BACTERIAL WILT SYMPTOMS ARE IMPACTED BY HOST AGE AND INVOLVE NET DOWNWARD MOVEMENT OF *ERWINIA TRACHEIPHILA* IN MUSKMELON

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ACKNOWLEDGEMENTS

This research was funded by a Specialty Crop Research Initiative (SCRI) Program Grant (2012-51181-20295) from the U.S. Department of Agriculture National Institute of Food and Agriculture (NIFA). We thank Jean C. Batzer and Xiaoyu Zhang for technical advice and assistance.

ABSTRACT

Cucurbit bacterial wilt, caused by *Erwinia tracheiphila*, is a damaging disease of cucurbit crops in the Midwest and Northeast U.S. Current management of bacterial wilt relies primarily on insecticide applications to control striped and spotted cucumber beetles (*Acalymma vittatum* and *Diabrotica undecimpunctata howardi,* respectively), which vector *E. tracheiphila.* Development of alternative management strategies is constrained by a lack of understanding of bacterial wilt etiology. The impact of host age on rate on symptom development and extent of bacterial movement in the xylem of muskmelon (*Cucumis melo* cv. Athena) was evaluated following wound inoculation of 2- to 8-week-old plants in growth chamber experiments. Wilting occurred more rapidly in plants after inoculating *E. tracheiphila* into 2- or 4-week-old plants than 6- or 8-week-old plants. Recovery of viable cells from stem segments revealed that vascular spread of *E. tracheiphila* was more extensive below than above the inoculation point. These findings provide experimental evidence that host age impacts the rate of symptom development in cucurbit bacterial wilt and that movement of the xylem-inhabiting pathogen *E. tracheiphila* within muskmelon plants occurs primarily in the downward direction.

KEYWORDS

Phytopathogenic bacteria; vascular wilts; vegetable diseases; ontogenic resistance

INTRODUCTION

Erwinia tracheiphila (Smith), the causal agent of cucurbit bacterial wilt, is transmitted by striped (*Acalymma vittatum* (F.)) and spotted (*Diabrotica undecimpunctata howardi* (Barber)) cucumber beetles. Bacterial wilt causes severe losses in many cultivated cucurbit crops, primarily in the genera *Cucurbita* and *Cucumis*. Economic losses of cucurbit crops from bacterial wilt can reach 75% (Zehnder et al. 1997), and muskmelon (*Cucumis melo* L.) and cucumber (*Cucumis sativus* L.) are among the most susceptible crops (Sherf 1986). Epidemics have occurred primarily in the Midwest and Northeast U.S. as well as in southern Quebec, Canada (Brust 1997, Fleischer et al. 1999, Toussaint et al. 2013); the disease has been restricted primarily to these areas with the exception of a recent report of bacterial wilt on pumpkin and watermelon in New Mexico (Sanogo et al. 2011).

Overwintering of *E. tracheiphila* occurs in the foregut and hindgut of striped cucumber beetles (Garcia-Salazar et al. 2000). It is transmitted when *E. tracheiphila*-infested frass of adult beetles comes into contact with fresh wounds on leaves, stems, or floral nectaries (Sasu et al. 2010) and the bacteria invade the xylem. *Erwinia tracheiphila* cells multiply in the xylem and can block water flow (Main and Walker 1971). Infected plants initially exhibit wilting of leaves near the infection site, followed by wilting of vines and eventual collapse and plant death.

Current management for cucurbit bacterial wilt relies primarily on insecticide applications (Cavanagh et al. 2009), but this approach is costly and potentially risks the health of humans, pollinators, insectivorous birds and other ecosystem service providers (Cavanagh et al. 2009, Potts et al. 2010). Although watermelon (*Citrullus lanatus* var. *lanatus*) is resistant to bacterial wilt and a few cultivars of cucumber (*Cucumis sativus*) are tolerant, resistant cultivars are not commercially available for most cucurbit crops. Therefore, a clearer understanding of the bacterial wilt infection process may yield insights to assist plant breeders in developing such cultivars. A starting point toward this goal is to understand the impact of host age on disease progress, as this may help breeders develop efficient protocols for screening candidate lines for resistance. Moreover, tracing patterns of pathogen movement in the xylem could result in a

deeper understanding of disease development.

The impact of plant age on wilt symptom development has been investigated for several xylem-inhabiting bacterial pathogens. Plant age affects bacterial wilt and canker of tomato caused by *Clavibacter michiganensis* subsp. *michiganensis*; tomato transplants up to the 17- to 18-leaf stage wilted and died after inoculation, whereas plants that were inoculated after that stage exhibited only mild symptoms (Sharabani et al. 2013). Similarly, *Ralstonia solanacearum* caused earlier symptoms, higher disease incidence, and greater disease severity of bacterial wilt of tomato in 2- to 3-week-old seedlings than in 5- to 6-week-old plants (Thomas and Upreti 2014). For cucurbit bacterial wilt, the relationship between plant age and symptom development has been examined only for the relatively resistant host genera *Cucurbita* and *Citrullus*. In pumpkin (*Cucurbita pepo* L.), which exhibits intermediate resistance to *E. tracheiphila* (Sherf 1986), seedlings inoculated at the cotyledon stage were more susceptible than older plants and resistance increased sharply with plant age (Brust 1997). Seedlings of watermelon (*Citrullus lanatus*), which is highly resistant to *E. tracheiphila* (Watterson et al.1971), exhibited more rapid symptom development than older plants and resistance increased with age (Watterson et al. 1971). However, no such relationships have been assessed for the highly susceptible cucurbits in the genus *Cucumis*, such as muskmelon or cucumber, which are at the greatest risk of economic losses from bacterial wilt.

The mechanisms by which *E. tracheiphila* causes bacterial wilt have not yet been investigated in detail. Absence of evidence for pectolytic enzyme production suggests that physical occlusion by the bacteria is the primary cause of xylem dysfunction (Main and Walker, 1971, Watterson, Williams, and Durbin, 1971). Moreover, the presence of strands of ooze as infected stems are cut and drawn apart suggests the presence of extracellular polysaccharides, which could also contribute to xylem blockage, as with wilt by *R. solanacearum, Pantoea stewartii* and *Xylella fastidiosa* (Ayers et al. 1979, Beck von Bodman et al. 1998, Saile et al. 1997). Factors that contribute to the virulence of other bacterial xylem pathogens, such as biofilm formation, quorum sensing, outer membrane vesicle production and motility (Herrera et al. 2008, Ionescu et al. 2014, Koutsoudis et al. 2006, Meng et al. 2005), have not yet been examined in *E. tracheiphila.* Recently, observational studies using a constructed bioluminescent strain of *E. tracheiphila* provided the first evidence that the bacterium can move both upward in muskmelon seedlings and downward into the roots following inoculation (Vrisman et al. 2016). The objectives of the present study were to i) quantify the impact of host age on development of wilt symptoms in muskmelon and ii) trace the movement of *E. tracheiphila* in the xylem following inoculation.

MATERIALS AND METHODS

Plant growth conditions. Muskmelon (*Cucumis melo* cv. Athena) seeds were planted in a 1:1:1 matrix of peat moss, coarse perlite, and Metro-Mix 300 (Sun Gro Horticulture, Vancouver, BC, Canada). A single seed was planted in each 650 cm^3 pot. The pots were incubated at 26°C under a daily regimen of 14 h light and 10 h darkness under ambient relative humidity (RH) in growth chambers. Plants were watered daily and fertilized weekly (NPK: 15-5-15: Miracle Gro®, The Scotts Co., Marysville, OH).

Bacterial strain and inoculum preparation. *E. tracheiphila* strain SCR3 (Saalau Rojas et al. 2012) was used in this study. This strain is a spontaneous rifampicin-resistant derivative of an isolate from a symptomatic muskmelon plant grown in Iowa, U.S. Pathogenicity of this isolate was confirmed by puncture inoculation of the first true leaf of 2-week-old muskmelon

plants in growth chamber trials (Saalau Rojas et al. 2012). To prepare cells for plant inoculation assays, SCR3 was recovered from -80°C storage on solid nutrient agar peptone medium (de Mackiewicz et al. 1998) that was amended with rifampicin (75 µg/ml) (NAP-Rif). Cells were grown at 27°C for 3 days, then transferred to fresh NAP-Rif medium and grown at 27°C for another 3 days. Bacterial suspensions were prepared by recovering SCR3 colonies from the surface of solid NAP-Rif medium and suspending them in 10 mM phosphate-buffered saline (PBS) to a concentration of approximately 2.5 x 10^8 CFU/ml, based on a standard curve relating cell density to optical density at 540 nm.

Symptom expression experiments. Muskmelon seeds were planted 2, 4, and 6 weeks before inoculation in the first experiment and 2, 4, 6 and 8 weeks before inoculation in a second experiment, in order to create multiple cohorts of plants that varied in age at the time of inoculation. In each experiment, plant age at inoculation was considered a treatment, and each treatment included four single-seedling replicates. A 100-µl droplet of inoculum was applied at the base of the adaxial surface of the youngest fully expanded leaf, followed by puncturing the leaf at the site of the droplet with a 28.6-mm-diameter, 60-pin florist's pin frog (Kenzan Pin Frog, sold by www.save-on-crafts.com). Next, the pipette tip was rubbed lightly against the punctured site in order to ensure maximal contact of the inoculum with the puncture wounds and an additional 100 µl of suspension was applied to the punctured site on the leaf, after which the pipette tip was again rubbed lightly on the site. Control plants were inoculated with PBS (10 mM) buffer in the same manner as described above. After inoculation, plants were incubated in a growth chamber at 26° C under a daily regimen of 14 h light and 10 h darkness and ambient RH. Numbers of wilted and asymptomatic leaves on each plant and their locations relative to the site of inoculation were determined daily until all *E. tracheiphila*-inoculated plants displayed wilt symptoms. Rate of wilting was determined by estimating the number of days for 50% of the plant to show wilt symptoms.

Pathogen movement experiments. Plants that were 2, 4, 6, and 8 weeks old were inoculated as described above, with plant age at inoculation considered as a treatment. On days 3, 7, 14, and 21 after inoculation, four plants of each treatment were chosen arbitrarily for destructive sampling. Stem segment samples (5 cm long and devoid of nodes) from each treatment were excised above and below the point of inoculation. In the first run of the experiment, all internodes were sampled for the plants that were 2 or 4 weeks old at inoculation (referred to here as 2-week-old and 4-week-old plants), whereas every third internode was sampled in older plants(referred to here as 6-week-old and 8-week-old plants). In the second run of the experiment, every internode was sampled from the 2- and 4-week-old plants, whereas every second internode was sampled from the 6- and 8-week-old plants. These internode stem segments were surface-sterilized by spraying them with 70% ethanol and then air dried on sterile paper towels. A stem segment was cut transversely at its midpoint, the cut surface was imprinted on the surface of NAP-Rif medium, and the resulting imprint was streaked for single colonies. The presence of colonies exhibiting *E. tracheiphila* morphology was recorded after 4 days of incubation at 27° C. The number of wilted and asymptomatic leaves per plant was also counted on each sampling date. Sampling was terminated in each treatment when all leaves had wilted. Bacterial movement in the upward direction vs. the downward direction was compared on the basis of the mean number of internodes from the site of inoculation to the furthest internode from which *E. tracheiphila* cells were recovered.

Data analysis. In the symptom expression experiments, disease progress was determined based on the area under the disease progress curve (AUDPC) using the trapezoidal method

(Simko and Piepho 2012) and the means were compared with a Fisher's least significant difference test using SAS 9.1 (SAS Institute Inc., Cary, NC, USA). For each treatment in the pathogen movement experiments, the mean number of internodes from which *E. tracheiphila* was recovered above vs. below the point of inoculation was compared using a Student's *t*-test.

RESULTS

Rate of wilting. Leaves on the inoculated muskmelon plants began to wilt as early as 4 days after inoculation. In both runs of the experiment, the inoculated leaves wilted rapidly regardless of plant age at inoculation (Table 1). In the first experiment, the plants that were 2 weeks old at inoculation wilted significantly ($p<0.05$) faster than those that were 4 or 6 weeks old, as reflected in the mean number of days from inoculation until wilting of 50% of leaves and area under the disease progress curve (AUDPC) (Simko et al. 2012). Similarly, plants that were 4 weeks old at the time of inoculation wilted significantly faster than those that were 6 weeks old. In the second run of the experiment, although the plants in each age group wilted more slowly than similarly-aged plants in the first experiment, the plants that were younger at the time of inoculation again wilted faster than those that were older. Specifically, the 2- or 4-week-old plants wilted significantly ($p<0.05$) faster than the 6- and 8-week-old plants based on the number of days required for plants within each treatment to display 50% of wilted leaves and on the AUDPC (Table 1). No wilting was observed on the control plants in either experiment.

Bacterial movement. In each of two replicate experiments to evaluate bacterial movement from the site of inoculation, *E. tracheiphila* cells were first recovered from stem samples 7 days postinoculation (dpi) and were isolated from sites both above and below the inoculation point (Fig. 1). There was evidence of upward movement of *E. tracheiphila* , determined by recovery of bacteria from stem segments above the inoculation site at 7, 14 and 21 dpi, and at sites as far as 5 and 8 internodes above the inoculation site in the first and second runs of the experiment, respectively. Stem growth above the inoculation point continued until 14 dpi on plants that had been 4, 6, or 8 weeks old at inoculation, but the pathogen was not recovered from the uppermost internodes of the stem even at 21 dpi, indicating that its movement in the upward direction was limited.

At each sampling time, *E. tracheiphila* moved much further downward than upward, with movement sometimes limited by reaching the lowest possible node (Fig. 1). In contrast to the upward movement to a maximum of 5 to 8 internodes above the inoculation site, bacteria moved downward as far as 9, 13, and 23 nodes by 7, 14 and 21 dpi, respectively, in the first run of the experiment regardless of plant age at inoculation (Fig. 1A), and to similar distances in the second run of the experiment (Fig. 1B). On plants inoculated at 6 or 8 weeks of age, *E. tracheiphila* cells were detected in nearly all of the stem segments sampled below the inoculation point at 21 dpi, and the distance of movement of the bacterium at 14 and 21 dpi was significantly ($p<0.05$) greater below than above the point of inoculation.

In both experiments, the 2-week-old and 4-week-old plants died before reaching 14 and 21 dpi respectively; thus, data for these treatments are not presented for those time points. By 21 dpi in both runs of the experiment, the stems of all of the plants that were 6 weeks old at inoculation had withered and died above the inoculation point; isolations from that portion of the plant in the first run were not attempted because the pathogen does not survive in dead host tissue (Latin 2000). In general, *E. tracheiphila* reached the lowest node of each plant by the time of the plant's death.

DISCUSSION

Our results provide the first experimental evidence that the rate of wilting of *Cucumis* sp. crop by *E. tracheiphila* is impacted by host age, with young plants developing wilt symptoms significantly faster than older ones based on the time required for 50% of the leaves to wilt. Although grower guides frequently state that young cucurbit plants are more susceptible to infection than older plants (Brust 1997a, Watterson et al. 1971), experimental evidence supporting this assertion is absent for *Cucumis* spp. In the Brust (1997a) study, more wilt was observed in pumpkin seedlings that had been inoculated at the cotyledon stage than at the later stage of ≥1 true leaf, although most of the seedlings with true leaves never showed wilt and recovered from the inoculation. The general trend observed in the Brust study – increasing resistance with increasing plant age – agrees with results of the present study of muskmelon.

Knowledge that susceptibility to bacterial wilt decreases as muskmelon plants age has important management implications. For example, our evidence that plants are more susceptible to bacterial wilt when they are young can help to inform optimal timing for reduced-insecticide management strategies such as the deployment of row covers as protective barriers against cucumber beetles (Mueller et al. 2006, Saalau Rojas et al. 2011). In particular, although row covers should be deployed during the most susceptible period to suppress bacterial wilt, deciding when to remove them should factor in both the probability of pathogen transmission by cucumber beetles (Saalau Rojas et al. 2011) and plant phenology related to resistance. In addition, clarifying responses to inoculation as a function of seedling age should help plant breeders to optimize screening assays for bacterial wilt resistance. For example, using plants that are 4 weeks old at inoculation might be a cost-effective option because they are relatively small and thus require minimal growth space, and moderate in susceptibility when compared with 2-, 6-, and 8-week-old plants. Moreover, their requirement for about 8 dpi to show symptoms enables sufficient observation time to compare symptoms among breeding lines. However, additional, season-long experiments in the field would be needed in order to comprehend impact on yield and fruit quality when plants become infected at later stages of the growing season.

The mechanism responsible for a decreased wilting rate as muskmelon plants age is unclear. Ontogenic resistance could result from factors such as increased production of phytochemicals and/or the development of physical barriers that slow disease progress (Panter and Jones 2002). Alternatively, the pathogen may be diluted if plant growth exceeds pathogen growth, which could slow the rate of wilting. The correlation of plant age with plant size makes it difficult to separate the impacts of age versus size; this is particularly true for cucurbit crops, most of which increase rapidly in size during the early part of the growing season. It is possible that both ontogenic and plant size-related factors may operate to slow wilting in older, larger plants. Further experimentation will be required to unravel the causal mechanisms of this phenomenon.

Our experiments are the first to document that internal movement of *E. tracheiphila* following infection is more rapid in a downward than upward direction. In common with other xylem-limited vascular wilt diseases, symptom expression as indicated by visible wilt progressed distally beyond the nodes from which the pathogen was recovered, presumably as sieve plates became blocked and water flow ceased (Holland et al. 2014, McElrone et al. 2003). Such a blockage of the upward movement of water inside the vascular system may help to explain our observation that *E. tracheiphila* moved in a primarily downward rather than upward direction from the inoculation point. Vrisman et al (2016) provided observational evidence that a

bioluminescent-labeled strain of *E. tracheiphila* moved not only upward from the inoculated leaf of a muskmelon seedling but also downward into the roots. The mechanism driving bacterial movement against the xylem flow is unclear, but is consistent with the movement exhibited by two other xylem-limited vascular wilt pathogens: *Xylella fastidiosa*, the causal agent of Pierce's disease of grape (Meng et al. 2005), and *Acidovorax avenae* subsp. *citrulli*, the causal agent of bacterial fruit blotch of cucurbits (Bahar et al. 2010). *X. fastidiosa*, a nonflagellated bacterial pathogen, was shown to spread in the xylem via motility mediated by type IV pili (Meng et al. 2005). Type IV pili, which are like grappling hooks, enable the bacterial cells to jerk forward along a surface in a form of motility known as twitching (Mattick 2002). *X. fastidiosa* mutants defective in these pili showed reduced downward colonization of the xylem (Meng et al. 2005). As a xylem-limited pathogen, *E. tracheiphila* may employ a similar mechanism of movement. This is consistent with the recent discovery that the putative type IV pili genes are conserved among *Erwinia* spp., including *E. tracheiphila* (Shapiro 2012). Our study, the first to quantify the relative movement of the pathogen upward and downward following infection, is a further step in understanding the movement of *E. tracheiphila* in the xylem and helps to set a foundation for evaluating the role of pilus genes in downward movement.

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Table 1. Impact of plant age at the time of inoculation with *Erwinia tracheiphila* on the rate of wilt and disease progression in muskmelon (*Cucumis melo* cv. Athena) plants in two growth chamber experiments.

Expt[a]	Plant age at inoculation (weeks)	Mean days to wilting of the inoculated leaf [b]	Mean days to wilting of 50% of the leaves[b,c]	AUDPC[b,d]
1	2	5.3 a	5.3 a	1,667 a
	4	5.0 a	11.3 b	1,062 b
	6	4.7 a	14.0 c	777 c
2	2	5.7 a	5.7 a	2,539 a
	4	6.0 a	8.5 a	2,289 a
	6	6.0 a	18.0 b	1,545 b
	8	7.3 a	20.7 b	1,240 b

[a] Two independent experiments were performed. Each experiment included four replicate plants per treatment.

[b] In each experiment, the data were subjected to Fisher's least significant difference (LSD) test to determine differences among the means using SAS 9.1 (SAS Institute Inc., Cary, NC, USA). Values in a column that are followed by the same letter do not differ significantly ($p<0.05$). n=4.

[c] Data include the inoculated leaf.

[d] AUPDC, area under the disease progress curve (Simko and Piepho 2012).

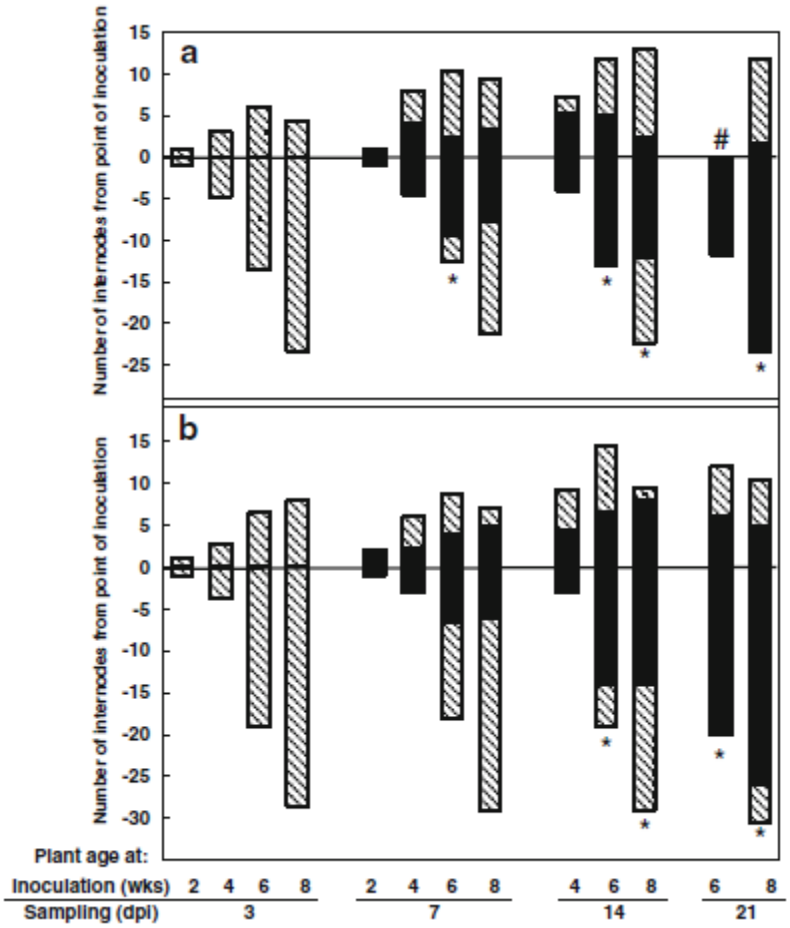

Fig. 1 Directional movement of *E. tracheiphila* in stems of muskmelon (*Cucumis melo* cv. Athena) plants inoculated at different ages. The results of two replicate experiments are shown in (a) and (b). Data shown are the mean number of internodes from the site of inoculation to the point where *E. tracheiphila* cells were recovered (black bars) or were not recovered (hatched bars) from stem segments. Plants were 2, 4, 6, or 8 weeks old when the youngest true leaf was inoculated. The zero point on the y-axis indicates the point of inoculation. Positive numbers on the y-axis indicate the number of internodes above the inoculation point, whereas negative numbers indicate number of internodes below the inoculation point. Data are means of four replicates per treatment. * indicates sampling times for which the bacterial movement from the site of inoculation was significantly (Student's *t*-test, $p<0.05$) greater in the downward than upward direction. n = 4. # = indicates samples that were too desiccated to attempt to recover *E. tracheiphila*.