Research

Bacteria Associated with Onion Foliage in Michigan and Their Copper Sensitivity

Kim E. Tho,^{1,2} Elizabeth Brisco-McCann,¹ Prissana Wiriyajitsomboon,^{1,3} George W. Sundin,¹ and Mary K. Hausbeck^{1,†}

¹ Department of Plant, Soil and Microbial Sciences, Michigan State University, East Lansing, MI 48824, U.S.A.

² Division of Research and Extension, Royal University of Agriculture, Phnom Penh 12401, Cambodia

³ Department of Microbiology, Faculty of Science, Kasetsart University, Bangkok 10900, Thailand

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Abstract

Bacterial stalk and leaf necrosis of onion, *Allium cepa* L., is a concern of Michigan producers. Plants with symptoms of bacterial stalk and leaf necrosis were collected in 2013 and 2014 from 17 fields in six Michigan counties. Bacterial isolates were identified using Biolog and confirmed by sequencing the 16s rDNA gene. From a total of 414 isolates, 10 bacterial species were identified. *Pantoea agglomerans* (42.5%), *Pantoea ananatis* (17.4%), and *Enterobacter cowanii* (7.5%) were most prevalent and were tested for pathogenicity on onion foliage and bulbs. More *P. ananatis* isolates (92%) were pathogenic on seedlings than *P. agglomerans* or *E. cowanii* isolates (approximately 50%). When 197 bacterial

isolates were tested for sensitivity to copper hydroxide (200 µg/ml), 41% of *P. agglomerans* isolates were found to be tolerant to copper hydroxide, whereas 19 and 22% of *P. ananatis* and *E. cowanii* isolates were tolerant, respectively. Identifying the bacterial species associated with foliar disease symptoms on onions in Michigan, their pathogenicity and tolerance to copper is an important step in developing improved disease management strategies.

Keywords: onions, bacteria, bacterial stalk and leaf necrosis, copper resistance

Bacterial pathogens incite disease on onion (*Allium cepa* L.) foliage and bulbs (Mark et al. 2002; Schwartz and Mohan 2008) with annual losses of 20 to 100%, depending on the pathogen species, cultivar, growing region, and environmental conditions (Schwartz and Mohan 2008). Symptoms of bacterial infection on onion foliage include blight, necrotic tips, streaks, water-soaked lesions, soft rot, bleached tissue, and occasionally plant collapse (Mark et al. 2002). Bulb rot in both the field and storage has caused significant economic losses for growers in Michigan (Hausbeck 2005) and in other U.S. production areas (Dutta et al. 2014). Bacterial diseases include *Xanthomonas* leaf blight (*Xanthomonas axonopodis* pv. *allii* [Dowson] Roumagnac et al. [syn. *Xanthomonas campestris* pv. *allii* (Kadota et al.) Roumagnac et al.]); bacterial leaf streak and bulb rot (*Pseudomonas viridiflava* [Burkholder] Dowson), bacterial soft rot (*Pectobacterium carotovorum* subsp. *carotovorum* [Jones] Waldee emend. Hauben et al., *Dickeya chrysanthemi* [Burkholder et al.] Samson et al. [syn. *Erwinia chrysanthemi* Burkholder et al.], *Pseudomonas marginalis* [Brown] Stevens, *Enterobacter rhapontici* [Millard] Burkholder), bacterial stalk and leaf necrosis (*Pantoea agglomerans* [Beijerinck] Gavini

et al. [syn. *Erwinia herbicola* (Löhnis) Dye; *E. agglomerans* Beijerinck]), center rot (*Pantoea ananatis* [Serrano] Mergaert et al. [syn. *Erwinia ananatis* Serrano]), slippery skin (*Burkholderia gladioli* pv. *alliicola* [Burkholder] Young et al. [syn. *Pseudomonas gladioli* pv. *alliicola* (Burkholder) Young et al.]), sour skin (*Burkholderia cepacia* [Palleroni and Holmes] Yabuuchi et al. [syn. *Pseudomonas cepacia* (ex Burkholder) Palleroni and Holmes]), and Enterobacter bulb decay (*Enterobacter cloacae* [Jordan] Hormaeche and Edwards) (Schwartz et al. 2015).

Bacterial pathogens causing disease in onion foliage and leaves vary in specific regions. For example, *Pantoea ananatis* (Gitaitis and Gay 1997), *P. agglomerans* (Edens et al. 2006), and *B. cepacia* (sour skin) (Dutta et al. 2015) have been reported in Georgia, where *Ps. viridiflava* (Guillebeau 2003) and *Ps. coronafaciens* sp. nov. (yellow bud disease) (Dutta et al. 2018; Gitaitis et al. 2012) are also significant onion pathogens. Major onion bacterial pathogens reported in Pennsylvania also include *P. agglomerans* and *P. ananatis*, as well as *Pe. carotovorum*, *Ps. marginalis*, and *B. gladioli*, with *P. agglomerans* among the most prevalent (Gugino 2016; Pfeufer et al. 2011). In New York, at least four bacterial species were associated with diseased onions, including *P. ananatis* (Carr et al. 2010), *Burkholderia* spp., *E. cloacae*, and *Rahnella* spp. (Beer et al. 2014). In Ontario, Canada, bacterial diseases in onions were reported to be slippery skin and sour skin, caused by *Burkholderia* spp. (syn. *Pseudomonas* spp.), and soft rot, caused by *Erwinia* sp. (syn. *Pectobacterium*) (Howard et al. 1994; OMAFRA 2009). *E. cloacae*, *B. cepacia*, and *B. gladioli* are significant pathogens causing bulb rot during storage in Washington (Schroeder and du Toit 2010; Schroeder et al. 2010a, 2010b). *B. cepacia*, *B. gladioli*, *Pe. carotovorum*, and *Xanthomonas axonopodis* pv. *allii* are all important pathogens in Colorado (Schwartz

[†]Corresponding author: M. K. Hausbeck; E-mail: hausbec1@msu.edu

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and Bartolo 1995). *P. ananatis* and *P. agglomerans* are also common in areas where temperature and relative humidity are high (Schwartz and Mohan 2008) and have both been reported to be associated with the insect vectors *Frankliniella fusca* (Dutta et al. 2016) and *Thrips tabaci* (Grode et al. 2019). In addition, the incidence of disease caused by *P. ananatis* can reach up to 70% in regions when plants are damaged by rainfall and hail (Schwartz and Otto 2000), although the rate of infection has been shown to vary, depending on the growth stage at the time of infection (Stumpf et al. 2017).

In Michigan, onions are grown on muck soils in the western and south-central regions of the state. In 2015, the onion crop yield in Michigan was approximately 40,000 tons valued at over \$10 million (USDA NASS 2018), consisting largely of long-day types that are pungent and suitable for storage (Schwartz 2013). In Michigan, *P. agglomerans* (Grode et al. 2019; Tho et al. 2015) and *P. ananatis* (Wiriyajitsomboon et al. 2014) are significant foliar pathogens, whereas *B. cepacia* complex was isolated from onion bulbs (Jacobs et al. 2008). *P. agglomerans* is widely distributed in nature and can be isolated from plant, water, soil, and animal specimens (Delétoile et al. 2009). *P. agglomerans* was first reported as a pathogen of onions in South Africa in 1981 (Hattingh and Walters 1981) but was not considered an onion pathogen in the United States until 2006, when it was reported in Georgia (Edens et al. 2006). Primary inoculum for *P. ananatis* can include weed species (Dutta et al. 2017; Gitaitis et al. 2002), insects (Gitaitis et al. 2003), and infested seed including onion (Walcott et al. 2002), sudangrass (Azad et al. 2000), and rice (Cother et al. 2004). *B. cepacia* (formerly *Ps. cepacia*) was first described as an onion pathogen causing sour skin and soft rot in New York in 1950 (Burkholder 1950). Ecological niches of *B. cepacia* include plant debris, soil, irrigation water, and volunteer plants (Ramette et al. 2005), although it is also known as a plant and human pathogen and has been used as biological control strains and biodegradation strains (Baldwin et al. 2005). *B. gladioli* pv. *alliiicola* primarily affects stored onion bulbs damaged before or during harvest (Schwartz and Mohan 2008). Bacterial soft rot pathogens, such as *Pe. carotovorum* subsp. *carotovorum*, are found in and disseminated

via irrigation water and can survive in contaminated soil and infested crop debris (Schwartz and Mohan 2008).

Owing to the prevalence of bacterial foliar diseases, Michigan growers are interested in applying copper-based products to their onion crops, despite reports indicating a lack of efficacy (Grode et al. 2019) and copper-tolerant *P. ananatis* strains (Nischwitz et al. 2007). Our objectives were to identify the bacterial species associated with onion foliar blight in Michigan, evaluate their pathogenicity on seedlings and bulbs, and determine their sensitivity to copper.

Isolate Collection and Identification

A total of 1,080 onion plants displaying foliar bacterial symptoms were sampled from production fields in six Michigan counties (Allegan, Ottawa, Ingham, Newaygo, Eaton, and Calhoun) every 2 weeks beginning when plants were at the two-leaf stage (June) through bulb enlargement (August) in 2013 and 2014 (Table 1). Fourteen yellow and two red onion cultivars grown in 17 different fields were included in the study. Each sampled field ranged in size from 2.0 to 5.0 ha. Symptomatic plants were collected, placed singly in individual plastic bags, labeled, and placed in a cooler with ice for transport to the laboratory for isolation. Each plant sample was rinsed under running tap water, placed into aluminum containers, and kept in a cold room (at 5.0 to 7.0°C) for 2 to 5 days until it was processed.

Onion extract medium (OEM) (Zaid et al. 2012) was prepared with chopped yellow onion bulbs (333 g) that were autoclaved in 500 ml of distilled water for 35 min at 121°C, cooled to room temperature, and filtered. The following ingredients were added to the filtered solution: 5.0 g of NaCl, 1 g of K₂HPO₄ (anhydrous), 3.8 g of KH₂PO₄ (anhydrous), 2.5 ml of crystal violet stock solution (75 mg/100 ml), 15.0 g of agar, and 250 mg of cycloheximide. The volume was then adjusted to 1.0 liter using distilled water and sterilized.

Approximately 1.0 cm² of symptomatic leaf tissue from each sample was surface sterilized with 70% ethanol for 30 s, rinsed twice in sterile distilled water, blotted dry on sterile tissue paper, and then crushed in a micro-Eppendorf tube containing 0.1 ml of sterile

TABLE 1
Total number and identity of bacterial species isolated from symptomatic onion foliar tissue sampled from six counties in Michigan in 2013 and 2014

Species	Counties												Total (%)
	Allegan		Ottawa		Ingham		Newaygo		Eaton		Calhoun		
	2013	2014	2013	2014	2013	2014	2013	2014	2013	2014	2013	2014	
<i>Pantoea agglomerans</i> ^y	21	20	7	26	1	26	3	27	1	11	6	27	176 (42.4) ^z
<i>Pantoea ananatis</i>	1	22	0	13	2	10	0	8	0	8	0	19	83 (20.0)
<i>Enterobacter cowanii</i>	3	5	0	2	1	1	0	3	1	4	3	8	31 (7.5)
<i>Pseudomonas fluorescens</i>	1	2	1	1	0	1	0	4	0	2	0	3	15 (3.6)
<i>Pantoea dispersa</i>	1	3	0	1	0	0	0	1	0	2	0	2	10 (2.4)
<i>Burkholderia ambifaria</i>	0	0	0	0	0	0	0	0	0	0	1	2	3 (0.7)
<i>Enterobacter cloacae</i>	0	0	0	0	0	0	0	2	0	0	0	0	2 (0.5)
<i>Rahnella aquatilis</i>	0	0	0	0	0	2	0	0	0	0	0	0	2 (0.5)
<i>Dickeya chrysanthemi</i>	0	0	0	0	0	0	0	0	0	1	0	1	2 (0.5)
<i>Burkholderia cepacia</i>	0	0	0	0	1	0	0	0	0	0	0	0	1 (0.2)
Other species	0	0	0	1	0	0	0	2	0	1	1	0	5 (1.2)
No identification	3	17	0	16	4	10	0	15	0	4	0	16	85 (20.5)
Total	30	69	8	60	9	50	3	62	2	33	11	78	415

^y Bacterial species were identified based on Biolog identification and sequencing of the 16s rDNA.

^z Percentage of each identified species compared with the total number of isolates.

distilled water with a sterile pestle. The resulting solution was plated on OEM agar and incubated for 48 h in the dark at 30°C. Single colonies were transferred to nutrient broth yeast agar (NBY) medium. Pure cultures were submitted to the Michigan State University (MSU) Diagnostic Clinic for identification using Biolog (Hayward, CA). The identified isolates were transferred to broth-glycerol cultures and maintained at -80°C for long-term storage.

Genomic DNA extraction was accomplished using the Wizard Genomic DNA Purification Kit (Promega, Madison, WI) using the gram-negative bacteria protocol. DNA samples were quantified using a NanoDrop-1000 spectrophotometer (Thermo Scientific, Wilmington, DE) and diluted to approximately 100 ng/μl. *P. ananatis* isolates could not be identified via Biolog because it is not available in the database, so an initial identification was accomplished with the diagnostic primers PanITS1 (5'-GTCTGATAGA AAGATAAAGAC-3') and EC5 (5'-TGCCAGGGCATCCACCG-3'), using the protocol described in Gitaitis et al. (2002). The 50-μl PCR mixture contained 10.0 μl of 5× Green GoTaq reaction buffer, 1.0 μl of dNTP mix (10 mM each), 0.25 μl of DNA Taq polymerase, a 0.2 mM concentration of each primer, and 5 μl of DNA suspension of each isolate, with autoclaved ultra-filtered water comprising the remaining volume. The PCR reactions were conducted in a Mastercycler thermal cycler (Eppendorf, Westbury, NY) with an initial denaturation of 5 min at 95°C, annealing for 30 s at 52°C, and elongation for 30 s at 72°C followed by another 30 cycles of denaturation at 95°C for 20 s, annealing at 52°C for 30 s, and elongation at 72°C for 30 s. The final extension step was for 7 min at 72°C, and resulting PCR products were stored at 4°C.

To confirm the species identified via Biolog or diagnostic primers as described above, the 16s rDNA gene was amplified using the method described by Vaneechoutte et al. (2000) and De Baere et al. (2004) and using the following universal primers: forward, 5'-AGTTTGATCTGGCTCAG-3'; and reverse, 5'-TACCTTGT TACGACTTCGTCCCA-3'. The PCR reaction was also performed using the protocol described above. The amplification reactions were performed with the following cycling parameters: 94°C for 5 min, followed by 3 cycles of 45 s at 94°C, 2 min at 50°C, and 1 min at 72°C and 30 cycles of 20 s at 94°C, 1 min at 50°C, and 1 min at 72°C, with a final extension at 72°C for 7 min. For *E. cowanii*, a subset of isolates was also amplified with the primers for *rpoB* gene using the method described by Brady et al. (2008) to confirm their identity. PCR products were visualized on a 1% agarose gel and cleaned using the ExoSAP enzymatic protocol (BioProducts, Rockland, ME) prior to sequencing of the 16s rDNA and *rpoB* genes. A total of 144 representative isolates were sequenced by Macrogen (Rockville, MD), and the results were compared with all known species in GenBank by using BLASTn (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) analysis.

Bacterial disease symptoms including water-soaked lesions were commonly observed on the more than 1,000 onion plant samples. Disease symptoms were observed as early as the two-leaf stage (approximately 4 to 5 weeks after planting). More frequently, bacterial disease symptoms were observed 6 to 7 weeks after planting.

A total of 415 bacterial isolates were obtained (Table 1). Based on Biolog identification and sequencing of the 16s rDNA, the majority of isolates were identified as *P. agglomerans* (176, 42.4%), *P. ananatis* (83, 20.0%), or *E. cowanii* (31, 7.5%). Other bacterial species identified included *Pseudomonas fluorescens* (15), *Pantoea dispersa* (10), *E. cloacae* (2), *Burkholderia ambifaria* (3), *D. chrysanthemi* (2), *Rahnella aquatilis* (2), and *B. cepacia* (1) (Table 1). In some instances, more than one species of bacteria were isolated from a single plant. *P. agglomerans*, *P. ananatis*,

E. cowanii, and *Ps. fluorescens* were found in all six counties (Table 1).

P. ananatis strains identified with PCR using primers PanITS1 and EC5 produced an amplicon of ~400 bp, similar to the study by Gitaitis et al. (2002). In addition to using these selective primers, nucleotide sequences of the 16s rDNA gene were compared with all known species in GenBank by using BLASTn, and high sequence identity to *P. ananatis* (97 to 99%) was determined. For other bacterial species, nucleotide sequencing of the 16s rDNA gene compared with GenBank showed 97 to 99% identity, usually similar to the identification through Biolog.

Pathogenicity and Virulence Testing

A subset of *P. agglomerans* (83), *P. ananatis* (51), and *E. cowanii* (24) isolates were selected to test for pathogenicity and virulence on onion seedlings and bulbs. The bacterial inoculum was prepared by plating bacterial strains from long-term storage onto NBY medium and incubating overnight at approximately 30°C. The bacterial suspension was prepared in sterile 15.0-ml Falcon tubes by using sterile deionized water and adjusted spectrophotometrically to approximately 1.0×10^8 CFU/ml. The same inoculum of each isolate was used for both seedling and bulb inoculations by separating 2.0 ml of each bacterial suspension into individual 2.0-ml Eppendorf tubes for bulb inoculations; the remaining bacterial suspension in each Falcon tube was used for plant inoculations.

'Bradley' onion seeds (Bejo Seeds, Oceano, CA) were planted into 98-square-cell flats containing a soilless medium (Hummert International, Earth City, MO), grown in the MSU Plant Sciences Research Greenhouses, and fertilized as needed with 200 ppm of 20-20-20 Peters water-soluble fertilizer (Scotts Company, Marysville, OH). Two-month-old seedlings were removed from the plant trays; their roots were trimmed to approximately 1.0 cm and rinsed under running water to remove the soilless medium. Each plant was disinfested with 5% sodium hypochlorite for 30 s, rinsed three times in sterile water for 10 s, and air dried in a laminar flow hood for 15 to 20 min. The leaves were trimmed to 12.0 cm using sterile scissors dipped in bacterial inoculum prepared as described above. Control plants were treated with sterile water. Inoculated and control plants were placed into sterile test tubes containing a moist cotton ball to promote high relative humidity and were covered with plastic caps. The pathogenicity screen was conducted once. Three seedlings were inoculated for each bacterial isolate and the control. The test tubes were arranged in a completely randomized design in test tube racks and incubated at room temperature (22.0 to 24.0°C) under continuous fluorescent light. Four days after inoculation, plants were visually assessed for disease symptoms. Isolates were identified as pathogenic if disease symptoms were detected in inoculated tissue. The virulence of each pathogenic isolate was determined by measuring disease severity using a scale from 1 to 5 as described by Schwartz (2013), where 1 = <1%, 2 = 1 to 10%, 3 = 11 to 25%, 4 = 26 to 50%, and 5 = >50% of leaves were collapsed.

Bradley onion bulbs, obtained from a commercial grower's storage facility located in Grant, MI, were inoculated using the bacterial isolates and inocula described above. Onion bulbs of similar size were selected and rinsed under running water to remove the soil. Bulbs were surface sterilized with 5% sodium hypochlorite for 2 min, washed three times for 1 min each with sterile water, and air dried under a laminar flow hood for 30 min. The bacterial suspension (0.5 ml), containing approximately 1.0×10^8 CFU/ml, was injected into the shoulder of each onion bulb, as described by Stumpf et al. (2018), with a 12.7-mm-long hypodermic needle and 1-ml gauge syringe. Control bulbs were injected with sterile water.

Following inoculation, the injection site was wiped two to three times with 70% ethanol and marked with a marker. Inoculated bulbs were incubated in a humid chamber for 2 weeks at room temperature (22.0 to 24.0°C) under continuous light. Three onion bulbs were used for each isolate and arranged in a completely randomized design. The assay was conducted once. Following incubation, onion bulbs were cut in half at the injection site lengthwise, and symptoms were evaluated. Pathogenicity was determined for each isolate as described above. Virulence was rated using a disease severity scale from 1 to 5 as described by Schwartz (2013), where 1 = <1%, 2 = 1 to 10%, 3 = 11 to 25%, 4 = 26 to 50%, and 5 = >50% of bulb tissue damaged. To confirm the identification of the pathogen, 10% of the symptomatic plants and bulbs were sampled and the resulting cultures grown on OEM medium and identified based on cultural characteristics (Zaid et al. 2012) and Biolog.

Disease severity data were analyzed by conducting an analysis of variance using SAS version 9.4 (SAS Institute, Cary, NC). PROC GLIMMIX was used to compare pathogenicity studies in onion seedlings and bulbs, using the Satterthwaite approximation of the degrees of freedom, because the number of isolates for each bacterial species was not equal. Means separation was performed using Student's *t* test (*P* < 0.05). Comparison of pathogenicity among locations (counties) was performed for *P. agglomerans* and *P. ananatis*.

Forty-seven (92%) *P. ananatis* isolates caused disease on seedlings with an average severity of 3.7 (Table 2). Approximately 50% of the *P. agglomerans* and *E. cowanii* isolates caused disease with a severity of 2.1 and 2.2, respectively. The disease severity resulting from *P. ananatis* on seedlings was significantly greater than that caused by *P. agglomerans* or *E. cowanii*. Significant differences in bulb pathogenicity were not detected among the bacterial species (*P* = 0.86), because all caused bulb rot symptoms 2 weeks postinoculation (Table 2). Disease severity among isolates collected from different locations was also compared for *P. agglomerans* and *P. ananatis*. Significant differences were not observed among locations for disease severity on either onion seedlings or bulbs for *P. agglomerans* (*P* = 0.31 and 0.77, respectively) or *P. ananatis* (*P* = 0.33 and 0.30, respectively).

Copper-Sensitivity Screening

Assays were conducted with 197 bacterial isolates, including *P. agglomerans* (117), *P. ananatis* (58), and *E. cowanii* (22). Each isolate was recovered from -80°C long-term storage, streaked

onto NBY medium, and incubated for 24 h at 30°C. Bacterial cells were collected using a loop, suspended in 1.0 ml sterile distilled water, and adjusted spectrophotometrically to approximately 1.0×10^8 CFU/ml. Casitone yeast extract glycerol medium (CYE-G), a low-nutrient medium with limited copper ion-binding capacity (Andersen et al. 1991; Sundin et al. 1989; Zevenhuizen et al. 1979), containing 1.7 g/liter of casitone, 0.35 g/liter of yeast extract, 2.0 ml of glycerol, and 15.0 g of technical agar (Dot Science, Burton, MI), was used for the assay. Filter-sterilized copper hydroxide stock solution (Kocide 3000, Dupont, Wilmington, DE) was added to the medium after autoclaving to achieve concentrations of 50, 100, 150, or 200 µg/ml. CYE-G medium containing no copper hydroxide was used as a control. A bacterial suspension was prepared as described above, and a 10.0-µl droplet was placed onto CYE-G medium plates and repeated three times for each copper hydroxide concentration. Each plate was divided equally into four sections, each section for one bacterial isolate. The plates were placed in a laminar airflow for 30 min to dry the bacterial droplets. The plates were incubated for 48 h at 30°C, and visible confluent growth of a bacterial strain on copper-amended plates was interpreted as tolerance for that copper concentration.

A subset of 197 isolates, consisting of *P. agglomerans* (117), *P. ananatis* (58), and *E. cowanii* (22) from different locations, was tested for copper hydroxide sensitivity (Fig. 1, Table 3). All isolates from the three species grew on CYE-G medium containing 50 and 100 µg/ml of copper hydroxide. When the copper hydroxide concentration was increased to 150 µg/ml, 85 to 90% of *P. agglomerans* and *P. ananatis* isolates produced confluent growth, whereas all of the *E. cowanii* isolates grew on this medium (Fig. 1). When CYE-G medium was amended with 200 µg/ml of copper hydroxide, 41% of *P. agglomerans* isolates (48) were tolerant (Fig. 1). Tolerant isolates of *P. agglomerans* were detected in all six counties in Michigan, but the majority (>50%) were collected from Allegan and Newaygo (Table 3). For *P. ananatis*, 19% of the isolates were tolerant to 200 µg/ml (11) and represented all six counties. Five *E. cowanii* isolates (22%) were copper hydroxide tolerant at 200 µg/ml and originated from Calhoun, Eaton, and Allegan Counties (Table 3).

Conclusions

We detected seven known bacterial onion pathogens, with *P. agglomerans*, *P. ananatis*, and *E. cowanii* representing the most frequently isolated species from the six counties that were sampled

TABLE 2
Pathogenicity and virulence of bacterial species *Pantoea agglomerans*, *Pantoea ananatis*, and *Enterobacter cowanii* from Michigan counties tested on onion seedlings and bulbs

Species	N ^w	Seedlings		Bulbs	
		Pathogenicity ^x	Virulence ^y	Pathogenicity ^x	Virulence ^y
<i>P. ananatis</i>	51	47	3.66 a ^z	49	3.08 a ^z
<i>E. cowanii</i>	24	12	2.20 b	21	3.00 a
<i>P. agglomerans</i>	83	40	2.14 b	79	3.00 a

^w Number of isolates tested.

^x Number of isolates found to cause disease symptoms on onion tissues. Onion seedlings and bulbs were inoculated with each bacterial species and evaluated for disease severity 4 days after inoculation. Seedlings were inoculated by dipping cut leaves into a bacterial suspension ($\sim 1.0 \times 10^8$ CFU/ml). Bulbs were inoculated by injecting inoculum ($\sim 1.0 \times 10^8$ CFU/ml) directly into the bulb.

^y Virulence was evaluated as a measure of disease severity using a scale of 1 to 5, where 1 = <1%, 2 = 1 to 10%, 3 = 11 to 25%, 4 = 26 to 50%, and 5 = >50% of leaves diseased or bulb rot. Average disease severity of each isolate was obtained from three replicated seedlings or bulbs.

^z Column means with a letter in common are not significantly different (*t* test LSD; *P* = 0.05).

across the onion growing regions of Michigan. In the United States, 14 bacterial species have been reported as common pathogens in onion (Schwartz and Mohan 2008), and three bacterial pathogens have been recently reported in Michigan as causing disease in

FIGURE 1
Frequency distribution of bacterial isolates *Pantoea agglomerans*, *Pantoea ananatis*, and *Enterobacter cowanii*, with confluent growth on casitone yeast extract glycerol medium containing 50 to 200 µg/ml of copper hydroxide (number of bacterial isolates tested: *P. agglomerans*, *n* = 117; *P. ananatis*, *n* = 58; and *E. cowanii*, *n* = 22).

onion: *P. agglomerans* (Grode et al. 2019; Tho et al. 2015), *P. ananatis* (Grode et al. 2017), and *B. cepacia* complex (Jacobs et al. 2008). In our current study, *Pantoea* spp. comprised the majority of the isolates obtained. *P. ananatis* and *P. agglomerans* are also common in areas where temperature and relative humidity are high (Schwartz and Mohan 2008), and *P. ananatis* is reported to proliferate under conditions of prolonged leaf wetness (Dutta et al. 2017). Disease incidence caused by *P. ananatis* can reach up to 70% in regions where plants are damaged by rainfall and hail (Schwartz and Otto 2000). *Pantoea* spp. have also been reported as bacterial pathogens of onions in Georgia (Edens et al. 2006; Gitaitis and Gay 1997), Pennsylvania (Gugino 2016; Pfeufer et al. 2011), and New York (Carr et al. 2010). *Pantoea* spp. were also the most frequently isolated bacteria from onion foliage in Puerto Rico (Bellido et al. 2012). *P. ananatis* and *P. agglomerans* are common epiphytic bacteria occurring on multiple weed species (Dutkiewicz et al. 2015; Dutta et al. 2017; Gitaitis et al. 2002). In addition to their association with more humid growing conditions, *Pantoea* spp. are also associated with onion thrips (*T. tabaci*), a common insect pest in Michigan (Dutta et al. 2014; Grode et al. 2017, 2019), which may also have contributed to the abundance of both *Pantoea* spp. observed in our study.

The third most abundant species we recovered, *E. cowanii*, had not been reported on onions in Michigan prior to this study. *E. cowanii* was first described by Inoue et al. (2000) as a new species in the Enterobacteriaceae and was reported as a clinical pathogen. Subsequently, the pathogen was determined to cause leaf blight and dieback of *Eucalyptus* in Uruguay (Brady et al. 2009) and bacterial leaf spot on a native forest species (*Mabea fistulifera*) in Brazil (Furtado et al. 2012). The related species *E. cloacae* is the causal agent of Enterobacter bulb decay (Schwartz et al. 2015) and has been reported in Washington (Schroeder et al. 2009) and New York (Beer et al. 2014). Only a single isolate of *E. cloacae* was detected in this trial. Asselin et al. (2016) suggested that it may be difficult to rely on genome sequences of some strains of this genus for accurate

TABLE 3 Copper sensitivity of bacterial isolates of <i>Pantoea agglomerans</i> , <i>Pantoea ananatis</i> , and <i>Enterobacter cowanii</i> from six Michigan counties evaluated at different concentrations of copper hydroxide embedded in casitone yeast extract glycerol (CYE-G) medium (<i>n</i> = 197)					
County	Pathogen	Number of bacterial isolates with confluent growth			
		50 µg/ml [†]	100 µg/ml	150 µg/ml	200 µg/ml
Allegan	<i>P. agglomerans</i>	33 (100) [‡]	32 (100)	29 (87)	17 (51)
	<i>P. ananatis</i>	16 (100)	16 (100)	13 (81)	3 (18)
	<i>E. cowanii</i>	8 (100)	8 (100)	8 (100)	1 (12)
Ottawa	<i>P. agglomerans</i>	20 (100)	20 (100)	18 (90)	6 (30)
	<i>P. ananatis</i>	8 (100)	8 (100)	8 (100)	3 (37)
	<i>E. cowanii</i>	2 (100)	2 (100)	2 (100)	0 (0)
Ingham	<i>P. agglomerans</i>	10 (100)	10 (100)	7 (70)	3 (30)
	<i>P. ananatis</i>	12 (100)	12 (100)	10 (83)	1 (8)
	<i>E. cowanii</i>	2 (100)	2 (100)	2 (100)	0 (0)
Newaygo	<i>P. agglomerans</i>	25 (100)	25 (100)	22 (88)	13 (52)
	<i>P. ananatis</i>	6 (100)	6 (100)	6 (100)	2 (33)
	<i>E. cowanii</i>	3 (100)	3 (100)	3 (100)	0 (0)
Eaton	<i>P. agglomerans</i>	13 (100)	13 (100)	11 (84)	5 (38)
	<i>P. ananatis</i>	6 (100)	6 (100)	6 (100)	1 (16)
	<i>E. cowanii</i>	2 (100)	2 (100)	2 (100)	1 (50)
Calhoun	<i>P. agglomerans</i>	16 (100)	16 (100)	13 (81)	4 (25)
	<i>P. ananatis</i>	10 (100)	10 (100)	9 (90)	1 (10)
	<i>E. cowanii</i>	5 (100)	5 (100)	5 (100)	3 (60)

[†] Concentration of copper hydroxide embedded in CYE-G medium.
[‡] Percentage of bacterial isolates tolerant to copper hydroxide.

identification. In their study, they developed a primer pair for *Enterobacter* sp. identification based on *Enterobacter* strains isolated directly from diseased onion samples to ensure the primer pair was relevant for detection of pathogenic *Enterobacter* strains (Asselin et al. 2016); however, they did not specify the species, and it is unknown whether the isolates they based their design on were *E. cloacae* or another pathogenic species such as *E. cowanii*. They also reported that recent work in Washington suggests that the species of *Enterobacter* most commonly associated with onion bulb decay may not be *E. cloacae* but another species of *Enterobacter* (Asselin et al. 2016).

The predominant bacterial onion pathogens in Ontario are described as *Pseudomonas* spp., responsible for slippery skin and sour skin, and *Erwinia* sp., responsible for soft rot; foliar bacterial pathogens are not widely reported (Howard et al. 1994; OMAFRA 2009). Although 15 isolates of *Pseudomonas* sp. were identified in this study across both years and originating from multiple counties across the state, the species associated with slippery skin and sour skin were not detected. *B. ambifaria*, *B. cepacia*, and *E. cloacae* are commonly associated with bulb infection in storage (Schwartz 2013; Schwartz and Mohan 2008) but were not frequently detected in our study. These pathogens are also common contributors to bulb rot in Washington (Schroeder and du Toit 2010; Schroeder et al. 2010a, 2010b) and Colorado (Schwartz and Bartolo 1995). *Erwinia* sp. (syn. *Pectobacterium* sp.) was not detected. A possible explanation for these differences could be that our isolates were generated from foliar disease samples, whereas slippery skin, sour skin, and soft rot are diseases of onion bulbs. *Erwinia* spp. are likely to enter bulbs through wounds created during harvest (Kado 2006). In Michigan, *Burkholderia* spp. are most commonly detected from the soil of the onion rhizosphere and roots (Jacobs et al. 2008). Therefore, our results do not mean that these pathogens are not present in Michigan onion bulbs, only that they were not detected in the foliar samples collected in this study.

In our study, *P. ananatis*, *P. agglomerans*, and *E. cowanii* caused disease on onion seedlings and bulbs. Disease severity on seedlings caused by *P. ananatis* was greater than that caused by *P. agglomerans* or *E. cowanii*. Carr et al. (2013) found that when onion leaves were inoculated with *P. ananatis*, the infection moved through the neck of the plant and caused center rot in storage. Our study did not allow for this progression; instead, onion bulbs were inoculated directly. All species were found to be pathogenic on onion bulbs; however, in contrast to inoculated seedling results, differences in onion bulb disease severity among pathogens were not observed in our study. Bulb rot symptoms occurred at the site of inoculation and were consistent to those described by Schwartz and Mohan (2008). Although this method is invasive, it has been used in a number of studies to determine the ability of bacterial pathogens to cause disease in onion bulbs (Schroeder and duToit 2010; Schroeder et al. 2010b; Stumpf et al. 2018; Vahling-Armstrong et al. 2016). It is possible that the results of our study may not be directly correlated to field results because of the inoculation method; however, Vahling-Armstrong et al. (2016) stated that this method produced results similar to those resulting from a natural infection of the bulb by *Pantoea* spp.

Copper resistance among plant-pathogenic bacteria has occurred worldwide (Martin et al. 2004; Shenge et al. 2014; Sundin et al. 1989; Voloudakis et al. 1993). Gitaitis (2006) reported that 60 to 80% of *P. ananatis* isolated from Georgia onions was copper tolerant. However, it was tested with copper sulfate pentahydrate (200 µg/ml) embedded in nutrient agar (NA) medium, which consists of approximately 25% metallic copper. In our study, 200 µg/ml of copper hydroxide, consisting of 46.1% metallic copper,

and CYE-G medium (a low-Cu²⁺-binding medium) were used. Therefore, the percentages of copper-tolerant isolates could be higher if the same concentration of copper sulfate and NA medium were used in our assay. Nischwitz et al. (2007) detected copper-tolerant strains of *P. ananatis* from field trials, in which *P. ananatis* strains tolerant to copper sulfate at 200 µg/ml were detected (Nischwitz et al. 2007). Our finding that 41% of *P. agglomerans* isolates were tolerant to copper hydroxide suggests that using copper hydroxide may not limit bacterial pathogen populations in Michigan onion fields. These results are consistent with a recent study examining control of bacterial stalk and leaf necrosis caused by *P. agglomerans* in Michigan onion fields (Grote et al. 2019). In this study, bacterial disease symptoms were only reduced when copper products were used in conjunction with insecticides for thrips control; copper products alone did not provide control of foliar symptoms of bacterial disease (Grote et al. 2019). A lack of efficacy of copper products can be explained if a high percentage of the pathogen population is resistant to copper, as our results demonstrate.

In summary, we found that the predominant bacterial pathogens of onion foliage in Michigan are *P. ananatis*, *P. agglomerans*, and *E. cowanii*. *P. ananatis* and *P. agglomerans* have been reported throughout onion production systems in the state in recent years; however, *E. cowanii* was previously unknown as a pathogen to onions. Seedling inoculation with *P. ananatis* resulted in higher levels of disease severity than seedling inoculation with either *P. agglomerans* or *E. cowanii*, although no differences were detected when bulbs were inoculated. Significant numbers of copper-tolerant isolates were identified for each of these species. Further studies should consider alternative chemistries or management strategies to limit these bacterial pathogens.

Acknowledgments

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