Epiphytic Survival of *Erwinia tracheiphila* on Muskmelon (*Cucumis melo* L.)

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Abstract

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Erwinia tracheiphila, the causal agent of bacterial wilt of cucurbits, is transmitted by striped (*Acalymma vittatum*) and spotted (*Diabrotica undecimpunctata howardi*) cucumber beetles. Transmission occurs when infested frass with *E. tracheiphila* is deposited on plant surfaces with fresh feeding wounds. However, it is unclear whether the pathogen can survive as an epiphyte on leaves. Experiments were conducted in controlled environments to monitor *E. tracheiphila* survival on muskmelon (*Cucumis melo*) leaves under various temperature and moisture conditions. In the first experiment, muskmelon seedlings that had been spray inoculated with a rifampicin-resistant strain of *E. tracheiphila* were incubated at 10, 15, 20, 25, 30, or 35°C (±2°C) at ≥95% relative humidity, and *E. tracheiphila* populations were monitored for 72 h. In the second experiment, *E. tracheiphila* was monitored during alternating 12-h wet and dry periods, or continuous wet or dry conditions for 48 h at 20°C. Survival of *E. tracheiphila* on wet muskmelon leaves depended on temperature ($P < 0.01$), with the greatest survival at 10 and 15°C and least at 30 and 35°C. Leaf wetness also impacted survival; an initial 12-h dry period resulted in a 1,000- to 10,000-fold reduction in population size, followed by stabilization of the surviving population. These results demonstrate that *E. tracheiphila* can survive on muskmelon leaves under a wide range of environmental conditions, suggesting that epiphytic populations might serve as a reservoir of inoculum for infections.

Bacterial wilt caused by *Erwinia tracheiphila* is a major challenge for cucurbit growers in the eastern half of the United States (11,23,24). Bacterial wilt impacts all cucurbit crops except watermelon. In muskmelon (*Cucumis melo* L.), for example, yield losses can be as high as 80% (23). The United States is one of the major producers of cantaloupe and melon (*Cucumis* spp.) worldwide, with a farm gate value of approximately $400 million in 2009 (31).

Striped (*Acalymma vittatum* (F.)) and spotted (*Diabrotica undecimpunctata howardi* (Barber)) cucumber beetles transmit *E. tracheiphila* (12,29,34). The pathogen overwinters in adult beetles (12,14,33). Transmission occurs when adults feed on plants; inoculum from infested frass enters freshly wounded tissues (5,6,22, 28,29,34) on leaves, stems, or nectaries of flowers (38). Bacteria enter the vascular system via these openings, multiply, and produce exopolysaccharides, which eventually block water flow to the rest of the plant (38). Symptoms include wilting of leaves and vines followed by collapse of the entire plant.

Management strategies against bacterial wilt focus primarily on controlling the vectors by insecticide applications (7,9,11,13). However, this dependency on insecticides can create hazards for growers, consumers, pollinators, and the environment (7,13,16,18). Alternative strategies such as row covers (19,29,37), perimeter trap crops (1,10,35), and kairomonal baits (17,27) can reduce the risk of bacterial wilt but they can be labor intensive and require further validation before they can be used reliably by growers.

Lack of understanding of *E. tracheiphila* ecology and epidemiology is a barrier to developing effective ecologically based alternatives for bacterial wilt management. One reason for this poor understanding is that *E. tracheiphila* is difficult to isolate and maintain in culture. Unlike several other important *Erwinia* sp. phytopathogens, *E. tracheiphila* grows slowly in culture and can rapidly lose viability (9). As a result, the potential role of epiphytic populations of *E. tracheiphila* in the disease cycle remains largely unexplored. Epiphytic populations can serve as primary inoculum for development of epidemics in many bacterial pathosystems (3,15,35), and characterizing the environmental biology of the epiphytic phase has facilitated development of more effective management strategies. For example, monitoring epiphytic populations of the fire blight pathogen *E. amylovora* has been essential for determining the risk of disease epidemics, improving biological control methods, and timing antibiotic applications (20,21,39,42).

Experiments by Brust (6) provided indirect evidence that *E. tracheiphila* might have an epiphytic phase on cucurbit crops. These experiments suggested that *E. tracheiphila* could survive for up to 6 h on muskmelon leaf surfaces, and that these populations could infect plants after leaves were wounded. Mitchell and Hanks (29) confirmed that *E. tracheiphila* could survive in beetle frass and retain viability as inoculum for up to 24 h after the bacterium was ingested by striped cucumber beetles.

The impact of environmental conditions on epiphytic survival of *E. tracheiphila* is also poorly understood. It is reasonable to assume that *E. tracheiphila* could be dispersed from frass deposits to other sites on leaf surfaces by rain or dew, potentially resulting in infection via wounds. Moist weather conditions may also favor infection by *E. tracheiphila* (34), as suggested by the common association of moist conditions with bacterial wilt development (41). The purpose of the present study was to evaluate whether *E. tracheiphila* survives on muskmelon leaves, and to assess how survival is influenced by temperature and fluctuating moisture conditions.

Materials and Methods

Plant growth conditions. Muskmelon seed ('Athena') were planted in 233-cm^3 pots (Nu-Pot 3; Summit Plastic Co., Akron, OH; 40% peat moss, 40% prepared substrate [Sunshine Mix SB300; Sun Gro Horticulture Canada Ltd., Vancouver, BC Canada], and 20% coarse perlite). They were incubated for 12 to 15 days at 25°C under a regime of 12 h of light and 12 h of darkness in a growth chamber (Model PGW36; Conviron, Winnipeg, AB, Canada) until unfolding of the first true leaf. After emergence, seedlings were thinned to one per pot.

Bacterial strain and growth conditions. A naturally occurring rifampicin-resistant strain of *E. tracheiphila,* SCR3, was isolated from a wilting muskmelon plant in Iowa in 2009. Colonies that resembled *E. tracheiphila* in morphology (9) were confirmed positive by polymerase chain reaction (PCR) using *E. tracheiphila*-specific primers ETC1and ETC2 (28). Colonies were selected for rifampicin resistance (75 µg/ml) and stored at –80°C in Luria-Ber-

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tani broth containing 25% glycerol. In order to fulfill Koch's postulates, 2-week-old muskmelon plants were puncture inoculated with SCR3 strain. Bacterial colonies were suspended in sterile 10 mM phosphate-buffered saline (PBS) solution (pH 7.4) and concentration was adjusted to 10^8 CFU/ml. A 100-µl aliquot of the suspension was spread on the first true leaf of seedlings and leaves were punctured using a pin frog. Wilt symptoms were assessed and the strain was reisolated from symptomatic plants.

Inoculum preparation. Strain SCR3 was recovered from –80°C 1 week prior to inoculum preparation, streaked onto nutrient agar peptone (NAP) plates amended with rifampicin at 75 µg/ml, and incubated at 26°C in darkness. Cultures were incubated for 4 days, then restreaked onto fresh NAP plates 3 days before inoculum preparation. Inoculum was prepared by suspending strain SCR3, grown on NAP, in 10 mM PBS solution (pH 7.4) and adjusting the concentration to approximately 10^8 CFU/ml. Cell concentrations were calculated based on optical density in a spectrophotometer at 540 nm according to a standard curve for *E. tracheiphila*.

Influence of temperature on epiphytic survival. Twelve pots, each containing one muskmelon plant, were evenly spaced in each of four 25-pot trays (Nu-tray 3-25; Summit Plastic Co.). Plants with a fully unfolded first true leaf were spray inoculated until runoff with inoculum using a hand-trigger sprayer (model 916CN; Contico International, St. Louis). Immediately after inoculation, trays were placed in dew chambers (model I60DL; Percival International, Perry, IA) and incubated for 72 h at 10, 15, 20, 25, 30, or 35°C (±2°C) and a regime of 12 h of light and 12 h of darkness under conditions (≥95% relative humidity [RH]) conducive to dew formation. The experimental design was a split-plot, randomized complete block in which temperature was the whole-plot treatment randomly assigned to a dew chamber in a run, and sampling time was the subplot treatment randomly assigned to a leaf in a tray. Temperature and RH were recorded hourly using WatchDog Data Loggers (model 150; Spectrum Technologies, Plainfield, IL). At each sampling time, two arbitrarily selected leaves, each from a different plant, were excised from each tray. In total, eight leaves per sampling time were removed at 0, 12, 24, 36, 48, and 72 h after inoculation. Each excised leaf was immersed in 20 ml of 0.1 M PBS (pH 7.4 + 0.01% peptone [wt/vol]) in sterile 50-ml polypropylene centrifuge tubes (Corning Inc., Corning, NY), then sonicated (model Branson 200; Branson Ultrasonic Corp., Danbury, CT) for 7 min and mixed by vortexing for 15 s. Fivefold dilutions of each leaf washing were plated onto NAP amended with rifampicin at 75 µg/ml and cycloheximide at 100 µg/ml. Leaves were removed from the centrifuge tubes and allowed to air dry on paper towels for 15 min before weighing. After 5 days at 26°C in darkness, colonies of *E. tracheiphila* were counted and expressed as CFU per gram of fresh leaf weight. The experiment was performed twice.

Epiphytic survival during intermittent wet and dry periods. To determine epiphytic survival of *E. tracheiphila* under wetting and drying conditions resembling those that occur under field conditions, plants were spray inoculated as previously described and maintained at 20°C throughout the experiment. In order to create different wetness regimes, plants were incubated for 48 h in

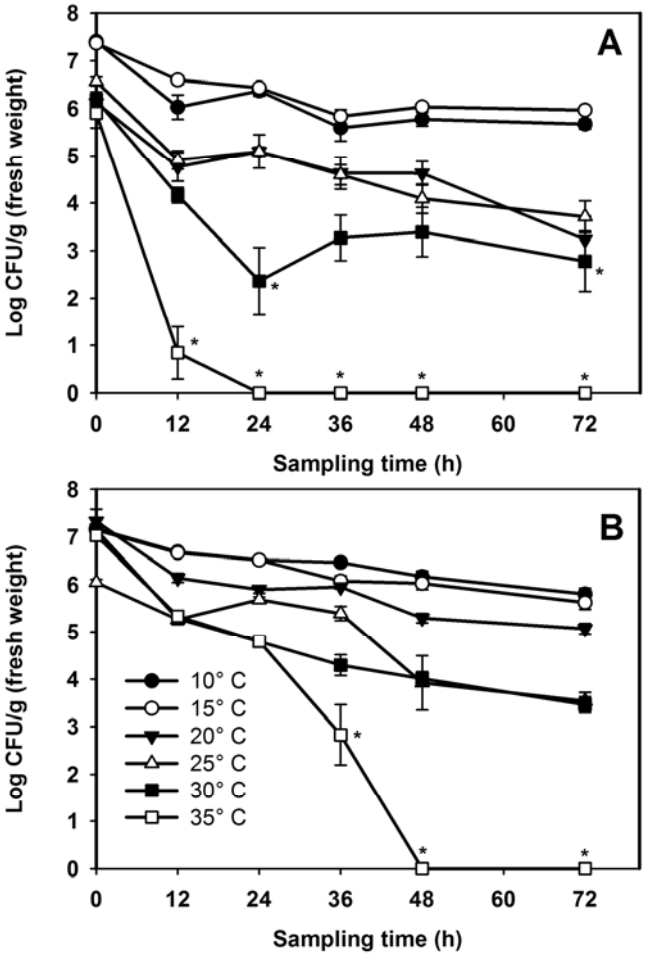

Fig. 1. Epiphytic populations (expressed as $\log_{10}$ CFU per gram of fresh leaf weight) of *Erwinia tracheiphila* strain SCR3 on muskmelon leaves incubated for 72 h under wet conditions at six different temperatures. The experiment was performed twice; each data point in **A**, first run and **B**, second run represents the mean ± standard error of eight leaves. Means below the detection limit 2.88 ($\log_{10}$ CFU/g fresh weight) are indicated by an asterisk (*).

Table 1. Summary of analysis of variance of epiphytic populations of *Erwinia tracheiphila* on muskmelon leaves under different temperature treatments and intermittent wet and dry periods[u]

Survival, source	df[v]	Error[w]	*F* value	*P* > *F*
Survival at different temperatures				
Run[x]	1	Run × temperature	8.56	0.0328
Temperature[y]	5	Run × temperature	19.93	0.0026
Sampling time[z]	5	Residual error	253.47	<0.0001
Temperature × sampling time	25	Residual error	8.23	<0.0001
Survival under intermittent wet/dry periods				
Run[x]	1	Run × treatment	18.00	0.0240
Treatment[y]	3	Run × treatment	83.89	0.0022
Sampling time[z]	3	Residual error	497.47	<0.0001
Treatment × sampling time	9	Residual error	47.14	<0.0001

[u] Mean square error with residual error values was 24.4 ± 1.47 for survival at different temperatures and 0.66 ± 0.28 for survival under different wetness regimes.
[v] Degrees of freedom.
[w] Error term used to test run of the experiment multiplied by temperature treatment and run of the experiment multiplied by wetness regime treatment.
[x] Run of each experiment.
[y] Temperature in growth chambers or dew chambers.
[z] Time at which leaves were sampled after inoculation.

a dew chamber (≥95% RH) in darkness or a growth chamber (≥30% RH) in the light as follows: continuous wet or dry conditions, 12-h wet periods alternated with 12-h dry periods, or 12-h dry periods alternated with 12-h wet periods. The experimental design was a split-plot, randomized complete block in which wetness regime was the whole-plot treatment randomly assigned to a chamber in a run, and sampling time was the subplot treatment randomly assigned to a leaf in a tray. In total, eight leaves were excised at each sampling time (0, 12, 24, and 48 h after inoculation). After sonication and vortexing, *E. tracheiphila* populations were enumerated as described above. The experiment was performed twice.

Statistical analysis. In each experiment, colony counts were normalized to CFU per gram of fresh leaf weight by $\log_{10}$ transformation. For both experiments, transformed data were compared by analysis of variance (ANOVA) using PROC MIXED (version 9.1; SAS Institute Inc., Cary, NC). The whole-plot treatment (temperature or wetness regime) was tested against the whole-plot error, run × temperature, split-plot treatment, and sampling time, and interactions were tested against the residual error. Area under the curve (AUC) values were estimated from average populations ($\log_{10}$ CFU/g fresh weight) determined at each sampling time in each experiment. AUC values were analyzed in a one-way ANOVA using PROC GLM (SAS Institute Inc.).

Results

Impact of temperature on epiphytic survival. Survival of *E. tracheiphila* on muskmelon leaves was impacted by temperature (Table 1). By 12 h after inoculation, population size had decreased significantly at all temperatures (Fig. 1) ($P < 0.01$). There was no significant change in population size at either 10 or 15°C between 12 and 72 h after inoculation. The population sizes at the midrange temperatures plateaued after 12 h but then decreased significantly by 36 h (25°C) and 72 h (20°C). *E. tracheiphila* populations declined rapidly and continuously for 24 h at 30°C and for up to 72 h at 35°C. Values of AUC indicated that survival at 10 and 15°C exceeded that at 20, 25, 30, and 35°C, whereas AUC values at midrange temperatures were similar to each other (Table 2).

Impact of intermittent wet and dry periods on epiphytic survival. Survival of *E. tracheiphila* on muskmelon leaves was impacted by wetness regime (Table 1). The presence or absence of wetness on leaves significantly impacted survival during the first 12 h after inoculation (Fig. 2). Survival was significantly higher under continuous wet conditions and lowest when plants were initially exposed to dry conditions (Table 2). During an initial 12-h dry period, populations fell by more than three orders of magnitude. A similar population decline occurred when plants initially exposed to wet conditions were subsequently exposed to dry conditions for 12 h. Population level stabilized after an initial decline associated with the first dry period, despite later recurrence of dry periods. No differences were observed between AUC values when plants were exposed to an initial 12-h dry period after inoculation or when they were exposed to dry conditions throughout the entire experiment.

Table 2. Comparison of area under the curve (AUC) values for epiphytic survival of *Erwinia tracheiphila* on muskmelon leaves under different temperature treatments and intermittent wet and dry periods

Treatment	AUC[s]
Temperature (°C)[t]	
10	450.3 a
15	446.0 a
20	378.0 b
25	341.9 bc
30	306.8 cd
35	259.5 d
LSD[u]	51.3
Intermittent wet/dry periods[v]	
12 h wet/12 h dry[w]	229.4 b
12 dry/12 h wet[x]	184.6 c
48 h wet[y]	285.7 a
48 h dry[z]	170.9 c
LSD	18.9

[s] $\log_{10}$ of AUC. Means in the same column followed by the same letter are not significantly different ($P = 0.05$).
[t] Under wet conditions (≥95% relative humidity) for 72 h.
[u] LSD = least significant difference ($P = 0.05$).
[v] At 20°C for 48 h.
[w] Schedule of 12-h wet periods alternated with 12-h dry periods.
[x] Schedule of 12-h dry periods alternated with 12-h wet periods.
[y] Continuous wetness.
[z] Continuous dryness.

Discussion

Our findings provide the first direct evidence that *E. tracheiphila* can survive for at least several days on muskmelon leaves. In the only previous study of epiphytic survival, Brust (6) demonstrated that placement of *E. tracheiphila* inoculum on muskmelon leaves followed by wounding up to 6 h later could result in wilting.

Although *E. tracheiphila* is known primarily as a vascular pathogen, our findings suggest that it is also persistent and resilient as an epiphyte. High temperatures and exposure to dry periods clearly reduced survival of *E. tracheiphila*, whereas continuous wetness at optimum temperatures (10 to 20°C) clearly favored survival. These surviving populations could become important sources of inoculum, particularly if leaves become wounded by subsequent events such as high winds or insect feeding. Relatively low survival at 30

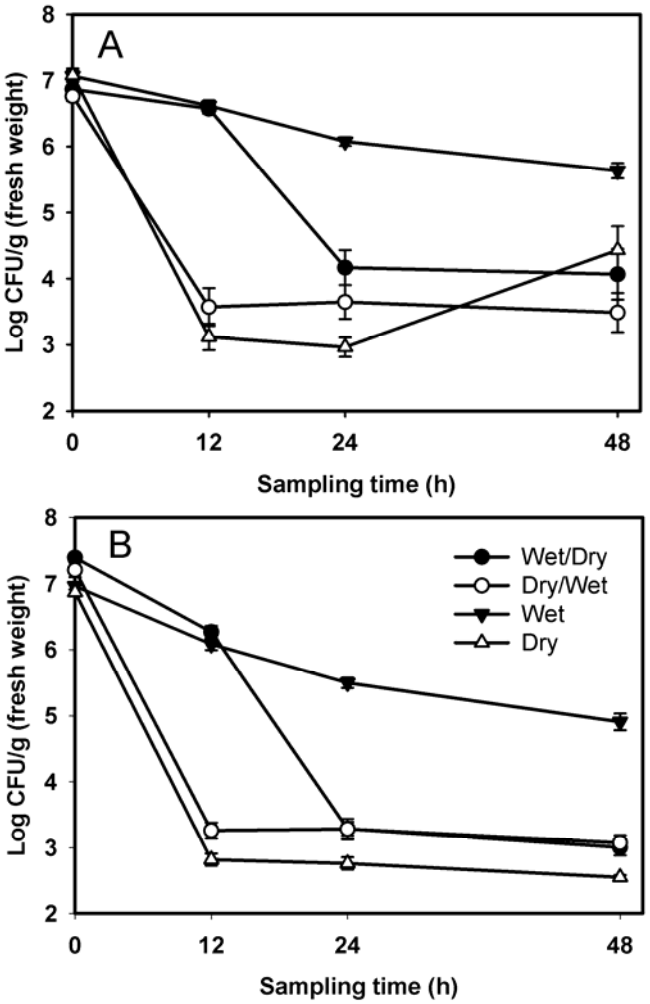

Fig. 2. Epiphytic populations (expressed as $\log_{10}$ CFU per gram of fresh leaf weight) of *Erwinia tracheiphila* strain SCR3 on muskmelon leaves incubated for 48 h at 20°C under alternating 12-h periods of wet and dry conditions. The experiment was performed twice; each data point in **A,** first run and **B,** second run represents the mean ± standard error of eight leaves.

to 35°C is consistent with studies of other epiphytic phytopathogenic bacteria (2). Although dry conditions can impose severe stress on epiphytic bacteria (2,26), *E. tracheiphila* populations stabilized at approximately 10^3 CFU/g of fresh leaf tissue after 2 days of continuous dryness. Survival of *E. tracheiphila* under dry conditions may have resulted from selection for a subpopulation having a relatively high ability to endure water limitation (4) or occupy protected microsites on the leaf surface (28).

Our study also demonstrates that *E. tracheiphila* can persist on leaf surfaces in the absence of frass. Mitchell and Hanks (29) demonstrated an association between infested frass of striped cucumber beetle deposited on cucumber leaf surfaces and transmission of *E. tracheiphila*. Although Mitchell and Hanks did not determine how long the pathogen survived in frass, they showed that *E. tracheiphila* could survive in the beetle gut and in frass produced up to 24 h after ingestion, and that puncture inoculation of infested frass on cucumber seedlings resulted in bacterial wilt infection.

Survival of *E. tracheiphila* in frass could play an important role in the pathogen bacterial wilt disease cycle. It is reasonable to assume that survival of *E. tracheiphila* could be extended by its association with frass if substances (e.g., water or organic compounds) in the frass conferred protection against desiccation. It is also reasonable to assume that *E. tracheiphila* could be dispersed from frass by rain splash, and that this dissemination could increase the probability of contact with leaf wounds and, thereby, facilitate entry of inoculum into the plant. Sasu and co-workers (38) provided clear evidence that accumulation of infested beetle frass in floral nectaries may also lead to bacterial wilt infection. This discovery confirmed a second route for entry of the bacterial wilt pathogen in addition to entry through leaf wounds, reaffirming the importance of understanding the environmental biology of *E. tracheiphila* on plant surfaces.

Although our experiments were performed under controlled conditions, we showed that *E. tracheiphila* can persist on muskmelon leaves under conditions that resemble environmental fluctuations occurring in the field. For example, alternating 12-h wet and dry periods at 20°C roughly approximate conditions that occur in muskmelon production fields in the Midwest United States during the early part of the growing season (40), when plantings are at highest risk for infection by *E. tracheiphila* (6,11,12,29,33). Taking into account the sporadic and poorly understood nature of bacterial wilt epidemics (37), a better understanding of the impact of environmental conditions on the risk of infection by epiphytic inoculum may improve management strategies and disease risk assessment.

To achieve a more realistic assessment of the role of epiphytic survival of *E. tracheiphila* in the bacterial wilt disease cycle, further investigation is needed (i) using more *E. tracheiphila* strains under a wider range of environmental conditions and longer periods, (ii) documenting survival of epiphytic populations of *E. tracheiphila* under field conditions, (iii) quantifying dispersal of *E. tracheiphila* on leaf surfaces by rain splash from frass, and (iv) explicitly associating circumstances (e.g., size of *E. tracheiphila* epiphytic populations or environmental conditions that damage leaves) with the risk of disease transmission. Our study has provided a foundation for taking the next steps in clarifying the role of epiphytic populations of *E. tracheiphila* in the epidemiology of this important pathosystem.

Acknowledgments

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