Characterization of the incompatible interaction between *Erwinia tracheiphila* and non-host tobacco (*Nicotiana tabacum*)

Eric S. Nazareno¹, Caleb M. Kersey², and C. Korsi Dumenyo^{1*}

¹Department of Agricultural and Environmental Sciences, Tennessee State University, Campus Box 9543, Nashville, TN, 37209, USA

²Department of Biological, Physical, and Human Sciences, Freed-Hardeman University, Henderson, TN, 38340, USA

*Corresponding author: cdumenyo@tnstate.edu

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Abstract

Bacterial wilt is one of the most destructive diseases affecting a wide range of crops in the Cucurbits family including muskmelon (*Cucumis melo*), cucumber (*Cucumis sativus*), and squash (*Cucubita pepo*). The disease is caused by *Erwinia tracheiphila*, a Gram-negative and xylem-inhabiting species of *Erwinia*, which pathogenic mechanism is poorly understood. Many Gram-negative phyto bacteria induce hypersensitive response (HR) in non-host plants, an immunity reaction triggered by pathogen recognition. With some exemptions, *Erwinia* species—notably *E. amylovora*, the causative agent of fire blight of rosaceous crops, and the reclassified soft rot pathogens, *Pectobacterium* and *Dickeya* species (formerly *E. carotovora* and *E. chrysanthemi*)—have been known to elicit HR in tobacco. However, concerning its pathogenic mechanism, the elicitation of classic HR has not been reported for some less-studied *Erwinia* species including *E. tracheiphila*. We characterized the induction of HR by the bacterial wilt pathogen in tobacco (*Nicotiana tabacum* cultivar ‘Xanthi’) using visual and physiological methods. We surveyed 21 *E. tracheiphila* strains and found that all of them elicited programmed cell death. Three strains (HCa1-5, UnisCu1-1, and MISpSq) fluorescently labeled with GFP could be visualized in the infiltrated leaves. We aligned the sequences of their HR-inducing protein, harpin (HrpN), predicted the secondary structures, and located the position of putative HR elicitors. We discovered differences between *Cucurbita* and *Cucumis* strains and found a close association of *E. tracheiphila* HrpN with those of *Pantoea* sp., *Erwinia piriflorinigra*ns, and *Erwinia pyrifoliae*. Pre-infiltration of tobacco leaves with a lower cell population prevented HR following a subsequent challenge at the same area with HR-inducing levels of inoculum. The selected strains induced leaf conductivity levels similar to the HR-inducing *E. amylovora* strain

40 E9, and their populations in the leaves decreased days after infiltration. Our results indicate that
41 *E. tracheiphila* induces a classic HR in tobacco just like other HR-inducing *Erwinia* species.

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43 Keywords: *Erwinia tracheiphila*; hypersensitive response; harpin (hrpN); bacterial wilt of
44 cucurbits; green fluorescent protein (GFP)

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Introduction

Bacterial wilt of cucurbits is one of the most destructive diseases of susceptible cucurbit species [1]. The disease is caused by an insect-transmitted, xylem-dwelling Enterobacterial phytopathogen, *Erwinia tracheiphila*. Despite its economic importance, the pathogen is one of the least studied *Erwinia* species—partly due to the success and popularity of insecticide-based bacterial wilt disease management and the difficulty of culturing the bacterium *in vitro* [2]. Since very little is known about this pathogen, there is a lot that the scientific community needs to know about not only its basic biology but also the various mechanisms of interaction with both compatible and incompatible host plants.

When a host plant is exposed to a pathogen, their interaction has one of two outcomes: disease or host resistance. A compatible interaction leads to disease while an incompatible one induces innate immunity from the plant. The two broadly defined and sometimes overlapping mechanisms of host defense against pathogens are pathogen-associated molecular patterns (PAMP)-triggered immunity (PTI) and effector-triggered immunity (ETI) [3]. Defined molecular motifs (PAMPs) such as flagellin, lipopolysaccharide, and peptidoglycan are signature pathogen molecules that interact with the surface of plant cells and induce PTI [4–6]. When the plant detects bacterial effectors—proteins that are directly injected into plant cells through the type III secretion system—effector-triggered immunity (ETI) is elicited [7]. A compatible interaction leading to disease occurs in the absence of the plant resistance (*R*) gene that recognizes the effector avirulence (*avr*) gene product from the pathogen, while resistance happens when the plant triggers programmed cell death through hypersensitive response (HR) as a result of pathogen recognition [5,8].

Many Gram-negative bacteria induce HR in non-host plants [9]. The visual characteristics of the response include a rapid and programmed death of few host cells (apoptosis) that prevent the spread of the pathogen. Physiologically, reactive oxygen intermediates and ion fluxes in the cells are observed during the process [10–13]. The first reported pathogen-associated cell-free HR elicitor (harpin) gene, *hrpN*, was cloned from *Erwinia amylovora* [14]. Harpins are glycine-rich, heat-stable proteins, which are reported to act both as PAMPs (targeted extracellular of plant cells and functions as translocators) and effectors to elicit plant immunity [3,4,15,16]. Although the *hrpN* genes from some erwiniae including *E. amylovora*, *Dickeya dadantii* (*E. chrysanthemi*), and *Pantoea stewartii* (*E. stewartii*) code for HR elicitors [17–19], some others including *E. billingae* and *Pectobacterium carotovorum* (*E. carotovora* subsp. *carotovora*) lack the ability to induce HR in the wild type [20,21]. However, gene knockout of a global regulator, *rsmA*, resulting in a de-repressed expression of *hrpN*, enabled *P. carotovorum* subsp. *carotovorum* to elicit HR in tobacco [20].

Because *E. tracheiphila* has received little research attention, we have insufficient knowledge about its mechanisms of interaction with its host(s). However, the pathogen shares some features with other species of *Erwinia* or *Pantoea*, which can be used to understand these mechanisms. First, *E. tracheiphila* produces copious amount of extracellular polysaccharide like *E. amylovora* and *P. stewartii* and demonstrates motility through the action of peritrichous flagella [22]. Secondly, *E. tracheiphila* induces wilting symptoms on the host resembling those caused by *E. amylovora* on rosaceous species, especially at the onset of the infection. These similarities have led us to hypothesize that *E. tracheiphila* also possesses some of the same mechanisms of host-microbe interactions with other erwiniae. Particularly, we believe that just like the other HR-inducing erwiniae, *E. tracheiphila* has a functional *hrpN*, whose product,

HrpN, will induce HR in a non-host plant. Our goals here are to gather evidence that *E. tracheiphila* induces classic HR in tobacco, compare the HrpN sequences of different strains of the pathogen, and determine the phylogenetic relationships of selected strains with the other members of Enterobacteriaceae based on HrpN.

Materials and Methods

Media and bacterial and plant growth conditions

Bacterial strains (Table 1) were grown on Nutrient Broth-Yeast Extract (NY) plates at 28 °C for 3-4 days as previously described [22]. When needed, antibiotics were supplemented to the media at the indicated rates, nalidixic acid (Nal) at 25 µg/ml and ampicillin (Ap) at 100 µg/ml). Tobacco cultivar ‘Xanthi’ seeds were sown in a tray with Fafard Mix No. 2 (Sun Gro Horticulture, MA, USA). One week after germination, the plants were transplanted to 6-inch diameter pots (Grower’s Solution LLC, Cookeville, TN, USA) and grown in a 14/10-hour light/dark cycle. Two weeks after transplanting, plants were fertilized with complete fertilizer (14-14-14) at a rate of 2 g/pot. The plants used in the experiments were at five- to six-leaf stage.

HR survey among *E. tracheiphila* strains

Twenty-one *E. tracheiphila* strains isolated from cucumber (*Cucumis sativus*), melon (*Cucumis melo*), and squash (*Cucurbita pepo*) were surveyed for elicitation of HR in tobacco. The strains were streaked on NY agar media and the growth on each plate was scraped and resuspended in 2 ml sterile distilled water. The cell densities were standardized at 0.5 (5×10^6 CFU/ml) with A₆₀₀ readings using a microplate reader (Synergy H1, Biotek, VT, USA). Sterile needleless 1 ml syringes (Henke Sass Wolf GmbH, Germany) were used to infiltrate the bacterial

suspension in the apoplastic region of pre-pricked tobacco leaves. Symptoms of HR were observed 24 hrs after infiltration. The experiment was conducted twice in four replicates.

Construction of GFP plasmid

The plasmid pCKD300 was constructed by amplifying the promoterless *gfp+* sequence (~1000 bp) from pRU1701 [23] using *Taq* polymerase and primers Pac1_GFP+P6 (GACTTAATTAAGCGGATTTGTCCTACTCAGG) and NotI_GFP+P5 (CTAGGCGGCCGCTCACAAAACGGTTTACAAGCA). The following PCR parameters were used: 95°C for 5 minutes, 1: 95°C for 30 sec, 2: 55°C for 30 sec, 3: 72°C for 1 minute x 30 cycles. The *gfp+* fragment was then ligated downstream of the *lac* promoter of plasmid pGEM[®]-T-Easy (Promega, Madison, WI). The ligation reaction was electroporated into *E. coli* XL 10 Gold and plated on LB Agar supplemented with ampicillin. A bright fluorescent colony was selected in which the *gfp+* gene is under the control of the *lac* promoter in the multiple cloning site of the vector pGEMT-Easy. The DNA of pCKD300 was purified using Wizard[®] Plus SV Minipreps (Promega, Madison, WI).

GFP detection *in planta*

The selected strains, HCa1-5N and UnisCu1-1N, (*Cucumis* isolates), and MISpSq-N (*Cucurbita* isolate) were labeled with green fluorescent protein (GFP) by transformation with the plasmid pCKD300. Transformants were selected in LB+Ap and GFP was observed using a handheld UV lamp (365nm, UVP, Upland, CA, USA). In a separate study, we determined that pGEM-T Easy plasmid replicon has almost 100% stability over three overnight sub-culturings in the *E. tracheiphila* strains used in this study (Prestwich and Dumenyo, unpublished). The labeled strains were infiltrated in tobacco leaves using the method described above. After 24 hours, leaves were exposed under UV radiation to visualize fluorescent bacteria in the infiltrated region.

Sequence alignment, protein prediction, and phylogenetic tree

The predicted harpin sequences of *E. tracheiphila* HCa1-5, UnisCu1-1, and MISpSq were pulled out from our draft sequences (unpublished data) and aligned with that of *E. tracheiphila* PSU-1 harpin (Genbank accession no. EOS94912.1) using CLUSTAL Omega (<http://www.ebi.ac.uk/Tools/msa/clustalo/>). The secondary structure of harpins were predicted using PSIPRED (<http://bioinf.cs.ucl.ac.uk/psipred/>), amino acid and isoelectric point using ExPASy ProtParam tool (<http://web.expasy.org/protparam/>), and putative HR elicitor using MUSCLE (<http://www.ebi.ac.uk/Tools/msa/muscle/>) alignment with *E. pyrifoliae* HR elicitor. Multiple sequence alignment of the harpin proteins of *E. tracheiphila* and other Enterobacteriaceae was conducted using COBALT (<http://www.stva.ncbi.nlm.nih.gov/tools/cobalt>). The alignment was downloaded as a Newick file and then uploaded to iTOL (<http://itol.embl.de/>) to generate the phylogenetic tree. All software analyses were carried out in default parameters.

Conductivity test

The procedures for the conductivity test were modified from those of Szatmári *et al.* (2014). Suspensions of GFP-labeled bacterial cultures, HCa1-5N/pCKD300, UnisCu1-1N/pCKD300, and MISpSq-N/pCKD300, were infiltrated in tobacco leaves using the method described above. Five leaf discs of 1 cm diameter were collected from the infiltrated area and suspended in 15 ml sterile distilled water in a 50 ml test tube. The leaf discs were gently shaken overnight at room temperature to allow electrolyte leakage. In four replicates, electrolyte leakage was measured using Orion 4 Star conductivity meter (ThermoFisher, MA, USA) calibrated with the appropriate standards. Data were analyzed in One-Way ANOVA using R statistical package.

Prevention of HR

Inocula for HR-inducing cell density (high) of $A_{600}=0.5$ (5×10^6 CFU/ml) and their non-HR-inducing 20-fold dilutions (2.5×10^5 CFU/ml) (low) were prepared. At Day 1, the low-density suspensions were infiltrated in the left sides of the first and second leaves (See Fig. 3, Panels 1 and 3) and the high HR-inducing concentration was infiltrated to only the right side of the first leaf (See Fig. 3, Panel 2). After 24 hours (Day 2), the side infiltrated with the low concentration on the first leaf (Panel 1) was challenged with the high HR-inducing concentration. To serve as control, the high inoculum concentrations were also infiltrated on the right side of the second leaf (Panel 4). The HR symptoms were observed 24 hours after the second day of infiltration. The experiment was replicated four times.

Populations in tobacco leaves

Transformed strains, HCa1-5N/pCKD300, UnisCu1-1N/pCKD300, and MISpSq-N/pCKD300 were infiltrated in tobacco leaves as described. After infiltration of 5×10^6 CFU/ml inoculum, four leaf discs (1 cm diameter) were collected from the infiltrated area and placed into 500 μ l of water in 2 ml microcentrifuge tubes. A 5 mm stainless steel bead was placed in each tube and the leaf discs were ground for 2 mins at 25 Hz using Tissue Lyser II (Qiagen, CA, USA). After grinding, the volume was raised to 1 ml and serial dilution was done up to 10^{-3} . From each dilution, 100 μ l was spread on NY agar plates with Nal (25 μ g/ml). Population sizes of each strain were estimated from samples 0, 1, and 3 days post-infiltration. The experiment was performed in three replicate leaves per strain and mean population sizes were analyzed using pairwise t-test in R. The experiment was performed twice.

Results

Induction of classical HR symptoms

182 Our main goal was to understand the mechanisms of *E. tracheiphila* host/non-host interactions.
183 We designed the first experiment to determine how the pathogen will react with non-host
184 tobacco, in which both *P. stewartii* and *E. amylovora* elicit classical HR. All of the 21 *E.*
185 *tracheiphila* strains (of *Cucurbita* and *Cucumis* origin) that we surveyed for HR induced
186 localized cell death in tobacco leaves (Table 1). Symptoms included rapid death (necrosis) of the
187 leaf area that had been in contact with the bacteria, which led to the arrested spread of the
188 pathogen. Nalidixic acid-resistant spontaneous mutants of selected strains (HCa1-5, UnisCu1-1,
189 and MISpSq) labeled with a plasmid carrying the green fluorescent protein (*gfp+*) gene showed
190 fluorescence on plates under UV radiation. When the GFP-labeled strains were infiltrated into
191 tobacco leaves, we detected fluorescence at the region of infiltration after 24 hours (Figure 1).

192

193 **Table 1.** Characteristics and HR profile of *E. tracheiphila* strains used in this study.

Bacterial strain	Host	Characteristics	HR in tobacco	Source/Reference
BoCu3-1	<i>Cucumis sativus</i>	Wild type	+	[22]
BoCu4-1	<i>C. sativus</i>	Wild type	+	[22]
FishCu1-3	<i>C. sativus</i>	Wild type	+	[22]
FishCu3-2	<i>C. sativus</i>	Wild type	+	[22]
HCu2	<i>C. sativus</i>	Wild type	+	[22]
UnisCu1-1*	<i>C. sativus</i>	Nal ^r	+	[22,25]
GHM2-1a	<i>Cucumis melo</i>	Wild type	+	[22]
HCa1-5*	<i>C. melo</i>	Nal ^r	+	[22]
MCa1-1	<i>C. melo</i>	Wild type	+	[25]
McM1-1	<i>C. melo</i>	Wild type	+	[25]
BuffGH	<i>Cucurbita pepo</i>	Wild type	+	[25]
BHKY	<i>C. pepo</i>	Wild type	+	[25]
Et3AG4Z**	<i>C. pepo</i>	Wild type	+	ISU
L1SumSq2**	<i>C. pepo</i>	Wild type	+	ISU
MISpSq*	<i>C. pepo</i>	Nal ^r	+	[25,26]
NYAcSq1	<i>C. pepo</i>	Wild type	+	[25]
PPHow1	<i>C. pepo</i>	Wild type	+	[25]
ATCC 33245	Unknown	Wild type	+	[22]
E. Salisbury CW	Unknown	Wild type	+	[22]
LW4	Unknown	Wild type	+	[22]
NJ11	Unknown	Wild type	+	[22]

194

195 *Nal^r derivatives obtained through spontaneous resistance to 25µg/ml nalidixic acid

196 **Obtained from M. Gleason, Iowa State University, Ames, IA, USA

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Ion fluxes and HR prevention as physiological responses

Classical HR is marked by increase in ion concentration in the tissues undergoing the reaction [13]. Therefore, having observed typical HR symptoms, we wanted to test if this was associated with the expected physiological changes by measuring electrical conductivity of infiltrated leaf discs to detect ion fluxes. We observed electrical conductivity at the leaf area of bacterial infiltration after 24 hours (Figure 2). The selected strains, HCa1-5N/pCKD300, UnisCu1-1N/pCKD300 and MISpSq-N/pCKD300, induced leaf conductivity levels that are not significantly different from *E. amylovora* E9 [27], a classical inducer of HR that was included as the positive control. On the other hand, the leaf areas infiltrated with *E. coli* DH5 α and distilled water had significantly lower conductivity.

Another hallmark characteristic of HR in plants induced by phyto**bacteria** is the ability of lower concentrations of the inoculum to prevent HR when infiltrated into a leaf prior to a challenge by HR-inducing concentrations of the HR-inducing organism [28]. To show that the reaction elicited by *E. tracheiphila* strains in tobacco is classical HR, we tested the ability of lower concentration inoculum of *E. tracheiphila* strains to prevent HR when pre-infiltrated into the challenged leaf area. We pre-infiltrated tobacco leaves with lower concentrations (2.5×10^5 CFU/ml) of inoculum at Day 1. As shown in Figure 3, the pre-infiltrated low concentration inoculum showed no HR (Panel 3) while the HR-inducing concentrations infiltrated at the same time (Panel 2) triggered programmed cell death. The pre-infiltrated area was followed up with a challenge with HR-inducing concentrations at the same sites of pre-infiltration after 24 hours (Day 2). In over 72 hours, we observed that HR was prevented in the pre-infiltrated regions (Panel 1). To show that the challenge inoculum could elicit HR, it was also infiltrated in area that had not previously been pre-infiltrated. The result again shows that this high inoculum could

readily elicit HR (Panel 4). The same inoculum that failed to elicit HR in pre-infiltrated areas elicited the response in regions of the same leaf that were not pre-infiltrated.

Figure 1. Detection of fluorescently labeled bacteria in infiltrated leaves. HCa1-5N (A), HCa1-5N/pCKD300 (B), UnisCu1-1N (C), UnisCu1-1N/pCKD300 (D), MISpSq-N (E), MISpSq N/pCKD300 (F); uninfiltrated leaf (G) and water-infiltrated leaf (H) as control. Leaves exposed under UV radiation showing no GFP (A, C, E, G, H)) and GFP fluorescence (B, D, F)) in the infiltrated region.

231 **Figure 2.** Electrical conductivity of leaves inoculated with *E. tracheiphila* strains HCa1-
 232 5N/pCKD300, UnisCu1-1N/pCKD300, MISpSq-N/pCKD300; positive control, *E. amylovora*
 233 E9; and negative controls, *E. coli* DH5α and distilled water. Conductivity was measured from
 234 leaf discs collected from the infiltrated region 24 hours after infiltration. Means of the same letter
 235 are not significantly different; error bars indicate standard deviation based on four replications
 236 ($P < 0.05$).

HrpN phylogeny and the association with other pathogens

Harpin (HrpN), the first known elicitor of HR, was first described in *Erwinia amylovora* and since then homologues have been characterized from many other phyto bacterial pathogens [14]. Using the available draft genomes of selected strains of *E. tracheiphila*, we wanted to see if these strains also have HrpN homologues and if they have any shared properties. The CLUSTAL alignment of predicted HrpN protein sequences of *E. tracheiphila* strains showed that HCa1-5 and UnisCu1-1 (*Cucumis* strains) HrpN are 100% identical. Similar results were obtained for MISpSq HrpN and PSU-1 (*Cucurbita* strains). The alignment also revealed that the two groups of strains are 97% identical. Phylogenetic analysis of the HrpN sequences indicated that *E. tracheiphila* HrpN is closer to those from the more distantly-related *Pantoea agglomerans* and *Pantoea stewartii* than to *E. amylovora*, and less related to the soft rot bacteria, *Pectobacterium* spp. and *Dickeya* spp. (Figure 4). The distance tree shows four distinct groups in the erwiniae HrpN: 1) non HR-inducing *Pectobacterium* spp.; 2) HR-inducing *Dickeya* spp.; 3) HR-inducing *Pantoea* spp. and *E. tracheiphila*; and 4) HR-inducing *E. amylovora*. Protein secondary structure predictions of *E. tracheiphila* HrpN show differences in amino acid composition of HrpNs from *Cucumis* and *Cucurbita* strains (Table 2). The location of the putative HrpN elicitor and the secondary structures are also different for each group (Figure 5).

Bacterial populations *in vivo*

We hypothesized that the bacterial cell counts would decrease through time after infiltration as a result of the death of the host cells—an indication that the plant has restricted the spread of the pathogen. To test this, we infiltrated HR-inducing concentrations of bacteria into

Table 2. Characteristics of *E. tracheiphila* HrpN. Amino acid composition and isoelectric point (pI) were predicted using ExPASy ProtParam tool.

Strain	Host	Total aa	Glycine (%)	Serine (%)	Leucine (%)	pI
HCa1-5	<i>Cucumis</i>	405	21.2	9.4	9.1	4.93
UnisCu1-1	<i>Cucumis</i>	405	21.2	9.4	9.1	4.93
MISpSq	<i>Cucurbita</i>	451	26.2	8.6	8.6	5.06
PSU-1	<i>Cucurbita</i>	454	26.4	8.6	8.6	5.06

Figure 4. Phylogenetic tree of predicted HrpN sequences from *E. tracheiphila* and other Enterobacteria. Clade 1: *Brenneria* sp. and *Pectobacterium* sp.; Clade 2: *Dickeya* sp.; Clade 3: *Pantoea* sp. and *Erwinia tracheiphila*; Clade 4: *Erwinia pyrifoliae*, *E. piriflorinigrans*, and *E. amylovora*. Supplementary Table 1 provides the NCBI accession numbers of all protein sequences used in the analysis.

279 **Figure 5.** Predicted secondary structures of HrpN from *E. tracheiphila* strains using PSIPREP. Putative HR elicitors were deduced by
 280 MUSCLE alignment with the 13-amino acid HR elicitor (GGLGGLGGDLGST) of *E. pyrifoliae* (sequence identity at 10/13, 77%).

tobacco leaves and recovered the bacteria from them. Average population sizes of infiltrated strains in tobacco leaves at Day 0 were 1.7×10^5 CFU/four leaf disc sample (HCa1-5N/pCKD300), 2.8×10^6 CFU/four leaf disc sample (UnisCu1-1N/pCKD300), and 7.5×10^5 CFU/four leaf disc sample (MispSq-N/pCKD300). The average bacterial population sizes of isolates UnisCu1-1N/pCKD300 and MISpSq-N/pCKD300 decreased significantly ($P < 0.001$) by 95% in tobacco leaf tissue one day after infiltration. Isolate HCa1-5N/pCKD300 also decreased significantly ($P < 0.001$) within one day after infiltration; the magnitude of the loss was 45%, which was not significantly different from the magnitude of loss by the other two isolates. After Day 1, average population sizes of each isolate remained stable and did not differ significantly ($P = 0.001$) comparing Day 3 populations to those from Day 1 (Figure 6).

Discussion

In this paper, we have shown for the first time that *Erwinia tracheiphila*, the bacterial pathogen that causes the wilting disease of cucurbits, elicits classic HR in non-host tobacco plants and that strains of the pathogen possess a copy of the *hrpN* gene, which specifies harpin, the elicitor of HR in Gram-negative phyto bacteria. First, we visually characterized the HR induced by *E. tracheiphila* in tobacco. Second, we showed that the physiological processes associated with classic HR occur in tobacco leaves infiltrated with the bacterium, which are typical HR symptoms. Lastly, we showed that *E. tracheiphila* HrpN is related to the HrpNs of other HR-inducing erwiniae.

Although a study recently reported the host-specific interaction of *E. tracheiphila* strains with different cucurbit hosts [25], the interaction of the pathogen with a non-host such as tobacco has not been characterized. We believe that the ability of *E. tracheiphila* strains to

induce HR is a determinant of pathogenicity, similar to the correlation of pathogenicity and HR induction in *E. amylovora* [14]. All of the strains tested positive for HR, suggesting that they could be pathogenic to compatible hosts as well. Indeed many of them were isolated from diseased hosts. We found that the selected strains labeled with GFP fluoresced in the necrotic area when leaves were exposed to UV, confirming that the bacteria were in the infiltrated region inducing the plant cell death. The transformation with the GFP plasmid did not affect the ability of the strains to induce HR, which also holds true for their pathogenicity (data not shown). Our success in labeling the strains with GFP opens the door for further

Figure 6. Bacterial populations in infiltrated leaf tissues. Percentage cell counts of HCa1-5N/pCKD300, UnisCu1-1N/pCKD300, and MISpSq-N/pCKD300 in tobacco leaves at 0, 1, and 3 days post infiltration. The average bacterial population count at Day 0 was 1.2×10^6 CFU/ml. Error bars indicate standard deviations based on three replications; high significance denoted by ‘***’; no significance by ‘ns’ ($P < 0.001$).

investigations of pathogen movement in both compatible and incompatible hosts.

The alignment of predicted HrpN protein sequences revealed slight differences between *Cucumis* and *Cucurbita* strains, consistent with the a previous observation that *Cucumis* strains and *Cucurbita* strains have distinct genetic and virulence profiles [25]. This provides additional evidence based on HrpN sequences that the genotypes of *E. tracheiphila* strains vary depending on which host they were isolated. In contrast to the present taxonomic relationships, the phylogenetic analysis shows that *E. tracheiphila* HrpN is more closely related to those of *P. stewartii* subsp. *stewartii* and *P. agglomerans* pv. *glysophilae* than to *E. amylovora* HrpN. Similar to the cucurbit wilt pathogen, *P. stewartii* subsp. *stewartii* also causes wilt in corn and survives in the gut of its vector, the corn flea beetle [29,30]. It is possible that the pathogenic mechanisms of these bacteria have something to do with the similarity of their HrpN.

Although most erwiniae have the *hrp* gene cluster, not all of them express a functional HrpN at high levels enough to induce HR in a non-host. *Pectobacterium* spp. exemplifies this case where the *hrp* cluster is under tight control of a global post-transcriptional negative regulator, RsmA, which controls the expression of *hrpN* and other virulence factors [20]. In another case, such as in *E. billingae*, the entire *hrp* gene cluster is absent in the genome, rendering the bacterium unable to induce HR [31]. However, recent studies have found that only a certain part of the HrpN protein can elicit HR [4]. For instance, in *E. pyrifoliae*, the 13-amino acid portion of its HrpN was found to be enough for HR elicitation [32]. Since *E. pyrifoliae* is the closest available reference for finding the location of the HR elicitor in *E. tracheiphila*, we used it to predict the position of the putative HR elicitor based on the alignment to the 13-amino acid sequence. Though putative, we believe that this is a good prediction due to the relative similarity of *E. pyrifoliae* and *E. tracheiphila* harpin sequences. More experimentation, such as the

construction of knockout portions of the HrpN protein, are required to determine the exact position of the HR elicitor in *E. tracheiphila* HrpN.

In terms of the physiological characteristics of classic HR, conductivity levels in the leaves undergoing HR indicated that ion concentrations increased after infiltration. Increased H^+ , K^+ , and Ca^{2+} concentrations along with fluxes in reactive oxygen species (ROS), nitric oxide, and salicylic acid (SA) is among the physiological changes that occur during HR [33,34]. Based on our data, *E. tracheiphila* strains induced an increase in ion concentrations similar to *E. amylovora* E9, which presented additional evidence that they elicit a classic immunity response from tobacco. Further, pre-infiltrating a leaf with lower inoculum concentrations prior to challenging with an HR-inducing concentration at the same site prevented HR elicitation. Prevention of HR by pre-infiltration with lower inoculum concentration has been described in classic HR. This prevention has been shown in strains that induce classic HR in tobacco including *P. syringae* pv. *pisi*, RsmA⁻ mutant of *P. carotovorum* subsp. *carotovorum*, and *Erwinia amylovora* [20,28,35]. Expectedly, the low inoculum concentrations did not elicit HR symptoms and instead provided a conditioning treatment. This prevention, in some ways, is similar to the concept of plant defense priming [36]. Both phenomena do not require viable cells, as defense priming occurs not only with pathogens but also with non-pathogens and chemicals such as herbicides. Similarly, HR prevention has also been documented with heat-killed bacterial cells in *Ralstonia*-tobacco pathosystem. In this case, pre-infiltration “primed” the plant against the subsequent encounter with both compatible and incompatible pathogens [37]. A plant in a primed state infiltrated with high inoculum concentrations at the same site of pre-infiltration will prevent the development of HR symptoms. We speculate that the priming induced by pre-infiltration strongly activated the plant physiologically to prevent the usual response in the

absence of priming. Finally, we observed a decrease in bacterial populations in the infiltrated region of the leaf after infiltration due to the collapse and death of plant cells during HR. The necrosis traps the bacteria within the infiltrated area and cuts the supply of water and nutrients needed for pathogen survival and proliferation.

In conclusion, our findings indicate that *E. tracheiphila* elicits classic HR in tobacco through its HrpN protein that triggers plant immunity. To verify the HR induction, we compared our strains with *E. amylovora*, a classical HR inducer, and other *Erwinia* species that were reported to induce HR. Based on typical HR symptoms, HrpN sequences alignment, and physiological tests, we report that *E. tracheiphila* elicits classic HR in tobacco like other known HR inducers in the erwiniae family.

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