IDENTIFICATION AND PATHOGENICITY OF *PSEUDOMONAS SYRINGAE* GENOMOSPECIES 1 PHYLOGROUP 2B CAUSING LEAF SPOTS AND FRUIT WARTS ON CUCURBITS IN WESTERN WASHINGTON, U.S.

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SUMMARY

Pseudomonas syringae pv. *lachrymans* and pv. *syringae* have been identified as pathogenic on cucurbit crops in the United States and based on molecular data, pv. *syringae* (genomospecies 1) are genetically distinct from pv. *lachrymans* (genomospecies 2). In 2015, leaf lesions, and bull's-eye lesions and raised warts on fruit were observed on 'Cinnamon Girl' pumpkins near Mount Vernon in western WA. PsAs_2015 was collected from leaf lesions and PsFt1_2015 and PsFt2_2015 were collected from fruit. The isolates were phenotypically and molecularly characterized. LOPAT results were consistent for *P. syringae*. Neighbor joining and Bayesian analyses of *gltA* and *gyrB* sequences clustered the three isolates within genomospecies 1 phylogroup 2b. 'Cinnamon Girl' seedlings inoculated with these isolates were spotted with small lesions having chlorotic borders. Seedlings and fruits of 11 pumpkin and squash cultivars were inoculated with PsAs_2015. Altogether, 100% disease incidence occurred on five cultivars (Baby Bear, Baby Boo, Cinnamon Girl, Lil' Pump-Ke-Mon, Spaghetti) of seedlings and six cultivars (Cinnamon Girl, Jack Be Little, Lil' Pump-Ke-Mon, Honeyboat, Spaghetti, Sweet Dumpling) of fruit. *gyrB* sequence data of isolates recovered after inoculation were comparable to PsAs_2015 on 33 to 100% of seedlings and fruit. The clustering of PsAs_2015, PsFt1_2015, and PsFt2_2015 with other genomospecies 1 pathovars confirms that they are distinct from *P. syringae* pv. *lachrymans* and represents a first report for Washington State. Moreover, the presence of wart symptoms on pumpkin fruit represents a newly observed symptom in the United States.

Keywords: leaf marginal necrosis, fruit warts, pumpkin

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INTRODUCTION

The genus *Pseudomonas* affects a number of agricultural food crops worldwide and members of *P. syringae* account for the majority of this genus (Lamichhane *et al.*, 2015). Reported epidemics caused by the pathovars of *P. syringae* on annual crops have been on the rise since 2000, with 72 reports on over 40 crops worldwide (Lamichhane *et al.*, 2015). Seven of these outbreaks have been documented on cucurbit species in five countries: Iran, Turkey, Italy, Serbia, and the United States (Lamichhane *et al.*, 2015). Studies on *P. syringae* that infect cucurbits have reported disease incidences varying from 10% in seedlings to 100% in field grown plants, with symptoms ranging from angular leaf spots and water-soaked lesions to leaf and stem blights, and necrosis (Harighi, 2007; Sedighian *et al.*, 2014; Aysan *et al.*, 2003; Mirik *et al.*, 2004; Balaž *et al.*, 2014; Langston Jr. *et al.*, 2003; Dutta *et al.*, 2016). On fruit, sunken lesions and dry cavities have been described on cantaloupe (Morris *et al.*, 2000) and raised warts and lesions have been reported on buttercup squash in New Zealand (Sharrock *et al.*, 1997).

Three pathovars of *P. syringae*, pv. *lachrymans*, pv. *aptata* and pv. *syringae*, and two other members of the *P. syringae* species complex, *P. viridiflava* and *chicorii* (Berge *et al.*, 2014; Lamichhane *et al.*, 2015) have been identified as pathogenic to cucurbit crops. *Pseudomonas syringae* pv. *lachrymans* first was described on cucumber in 1913 (Burger, 1913) while *P. syringae* pv. *aptata* and pv. *syringae* only have been recognized as infecting cucurbits since 2003 (Sedighian *et al.*, 2014; Langston Jr. *et al.*, 2003). Of the three pathovars, only *P. syringae* pv. *lachrymans* and pv. *syringae* have been identified in the United States (Burger, 1913; Langston Jr. *et al.*, 2003; Dutta *et al.*, 2016). *Pseudomonas syringae* pv. *lachrymans* is considered to be primarily a pathogen on cucurbits (Young, 2010), although in 2005 it was described as causing water-soaked leaf spots on white *Strelitzia augusta* Thunb. in Italy (Polizzi *et al.*, 2005). Pathovars *aptata* and *syringae* have a wider host range. In one study, *P. syringae* pv. *aptata* caused symptoms on sugar beet, for which pv. *apata* is most commonly associated, but also on barley, cantaloupe, cucumber, corn, sorghum, and soybean (Morris *et al.*, 2000). In the same study *P. syringae* pv. *syringae* caused symptoms on bean, cantaloupe,

eggplant, lettuce, onion, pea, pepper, soybean, sorghum, sugar beet, sunflower and tomato. Although leaf lesions were observed on some cucumber plants inoculated with *P. syringae* pv. *syringae*, pathogenicity could not be confirmed since compatible reactions were not observed in both inoculation trials (Morris *et al.*, 2000).

Pathovars of *P. syringae* have been partitioned into genomospecies based on DNA-DNA hybridization (Gardan *et al.*, 1999). *Pseudomonas syringae* pv. *aptata* and pv. *syringae* are placed within genomospecies 1 phylogroup 2b which also includes *P. syringae* pv. *lapsa*, *P. syringae* pv. *papulans*, *P. syringae* pv. *atrofasciens*, and *P. syringae* pv. *pisi*. Genomospecies 1 pathovars are genetically distinct from *P. syringae* pv. *lachyrmans*, which falls within genomospecies 2, group C and phylogroup 3 (Bull and Koike, 2015).

Pseudomonas syringae pv. *lachrymans* has been one of the primary causes of bacterial diseases of cucurbits in western Washington in the United States, and infections result in angular leaf spots typical of bacteria (Pscheidt and Ocamb, 2008). However, in 2015 and 2016, marginal necrosis (Fig. 1A), often surrounded by chlorosis was observed on *Cucurbita pepo* 'Cinnamon Girl' leaves in a field research trial on biodegradable plastic mulch films for agriculture. Morphologically distinct lesions and raised warts also were observed on the pumpkin fruit in both years. Fruit lesions were round, with a necrotic spot located in the center of the lesion, giving a bull's-eye appearance (Fig. 1B). The warts that were observed appeared similar to those described by Sharrock *et al.* (1997), where the rind cracked near wart edges and either discoloration or a sunken dimple was observed at the center (Fig. 1C). Thus, the objectives of this study were to (i) characterize and identify the pathogen; (ii) confirm pathogenicity and (iii) determine cultivar susceptibility.

MATERIALS AND METHODS

Isolations from lesions on leaves and bull's-eye lesions and warts on fruit. Pumpkin foliage with chlorotic and necrotic lesions as well as fruit exhibiting erumpent warts and/or bull's-eye type lesions were collected from an experimental field trial at the Washington State University Northwestern Research & Extension Center near Mount Vernon, WA in summer 2015. Foliar samples were excised and placed into wax-lined sandwich paper bags. Two fruit lesions and warts were cored from fruit to a depth of approximately 1.5 cm with 1.4-1.6 mm diameter cork borers before placement into 6.5 cm × 8.2 cm plastic bags. Between successive samples, scissors and cork borers were surface disinfested with 70% ethanol until run-off, blotted with clean paper toweling, and air-dried. Tissue was transported to the laboratory. Leaves and fruit sections were surface-disinfested by dipping into 70% ethanol for 30 s, blotted with a paper toweling, and air-dried. Lesions and warts were excised and macerated in sterile, deionized water with a sterile mortar and pestle. Macerate was streaked onto a nutrient broth and yeast (NBY) agar medium (Becton, Dickinson and Company, US) and King's B agar medium (King *et al.*, 1954) then incubated at 27°C for two days. Isolates were stored in pure culture after streaking individual colonies three times on NBY agar, in 30% glycerol at −80°C for long-term storage.

Phenotypic characterization. Isolate PsAs_2015 was collected and isolated from 'Cinnamon Girl' pumpkin leaves with necrotic lesions in 2015, and PsFt1_2015 and PsFt2_2015 were collected and isolated from bull's-eye type lesions from fruit. Fluorescence was assessed on King's B medium. Further phenotypic characterization was assessed by determining the LOPAT profile (Lelliott *et al.*, 1966).

DNA extraction, PCR amplification and sequencing. All three isolates were grown in liquid culture by spiking NBY broth with a pure culture of the isolate. After shaking on a G10 rotary shaker (New Brunswick Scientific, US) at 150 rpm for 12 h at room temperature, genomic DNA was extracted from isolates according to the MoBio Ultraclean Microbial Kit protocol (Qiagen, US).

Initial identification of all three isolates was assessed by PCR amplification and sequencing of 16S ribosomal RNA (rRNA) using primers 27F (5'- AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-CGGTTACCTTGTTACGACTT-3') (Turner *et al.*, 1999; Lane, 1991). Reactions were performed using 20 µl reaction volumes in thin-walled, 0.2 ml PCR strip tubes. Reaction mixes were comprised of 2.0 µl of 10X PCR buffer (NEB, US), 0.6 mM $MgCl_2$, 30 µM dNTPs (ThermoFisher Scientific, US), 0.15 µM of each primer (Invitrogen Life Technologies, US), 1 unit of *Taq* polymerase (NEB), and 20 ng DNA. Cycling was performed in a Techne TC-3000X Thermal Cycler (Burlington, US). Cycles consisted of two min at 95°C, followed by 29 cycles of 30 s at 95°C, 30 s at 53°C, and two min at 72°C; with a final extension cycle of five min at 72°C. Amplicons were run on a 1% agarose gel to confirm amplification. PCR product cleanup was performed by adding 2.5 µl of Affymetrix Exosap-It (Thermo Fisher Scientific) to 8 µl of PCR product and incubating the mix at 37°C for 15 min followed by incubation at 80°C for 15 min. DNA sequencing was performed by Eurofins Genomics (Eurofins Operon, US). Basic Local Alignment Search Tool (BLAST) searches were done to identify the closest matches available in NCBI GenBank (Benson *et al.*, 2013).

A 752 bp fragment of the syringomycin B gene was amplified for PsAs_2015 using primers *syrB*-B1 (5'-CTTTCCGTGGTCTTGATGAGG-3') and *syrB*-B2 (5'-TCGATTTTGCCGTAATGAGTC-3') (Sorensen *et al.*, 1998) as an additional method to distinguish among *P. syringae* genomospecies 1 phylogroup 2 isolates and *P. syringae* pv. *lachrymans*. Pathovars of genomospecies 1

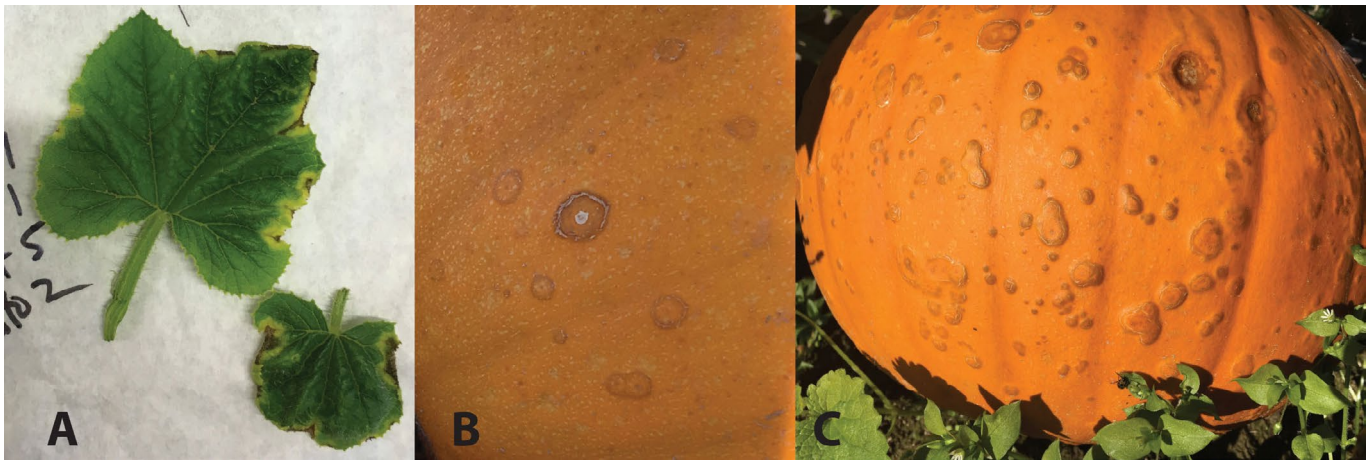

Fig. 1. Leaf lesions surrounded by chlorosis on *Cucurbita pepo* 'Cinnamon Girl' leaves (A) and bull's-eye lesions (B) and raised warts (C) on fruit. Photo (C): P. Morgan.

phylogroup 2 produce syringomycin whereas *P. syringae* pv. *lachrymans* does not (Young, 2010; Young and Triggs, 1994). Reactions were prepared as described above. Cycles consisted of five min at 95°C followed by 35 cycles of one min at 95°C, one min at 58°C, three min at 72°C; with a final extension cycle of 10 min at 72°C. PCR product was prepared for sequencing by Eurofins Genomics as described above to validate amplification of the *syrB* region.

A multilocus sequence analysis was carried out by amplifying and sequencing *gyrB* and *gltA* loci for PsAs_2015, PsFt1_2015, and PsFt2_2015 using previously described primers (Hwang *et al.*, 2005). Cycles consisted of two min at 95°C followed by 29 cycles of one min at 95°C, one min at 56°C, one min at 72°C, with a final extension cycle of 10 min at 72°C. For *gltA* amplification, the annealing temperature was adjusted to 57°C. Amplicons were run on a 1% agarose gel to confirm amplification. Sequences were aligned using the Clustal W package (EMBL-EGI, UK) with reference sequences of *P. syringae* pathovars identified by Dr. Carolee Bull, Department of Plant Pathology, The Pennsylvania State University, and obtained from the Plant Associated and Environmental Microbes Database (Table 1) (Almeida *et al.*, 2010). Sequences were trimmed to equal lengths using MacVector 15.1.5 (MacVector Inc., US). PsAs_2015 sequence data for *16S* (accession No. MF074184), *gltA* (MF074187), *gyrB* (MF074185), and *syrB* (MF074186) were deposited into GenBank.

Phylogenetic analysis. To assess the phylogenetic relationship of PsAs_2015, PsFt1_2015, and PsFt2_2015 to the reference PAMDB *P. syringae* pathovar isolates, a neighbor joining analysis was conducted in PHYLIP 3.695 (Felsenstein, 1993) for each locus amplified. jModelTest 2.1.7 (Posada, 2008) was used to determine the best evolutionary model for phylogenetic analysis. The number of substitution schemes was set at five, which used algorithms to test the appropriateness of the JC, HKY, TN, TPM1, and GTR models. The algorithms also were set to evaluate base frequencies (+F), the proportion of invariable sites (+I), and the rate of heterogeneity among sites (+G). The number of rate categories selected was set at four categories.

Seqboot, part of the PHYLIP 3.695 package, was used to estimate a phylogeny independently from *gltA* and *gyrB* sequences. Default settings were used, with the exception of the number of replicates (R), which was adjusted to 1000. The PHYLIP program dnadist was used to calculate the distance matrix for the neighbor-joining analysis. The F84 and F84+G models were selected for *gltA* and *gyrB* analyses, respectively. Transition/transversion ratios (T) were reset to 3.3909 for the *gltA* analysis and 4.6619 for *gyrB*. The gamma ratio was set at 1.56 for the *gyrB* analysis. The neighbor-joining analysis, using PHYLIP program neighbor, instructed the program to randomize the input order of the species using the jumble setting (J). The tree construction results are input-order dependent for many of the programs in PHYLIP (Felsenstein, 2008), so the jumble function was activated to ensure that the best tree found was independent of the input order. One thousand bootstrapped datasets were used to estimate support for the nodes of the phylogeny. A majority rule consensus tree was determined using the program consense. The consensus tree was viewed using FigTree v.1.2.2 (Rambaut *et al.*, 2008) and *P. fluorescens* was used to root both phylogenies. Trees were saved as PDF files and final tree editing was completed using Adobe Illustrator CS6 (San Jose, CA, US).

A Bayesian analysis for each of the two loci was conducted using MrBayes v.3.2.6 (Ronquist and Huelsenbeck, 2003). The SYM+I+G model was determined to be the best fit model of evolution using jModeltest v.2.1.7 (Posada, 2008) for both *gltA* and *gyrB* analyses. All default priors were used except nucleotide frequencies were fixed and the gamma shape parameters was set to 0.1, 50. *P. fluorescens* Pf-5 was designated as the outgroup. Analysis was conducted with a Markov chain Monte Carlo process with three heated chains simultaneously run with one cold chain. Two independent

Table 1. *Pseudomonas syringae* complex PAMDB reference isolates used for *gltA* and *gyrB* neighbor-joining and Bayesian analyses.

Pathovar	Isolate	Genomospecies[a]	Pathovar	Isolate	Genomospecies[a]
P. syringae			*P. syringae*		
pv. *aceris*	LMG2106^{PT}	1(2d)	pv. *persicae*	NCPPB2761^{PT}	3(1a)
pv. *actinidiae*	NCPPB3739^{PT}	3(1b)	pv. *photiniae*	NCPPB3688^{PT}	2(B)
pv. *aesculi*	NCPPB3681^{PT}	2(A)	pv. *pisi*	NCPPB2585^{PT}	1(2b)
pv. *antirrhini*	CFBP1620^{PT}	3(1a)	pv. *primulae*	LMG2252^{PT}	6(7a)
pv. *apii*	LMG2132^{PT}	3(1a)	pv. *rhaphiolepidis*	NCPPB3618^{PT}	2(A)
pv. *apii*	NCPPB1626^{PT}	3(1a)	pv. *ribicola*	LMG2276^{PT}	6(8)
pv. *aptata*	CFBP1617^{PT}	1(2b)	pv. *savastanoi*	LMG2209^{PT}	2(3)
pv. *atrofaciens*	LMG5095^{PT}	1(2b)	pv. *sesami*	LMG2289^{PT}	2(B)
pv. *atropurpurea*	LMG5030^{PT}	4(4)	pv. *solidagae*	ICMP16925^{PT}	1(2d)
pv. *berberidis*	CFBP1727^{PT}	3(1a)	pv. *spinaceae*	ICMP16929^{PT}	3(1a)
pv. *broussonetae*	ICMP13650^{PT}	2(B)	pv. *syringae*	LMG1247^{PT}	1(2b)
pv. *cerasicola*	CFBP6109^{PT}	2(B)	pv. *tabaci*	NCPPB1427^{PT}	2(A)
pv. *ciccaronei*	LMG5541^{PT}	2(A)	pv. *tagetis*	LMG5090^{PT}	7(6)
pv. *coronafaciens*	NCPPB600^{PT}	4(4)	pv. *theae*	LMG5092^{PT}	3(1b)
pv. *coryli*	NCPPB4273^{PT}	1(2b)	pv. *tomato*	CFBP2212^{PT}	3(1a)
pv. *cunninghamiae*	ICMP11894^{PT}	2(A)	pv. *ulmi*	LMG2349^{PT}	2(A)
pv. *daphniphylli*	NCPPB3617^{PT}	2(C)	pv. *viburni*	LMG2351^{PT}	3(1b)
pv. *delphinii*	LMG5381^{PT}	3(1b)	pv. *zizaniae*	NCPPB3690^{PT}	4(4)
pv. *dysoxyli*	LMG5062^{PT}	1(2a)			
pv. *eriobotryae*	LMG2184^{PT}	2(A)	*P. avellanae*	LMG3487^{T}	3(1b)
pv. *garcae*	LMG5064^{PT}	4(4)			
pv. *helianthi*	LMG5067^{PT}	7(6)	*P. cannabina*	CFBP2341^{T}	9(5)
pv. *hibisci*	NCPPB3682^{PT}	2(A)	pv. *alisalensis*	CFBP6866^{PT}	9(5)
pv. *lachrymans*	CFBP6463^{PT}	2(C)	pv. *coriandricola*	ICMP12471^{PT}	9(5)
pv. *lapsa*	LMG2206^{PT}	1(2b)	pv. *philadelphi*	NCPPB3257^{PT}	9(5)
pv. *maculicola*	CFBP1657^{PT}	3(1a)			
pv. *mellea*	LMG5072^{PT}	2(B)	*P. savastanoi*		
pv. *mori*	LMG5074^{PT}	2(B)	pv. *glycinea*	LMG5066^{PT}	2(B)
pv. *myricae*	LMG5668^{PT}	2(A)	pv. *phaseolicola*	LMG2245^{PT}	2(B)
pv. *oryzae*	LMG10912^{PT}	4(4)			
pv. *papulans*	LMG5076^{PT}	1(2a)	*P. fluorescens*	Pf-5	
pv. *passiflorae*	LMG5185^{PT}	3(1b)			

[a]Genomospecies defined by Gardan *et al.* (1999). Phylogroups, in parentheses, defined by Hwang *et al.* (2005) and shown by Bull and Koike (2015).
^{T}indicates type species.
^{PT}indicates pathotype.

runs were conducted and stationarity was assessed in Microsoft Excel for Mac v. 15.26 (Redmond, US) with a scatterplot of likelihood plotted by generation. All trees prior to the burnin fraction of 0.25 were discarded. Remaining trees were summarized to develop a 50% majority rule consensus tree and posterior probabilities were estimated. The consensus tree was viewed using FigTree v.1.2.2 (Rambaut *et al.*, 2008). Trees were saved as PDF files and final tree editing was completed using Adobe Illustrator CS6.

A partition homogeneity test was run using PAUP* v.4.0a152 for Mac (Swofford, 2003) to determine if the tree topologies from the previous analyses were congruent by using two partitions: 528 nucleotides from the *gltA* locus and 506 nucleotides from the *gyrB* locus. The topologies were significantly different from each other ($P=0.01$); however, since the three study isolates clustered within the same clade in both trees (data not shown), sequences were concatenated and an additional Bayesian analysis was run, with two partitions, using the SYM+I+G model of evolution.

Pathogenicity of isolates. Pathogenicity assays with PsAs_2015, PsFt1_2015, and PsFt2_2015 were conducted on 'Cinnamon Girl' seedlings. The isolates were grown in liquid culture by spiking NBY broth with pure cultures of each bacterium. The cultures were shaken on a rotary shaker at 150 rpm at room temperature for 12 h. Following, cells were extracted from NBY broth by centrifugation at 8,000 rpm for five min, then washed two times in sterile deionized water by vortexing until resuspended in the buffer, and finally collected by centrifugation again at 8000 rpm for five min. Washed cells were suspended in sterile deionized water, the inoculum concentration was assessed by spectrophotometry, and the concentration adjusted to an A_{620} of 0.3 or approximately 10^8 CFU/ml.

To insure pathogen-free 'Cinnamon Girl' plants, seeds were treated by soaking in 50°C water for 20 min and then dried in a laminar flow hood. Seeds then were planted into Sunshine Redi Earth Plug and Seed Mix (Sun Gro Horticulture, Agawam, MA) in a greenhouse at 72°C. Five two-week old pumpkin seedlings were either spray inoculated with a bacterial isolate or sterile deionized water, which

served as a negative control, until runoff. Seedlings were maintained in a humidity chamber for six days and evaluated for disease every two days.

Symptomatic tissue from seedlings was photographed, excised, and macerated in sterile deionized water. Macerate was plated onto NBY medium and identified based on colony morphology on NBY and growth on King's B medium. Reisolated bacteria were put into pure culture as described above. DNA was extracted from pure cultures and the *gyrB* locus was amplified and sequenced as described above. Clustal W was used to align recovered isolate sequences with that of PsAs_2015 to validate recovery.

Koch's postulates and cultivar susceptibility. Pathogenicity assays were conducted on plants of 11 pumpkin and squash as well as fruit using PsAs_2015. These cultivars are sometimes planted in western Washington. Six pumpkin cultivars (Baby Bear, Baby Boo, Cinnamon Girl, Jack Be Little, Orange Cutie, and Lil' Pump-Ke-Mon) and five squash cultivars (Acorn, Honeyboat, Kabocha, Spaghetti, and Sweet Dumpling) were evaluated. Isolate PsAs_2015 was selected as the representative isolate, based on identical phenotypic and sequence data, to fulfill Koch's postulates and determine cucurbit cultivar susceptibility. Isolate PsAs_2015 was inoculated onto both seedlings and fruit. Inoculum was prepared as described above except cells were washed and suspended in phosphate buffered saline (PBS) (10 mM PO_4^{-3}, 137 mM NaCl, 2.7 mM KCl). Pumpkin seeds were treated by submerging seeds in 1% HCl for 20 min. Seeds then were dried in a laminar flow hood and planted into Sunshine Redi Earth Plug and Seed Mix. After 11 days, seedlings were transplanted into Sunshine Mix #1 and when seedlings were 17 day old, plants were either spray inoculated with PsAs_2015 inoculum or sterile PBS only. Plants were placed inside a humidity chamber and arranged in an RCBD with three replications and one plant per replication. Plants were evaluated for disease symptoms every two days for 6 days. The trial was repeated.

Field-grown pumpkin and squash fruit of the 11 selected cultivars were harvested in October 2016 and stored in a pole barn at ambient temperatures for one month prior to inoculation. PsAs_2015 inoculum was prepared as described above. Fruit were rinsed in deionized water to wash off any field soil, and submerged in a 1% sodium hypochlorite solution for 2 min. Following, fruit were rinsed three times in sterile deionized water and air dried in a laminar flow hood. Fruit were each submerged in either PsAs_2015 inoculum or sterile PBS for 20 min before arranging in clean crates lined with paper toweling so that fruit were not touching each other. Fruit were arranged in an RCBD with three replications and one fruit per cultivar per replication. Crates were loosely sealed in plastic bags and stored in the dark for 20 days. Due to limitations in fruit availability, only one trial was conducted.

Symptomatic tissue from inoculated seedlings and fruit was photographed, excised, surface-disinfested in 70% ethanol for 30 s, and macerated in sterile deionized water. One to two lesions per seedling and between and four to six lesions per fruit were sampled. Since bull's-eye type lesions were not observed on 'Acorn', 'Kabocha', and 'Orange Cutie' cucurbits, tissue without lesions, or appearing discolored, or surrounding calluses that were a result of being grown in the field, were collected instead to assess if *Pseudomonas* could be recovered. Macerate was plated onto NBY medium. Reisolated bacteria were put into pure culture and Koch's postulates were fulfilled for both seedlings and fruit as described above.

On fruit, disease incidence was calculated as the number of observed bull's-eye type lesions. Since the assumption of normality for this data set could not be met, data were analyzed with a Kruskal-Wallis test using Proc npar-1way in SAS University (SAS Institute, Cary, NC, US). Pairwise comparisons were conducted by a Wilcoxson rank sum test also using PROC NPAR1WAY. Pearson's r correlation was estimated to determine the degree of association between lesions on leaves and percent *P. syringae* genomospecies 1 reisolated from bull's-eye type lesions.

RESULTS

Phenotypic characterization. None of the three Washington isolates collected in 2015 fluoresced on King's medium B. Isolates were all positive for the Levan test, negative for the oxidase test, negative for the potato slice test, negative for arginine dihydrolase test, and positive for causing a hypersensitive response on tobacco. Results of the LOPAT scheme are consistent with the identification of the species as *P. syringae*.

Multilocus sequence analysis and genotypic characterization. 16S rRNA sequence data of PsAs_2015, PsFt1_2015, and PsFt2_2015 were 99% identical to that of *P. syringae* pv. *syringae* (JQ071937). Phylogenies estimated from neighbor joining *gltA* and *gyrB* sequence analyses clustered PsAs_2015, PsFt1_2015, and PsFt2_2015 with *P. syringae* pv. *syringae* PT LMG 1247 within the genomospecies 1 phylogroup 2b clade, and provided significant branch support (data not shown). Phylogenies estimated from the Bayesian analyses of *gltA*, *gyrB* (data not shown), and concatenated sequences (Fig. 2) also clustered the three isolates within the genomospecies 1 phylogroup 2b clade with a significant branch support of 100% (posterior probability). The *syrB* primers successfully amplified a 752 bp fragment of *syrB* gene indicating that PsAs_2015 is capable of producing syringomycin, a characteristic of pathovars within genomospecies 1. Sequences were 100% identical to *P. syringae* pv. *syringae* (CP006256.1). Based on these findings, isolate PsAs_2015, PsFt1_2015, and PsFt2_2015 were identified as *P. syringae* genomospecies 1 phylogroup 2b.

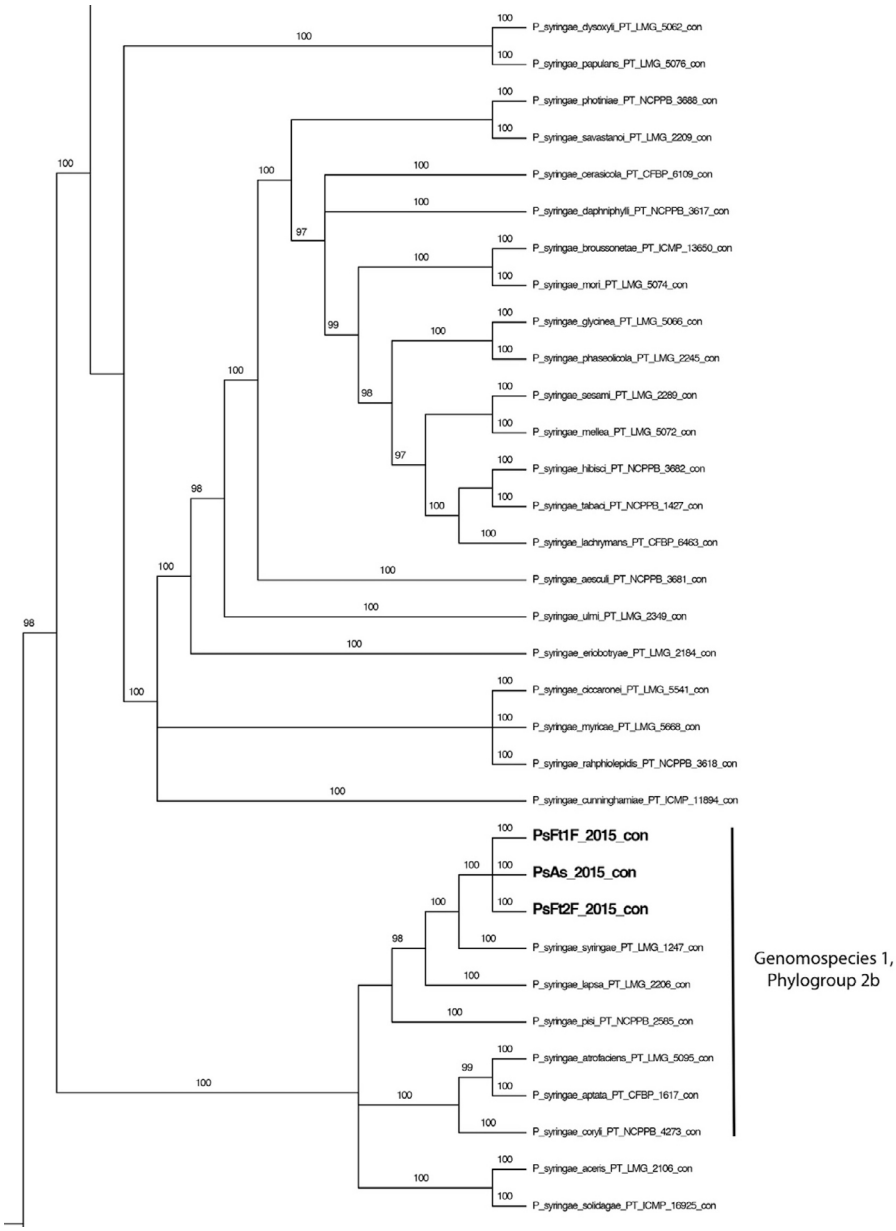

Fig. 2. Phylogenetic tree generated from Bayesian analysis of concatenated *gltA* and *gyrB* sequence data from three *P. syringae* isolates collected from Mount Vernon, WA in 2015 (in bold) and 58 *P. syringae* pathovar PAMDB reference isolates (Almeida *et al*., 2010). The tree has been reduced to show the genomospecies 1 phylogroup 2 and the four next closest clades. Node support values indicate Bayesian posterior probability (> 95%).

Pathogencity of isolates. PsAs_2015, PsFt1_2015 and PsFt2_2015 all caused symptoms on inoculated 'Cinnamon Girl' pumpkin seedlings. Small, diffuse necrotic lesions each surrounded by yellow halos were observed, consistent with the symptoms observed in the original field grown pumpkin plants. *P. syringae* colonies were re-isolated from macerated seedling tissue and identities confirmed on NBY and growth on King's B medium based on colony morphology. No lesions were observed on plants inoculated with water.

Determination of host range and Koch's postulates. Plant inoculations with PsAs_2015 resulted in leaf symptoms ranged from necrotic lesions, each surrounded by a chlorotic halo, to marginal necrosis, to watersoaking at the leaf margins. Lesions were observed on at least one seedling of all cultivars (Table 2) and five cultivars (Baby Bear, Baby Boo, Cinnamon Girl, Lil' Pump-Ke-Mon, and Spaghetti) had 100% seedling disease incidence. The squash cultivar, Honeyboat, had the lowest disease incidence at 33%.

Symptoms on fruit resulted in a lesion that had a bull's-eye appearance (Fig. 1B), where a center lesion was surrounded by concentric rings of necrosis. On some cultivars, such as 'Lil Pump-Ke-Mon', lesions were dark and sunken. Only 33% of 'Baby Bear' and 'Baby Boo' inoculated fruits exhibited symptoms while 0% of 'Acorn', 'Kabocha', and 'Orange Cutie' showed symptoms (Table 2).

Table 2. Number of seedlings and fruits observed, by cultivar, with lesions after inoculation with *Pseudomonas syringae* genomospecies 1 isolate PsAs_2015 and percent seedlings and fruit, by cultivar, on which *P. syringae* colonies with identical *gyr*B sequences to *P. syringae* genomospecies 1 isolate, PsAs_2015 were recovered during this study.

Host cucurbit	% Seedlings with lesions[a]	% Seedlings with *Ps*[b] recovered	% Fruit with lesions[a]	% Fruit with *Ps*[b] recovered
Pumpkin cultivar				
Baby Bear	100 (100, 100)[c]	100 (100, 100)	33	33
Baby Boo	100 (100, 100)	67 (33, 100)	33	33
Cinnamon Girl[d]	100 (100, 100)	83 (100, 67)	100	50
Jack Be Little	83 (67, 100)	67 (33, 100)	100	67
Lil' Pump-Ke-Mon	100 (100, 100)	67 (67, 67)	100	100
Orange Cutie[e]	60 (100, 33)	60 (50, 67)	0	30
Squash cultivar				
Acorn	67 (67, 67)	83 (67, 100)	0	67
Honeyboat[f]	33	33	100	33
Kabocha	67 (67, 67)	67 (33, 100)	0	100
Spaghetti	100 (100, 100)	87 (67, 100)	100	100
Sweet Dumpling[f]	100	67	100	100

[a] A total of six seedlings or three fruit were inoculated as described in the main text.
[b] *Ps* defined as *Pseudomonas syringae* genomospecies 1 phylogroup 2b isolate PsAs_2015.
[c] Results separated by trial (Trial 1, Trial 2).
[d] Only two fruits were inoculated due to limitations in availability.
[e] Only five seedlings were inoculated due to limitations in availability.
[f] Only three seedlings were inoculated due to limitations in availability.

Overall, significant differences in disease incidence on fruit were observed among cultivars ($P = 0.0089$) (Fig. 3). Although not significant at $P < 0.05$, fruit disease incidence was greater on 'Cinnamon Girl' fruit than on fruit of 'Acorn', 'Honeyboat', 'Kabocha', and 'Orange Cutie' ($P = 0.053$) (Fig. 3).

To fulfill Koch's postulates, *gyrB* sequence data of re-isolates from inoculated seedlings and fruit were compared to the *gyrB* sequence of PsAs_2015. Percent seedlings and fruit from sequences of recovered isolates were identical to PsAs_2015 and ranged from 33 to 100%. Percent seedlings of cultivars with the least *Pseudomonas* recovery on seedlings included 'Honeyboat' (33%) and 'Orange Cutie' (60%). *Pseudomonas syringae* was not recovered from any seedlings inoculated with PBS. On fruit, cultivars with the lowest *Pseudomonas* recovery were 'Baby Bear' (33%), 'Baby Boo' (33%), 'Honeyboat' (33%), and 'Orange Cutie' (30%) (Table 2). No significant correlation was observed between number of seedlings with lesions and percent *Pseudomonas syringae* genomospecies 1 isolate recovery ($r = 0.02$, $P = 0.89$).

DISCUSSION

Infection of pumpkin and other cucurbit crops by *P. syringae* pv. *lachrymans*, a genomospecies 2 pathovar, has been well documented in the United States since the early 1900s and only recently have reports of *P. syringae* pv. *syringae*, a genomospecies 1 pathovar, infecting cucurbits emerged. While identification of *P. syringae* isolates down to the genomospecies group level is possible with molecular methods (Bull *et al.*, 2011), pathovar designations must follow established standards, which includes differentiation among pathogenicity profiles (Dye *et al.*, 1980; Newberry *et al.*, 2016). The clustering of the three study isolates collected from Mount Vernon, WA with the PAMDB genomospecies 1 reference pathovar isolates, along with the high branch support data, confirm that these isolates are distinct from *P. syringae* pv. *lachrymans*, which is commonly found in United States' cucurbit fields. Moreover, molecular data show that the three isolates studied here are genetically identical and that the isolate PsAs_2015, collected from lesions on leaves, caused lesions when inoculated onto fruit, and vice versa. This is a first report for Washington State and the presence of wart symptoms on pumpkin fruit represents a newly reported symptom. Until the pathovar assignment of isolates PsAs_2015, PsFt1_2015, and PsFt2_2015 can be determined, identification of this bacterium must be restricted to its genomospecies designation. Pathogenicity assays to determine isolates to pathovar are currently underway on hosts of other common genomospecies 1 phylogroup 2b pathovars, including *Zea mays* cvs Jubilee and Golden Beauty, *Pisum sativum* cv. Kelvedon Wonder, *Syringae* cv. Mme Lemoine, *Beta vulgaris* cv. Red Ace, and *Triticum aestivum* cv. Norwest 553. Results will be published in a future manuscript.

Cucurbit growers in western Washington and elsewhere typically grow a number of different pumpkin and winter squash cultivars in close proximity. Therefore, investigation of cucurbit host range is critical, particularly since the epidemiology of the pathogen presently is unknown. Overall, pumpkin and squash fruit were less susceptible to infection than seedlings. This result is not unexpected since contaminated seeds can be a primary inoculum source for *P. syringae* (Agarwal and Sinclair, 1996; Lamichhane

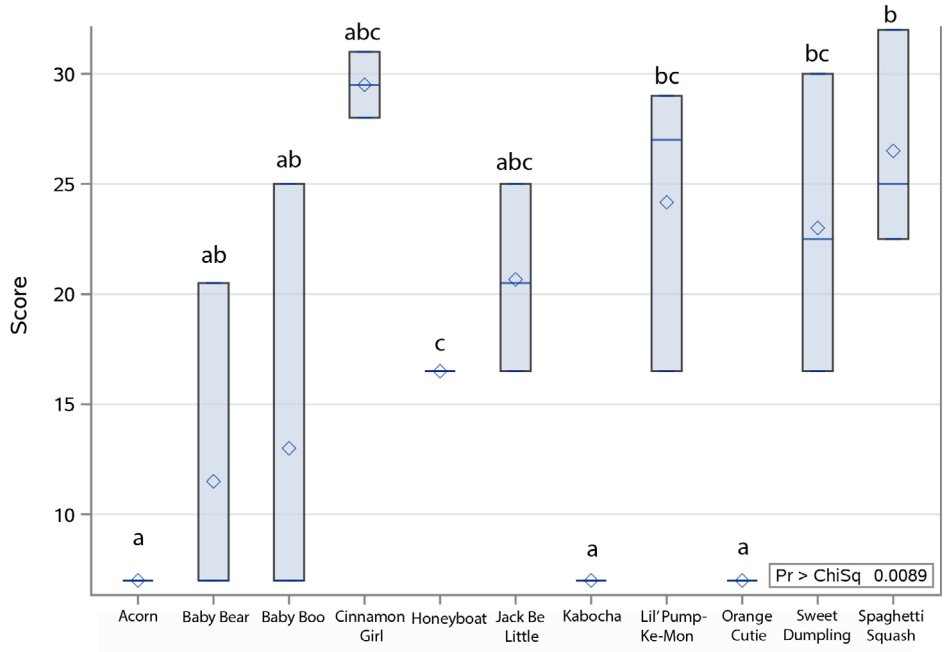

Fig. 3. Distribution of the Wilcoxson scores for disease incidence of 11 cucurbit fruit cultivars inoculated with *P. syringae* genomospecies 1 isolate, PsAs_2015. Disease incidence was calculated as the number of lesions per three inoculated fruit per cultivar. Values with the same letter are not significantly different at $P = 0.05$.

et al., 2015; Refshauge *et al.*, 2010) and the tender tissue of seedlings is ideal for bacterial penetration, infection, and colonization.

The procedure used for fruit inoculation in this study was as described in Sharrock *et al.* (1997) where warts were observed on the inoculated fruit of *C. maxima* after 11 days. In this study, although lesions that were consistent with early infection were observed on inoculated fruit, no warts were observed. One possible hypothesis explaining the lack of warts may be due to the increased storage time of the cucurbit fruit in this study prior to inoculation (one month post harvest) versus the time before inoculation used by Sharrock *et al.* (1997) (one week postharvest). The longer time in storage of the Mount Vernon fruit provided the opportunity for fruit curing, which makes rinds harder and potentially less susceptible to infection (Pessarakli, 2016). This observation also suggests that timing of fruit infection may be important in development of symptoms, and fruit disease incidence and severity. Rind characteristics may be a factor in fruit resistance to infection as well. No obvious lesions were observed on 'Acorn', 'Kabocha', and 'Orange Cutie' fruit. The rinds of these three cultivars differ from those of the other inoculated cultivars in that they are waxy and thick, potentially inhibiting initial penetration into the fruit and epiphytic colonization. Although there are no studies examining the effects of rind texture on bacterial epiphytic colonization, a previous study observed that extra glossy leaf cuticles of mutant maize plants discouraged epiphytic bacterial colonization (Lindow and Brandl, 2003).

Isolates of *P. syringae* genomospecies 1 were recovered from all cultivars of inoculated cucurbit seedlings and recovery was lowest from 'Honeyboat' even though the number of seedlings with lesions did not differ from that of other cultivars. The low incidence from 'Honeyboat' may be due to only three seedlings being inoculated in this study due to lack of seed and therefore more inoculations should be carried out in the future. No significant correlation was found between number of seedlings with lesions and percent of seedlings in which *P. syringae* genomospecies 1 was reisolated from lesions. While the high humidity and warm temperatures at which the seedlings were maintained post inoculation were conducive to *P. syringae* infection, these conditions may have been additionally optimal for a number other epiphytic and endophytic bacteria which colonize leaves. Some other genera isolated from lesions were *Pantoea* spp. and *Bacillu*s spp. (data not shown), although they were in such low frequency that Koch's postulates were not attempted for isolates of these bacteria.

Pseudomonas syringae genomospecies 1 were recovered from all cultivars of inoculated cucurbit fruit even though disease symptoms were not observed post-storage on 'Acorn', 'Kabocha', or 'Orange Cutie' fruit. Samples were taken from areas of fruit with rind discoloration or calluses, but lacking the obvious bull's-eye symptoms or sunken cankers observed on other cultivars. The waxy skin textures and dark or mottled coloration of the rinds of these three cultivars may lead to different symptom characteristics or make it more difficult to detect lesions than on cultivars with smoother, thinner skins such as

"Spaghetti' squash or 'Cinnamon Girl' pumpkins. Thus, further inoculation studies need to be carried out to fully assess infection by isolates of *P. syringae*, genomospecies 1 and symptomology on different species and cultivars of cucurbits.

A recent study by Newberry *et al.* (2016) assessed the genetic diversity of *P. syringae* strains causing angular leaf spot on watermelon from California, Georgia, and Florida; on cantaloupe from Florida; and on squash from California and Georgia. A phylogenetic analysis showed that all clustered within three clades of genomospecies 1 phylogroup 2. Isolates collected from Georgia and California squash clustered within phylogroup 2a clade while most isolates collected from Florida and Georgia watermelon clustered within phylogroup 2b clade. A comparison of these results with those in this study indicate that the three isolates collected from pumpkin in Washington are more closely related to isolates collected from Florida and Georgia watermelon than to the squash isolates.

While contaminated seed can be the primary inoculum source of *P. syringae* in the field, little else is known about the epidemiology of *P. syringae* genomospecies 1 phylogroup 2b on cucurbits. A number of pathovars within this genomospecies 1 group are generalists and infect host plants ranging from lilac to beans, all of which are grown nearby to cucurbit production fields in western Washington and elsewhere. Hosts of other *P. syringae* genomospecies I pathovars, including beet (pv. *aptata*), pea (pv. *pisi*), wheat and corn (pv. *atrofasciens* and pv. *lapsa*), also are grown in the region as in other cucurbit growing areas and additional pathogenicity assays are needed to identify the three study isolates to the pathovar level. Nevertheless, the results of this study were able to confirm that the bacterium causing necrotic lesions on leaves are genetically identical to those causing bull's-eye-type lesions and warts on fruit, a symptom which has not been previously described in the United States. Furthermore, the isolate PsAs_2015 not only caused necrotic leaf lesions on a number of pumpkin and squash cultivars, but also on fruit, which can have implications for cucurbit growers who produce a number of different cultivars at the same time and in close proximity to each other. Lastly, the genetic similarity of the western Washington pumpkin isolates with Florida and Georgia watermelon isolates rather than to California squash isolates indicates that the geographic range of this pathogen may be widespread and further studies will be helpful in more fully assessing the distribution.

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