segments from any of the four areas tested, and no bacteria were observed in the stem vascular bundles by SEM.

Fig. 1. Localization and immunological identification of *Erwinia tracheiphila* in vascular tissues of infected cucumbers. (A) Light micrograph of a 0.25-μm-thick tangential section through a petiole vascular bundle from an *E. tracheiphila*-inoculated cucumber 14 days after inoculation. Bacterial cells (B) and associated materials accumulate in the lumen of the vessel elements (VE). Xylem parenchyma cells (P) remain uninfected (×470, bar = 40 nm). (B) Higher magnification scanning electron microscopy of aggregated material observed in VE lumen from infected cucumber showing that the aggregates consist almost exclusively of rod-shaped bacterial cells (×5,600, bar = 4 nm). (C) Transmission electron micrograph (TEM) of vessel element contents immunolabeled with antibodies to *E. tracheiphila* and protein A-conjugated 10 nm gold. Gold concentrated around bacteria; arrow indicates low-level background nonspecific gold (×26,000, bar = 0.4 nm). (D) Higher magnification TEM of an immunogold-labeled *E. tracheiphila* cell in section showing specificity of antibody-gold (G) for the area associated with bacterial cell wall (CW) (×50,800, bar = 0.2 nm).

Detection of nonviable bacteria. Serological evidence of *E. tracheiphila* was obtained from field-collected plants and greenhouse tests of potential herbaceous weed hosts 3 wpi. However, we failed to reisolate *E. tracheiphila* from any of these species by different isolation methods. We considered the possibility that inoculated *E. tracheiphila* might initially spread or multiply in the plant, but then die or be inactivated by plant defense mechanisms.

When live *E. tracheiphila* were stab inoculated into the stems of cucumber seedlings, the bacteria caused wilt symptoms in all of the plants tested and were easily detected at various distances from the inoculation point 3 wpi by both direct tissue blots (not shown) and by ELISA (Fig. 2A). ELISA absorbance values were equally high for all stem segments tested above and below the inoculation site and *E. tracheiphila* was reisolated from all live-bacteria-inoculated cucumbers. When fixed *E. tracheiphila* were stab inoculated to cucumbers in a parallel treatment, these plants also tested positive by DAS-ELISA and immunoblot 3 wpi (Fig. 2B). Serological response, however, decreased as distance from the inoculation site increased (compare Fig. 2A with 2B) and no bacteria were reisolated. In the live-bacteria treatment, all sites had high serological responses. In the fixed-bacteria treatment, highest serological responses occurred at the inoculation site and within 1 cm above the inoculation site. Results indicate that fixed bacteria systemically spread in vascular bundles at least 3 cm from the inoculation site in both directions, but they elicited lower serological responses as the distance from the inoculation point increased. Immunoblots of cucumber stem

tissue indicated that both the live and the fixed bacteria were localized in xylem elements of the vascular bundles. No positive responses were detected with immunoblots of cucumber plants mock inoculated with a sterile needle in the stem. Therefore, DAS-ELISA was not performed on these wounded plants. We were never able to reisolate bacterial from the wounded cucumbers or the healthy control plants. Uninoculated and unwounded cucumbers were always negative in stem blots and DAS-ELISA.

There was little difference in the pattern of serological response when live or fixed *E. tracheiphila* were inoculated into the goldenrod stems (Fig. 3A and B). In both treatments high absorbance values were recorded only at the inoculation site. In some of the goldenrod plants of both treatments, live and fixed *E. tracheiphila* could be detected at low levels more than 15 cm from the inoculation point with stem blots and ELISA. Absorbance values for mock-inoculated, wounded goldenrod plants were very low and not different from those of healthy controls.

No *E. tracheiphila* could be reisolated from any part of any of these goldenrod plants in any of the treatments, but cantaloupe seedlings inoculated with the same live *E. tracheiphila* inoculum did wilt. Cantaloupe seedlings inoculated with the fixed bacteria did not wilt and fixed *E. tracheiphila* did not grow when streaked on NAP.

DISCUSSION

Although *E. tracheiphila* is known to be transmitted by cucumber beetles, little is known about the specifics of the disease cycle. Recent work quantitated the corre-

lation between beetle incidence in cucumber fields and disease severity and verified the importance of the vector (36). It is assumed that *E. tracheiphila* can infect plants only after wounds allow ingress into the vascular xylem tissue. Whether beetles can transmit the bacteria directly to the plant during feeding is not known. Brust (6) recently showed that plants were more likely to become infected if inoculum was placed on the plant epidermis prior to leaf wounding, and that large wounds were more likely than smaller wounds to result in infection. This information is compatible with the idea that the beetle feces are a source of inoculum on the plant surface (24) and that increased beetle feeding damage is associated with increased disease. How the first beetles moving into cucurbit fields in late spring acquire the bacteria is not known. It is assumed that the bacteria survive over the winter in adult beetles that have acquired *E. tracheiphila* the previous growing season. Evidence for *E. tracheiphila* overwintering in beetles is circumstantial and has never been experimentally verified. Although the hypothesis is rational, it is based on the ability of a small percentage of beetles captured in late spring to transmit *E. tracheiphila* to test seedlings. In work by Doolittle (9), beetles were field collected primarily during the month of May, 1917, before cucumbers were planted, and 50 beetles caged on an unspecified number of seedlings in the field. Four seedlings became wilted. This experiment was repeated in 1919, with 3 of 22 seedlings becoming wilted. Doolittle assumed the beetles carried the bacteria over winter and could not have acquired bacteria from another source. However, he noted that the beetles were observed feed-

Fig. 2. Double antibody sandwich-enzyme-linked immunosorbent assay absorbance from *Cucumis sativus* inoculated with (A) live and (B) fixed *Erwinia tracheiphila*. Data show mean absorbance from seven stem sections representing inoculation site and incremental distances from that site.

ing on wild cucumbers and other weed species in the area at the time of collection, but that these plants never showed symptoms of wilt. Wild cucumbers are susceptible to *E. tracheiphila* (35). When Rand and Cash (24) collected beetles from young cucurbit seedlings in the field from 19 May to 6 June and wound inoculated cucumber seedlings with individual dissected beetle guts as a bioassay for *E. tracheiphila*, inoculum from viscera of 4 of 1,240 beetles resulted in wilt. These authors also assumed that the beetles had acquired *E. tracheiphila* the previous season from infected crops. Further supporting this suggestion, Rand and Enlows (25) demonstrated that beetles fed on infected cucumbers could retain viable *E. tracheiphila* for up to 6 weeks in the laboratory at 4 to 10°C. However, this experiment was not repeatable, and attempts to test for *E. tracheiphila* overwintering in over 11,500 field collected beetles were equally unsuccessful in several attempts over a 2-year period (26).

The hypothesis that *E. tracheiphila* must survive overwinter in the beetle vector was based on a few observations of the ability of a small percentage of beetles captured in late spring to transmit *E. tracheiphila* to test seedlings. It was assumed that these beetles could not have acquired *E. tracheiphila* from any other source. Although these beetles prefer cucurbits, they are capable of feeding on 68 species of plants in 20 plant families in early spring before cucurbit crops are planted (14), and the beetles often emerge 40 to 50 days before cucurbit crops are available. Several authors (11,14,29,31,35) have questioned the existence of alternative weed hosts as an im-

portant component of *E. tracheiphila* epidemiology. Using immunofluorescence microscopy, Bassi (2) detected *E. tracheiphila* in goldenrod and johnsongrass. This supported the idea that *E. tracheiphila* could infect and survive overwinter in perennial weed species and that these weeds could be a source of bacteria for the beetles in the spring. Our work was designed to test this hypothesis.

Results of our study do not support the hypothesis that herbaceous weeds may support ecologically important reservoirs of *E. tracheiphila*. We obtained serological evidence of *E. tracheiphila* in several weed species collected from a cucurbit field in the fall of 1994, but we were not able to reproduce these results in the spring or fall of 1995. Also, we were unable to isolate *E. tracheiphila* from any of the serologically positive weeds. Initially, we attributed this inability to reisolate bacteria to low titers in field-collected weed tissue. Therefore, we initiated greenhouse tests with mechanical inoculation of *E. tracheiphila* to six weed species common to cucurbit fields. Three weeks after inoculation, 40 to 73% of the plants in five of the six species inoculated were ELISA positive for *E. tracheiphila*. However, we again failed to re-isolate *E. tracheiphila* from these plants. To overcome what we perceived as a problem in bacterial concentration in plant tissues, we developed a variation of immunoaffinity isolation capable of isolating *E. tracheiphila* from 200- μ l samples containing as little as 2 CFU. When applied to the inoculated ELISA positive weed species, we again failed to recover bacteria.

This conflict in detection between serology and isolation was examined more

closely by comparing susceptible cucumbers and the weed goldenrod (*S. altissima*) inoculated with either live or dead (fixed) *E. tracheiphila*. When we inoculated live bacteria in susceptible cucumbers, we found, as expected, strong ELISA positives at a series of distances from the inoculation point. Also, as expected, we were able to reisolate when we used live bacteria, and unable to reisolate when we used dead bacteria. However, we were surprised to find serological evidence for *E. tracheiphila* in cucumbers 3 weeks after they were inoculated with dead cells. The ELISA absorbance values obtained with dead *E. tracheiphila* in cucumber followed a strong pattern of decreasing values with increasing distance from the point of inoculation.

In goldenrod inoculated with live bacteria, we were able to find serological evidence for *E. tracheiphila*, but we were unable to reisolate the bacteria. Also, the serological results followed the same pattern with respect to the inoculation point as shown when dead cells were inoculated in susceptible cucumbers. Furthermore, this decrease in the serological response with respect to the inoculation site occurred in goldenrod regardless of whether live or dead cells were introduced. These results indicated that *E. tracheiphila* did not survive following inoculation to goldenrod. Limited systemic spread of the bacterial cells in the xylem before cell death could explain the low serological values detected distant from the inoculation point. Thus, we conclude that our immunoassays and our isolation techniques were effective, and that DAS-ELISA can detect dead bacterial cells systemically spread in vascular elements of plants 3 weeks following intro-

Fig. 3. Double antibody sandwich-enzyme-linked immunosorbent assay absorbance from *Solidago altissima* inoculated with (A) live and (B) fixed *Erwinia tracheiphila*. Data show mean absorbance from four stem sections representing inoculation site, top of plant, base of plant, rhizome, and roots.

duction. These results may also explain why Bassi (2) was able to visualize a low number of *E. tracheiphila* cells in plant sap, but was unable to isolate them. The cells may have been dead but still able to bind fluorescent antibodies.

Our histological survey of cucumber vascular tissues verified that *E. tracheiphila* was specifically limited to xylem vessel elements in stems and petioles of severely wilted cucumbers up to 14 dpi, after which time the plants usually collapsed. As reported for *E. stewartii* (5), there was no ultrastructural evidence of enzymatic degradation of vessel element cell walls associated with bacterial aggregates. These observations supported results from nitrocellulose stem blots of infected plants showing bacterial detection limited to vascular bundles throughout the plant. Bacterial cell aggregates were occasionally observed blocking some xylem elements in cucumber, and earlier studies concluded that mechanical plugging was probably the primary cause of wilting in infected plants (17). However, not all vessels were completely filled with bacteria and most vessel elements in a bundle were not blocked, even in severely wilted seedlings. Although these observations may be influenced by fixation artefacts or variability in tissues infected, it leaves open the possibility that wilting may be attributed to the release of bacterial metabolites (33).

Our investigation with serological assays, immunoaffinity isolations, and bioassays does not support the hypothesis that herbaceous weeds may function as ecologically important reservoirs of *E. tracheiphila*. Serological evidence (ELISA) indicated that weeds collected in some cucurbit fields, and some mechanically inoculated weeds, contained *E. tracheiphila* cells, but we had no evidence that these cells were alive. In order to prove that the weed species we tested were a significant source of inoculum, it would have been necessary to reisolate this pathogen from the weeds and then reproduce the transmission from weeds to cucurbits, but we were never able to isolate the bacteria. Our repeated failures to reisolate this pathogen with multiple isolation techniques and our evidence that we can detect dead (fixed) *E. tracheiphila* have led us to conclude that the weed species we tested are not important reservoirs of inoculum. As a result of this study, our current work is refocused on examining potential mechanisms of bacterial survival in beetle vectors.

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