SURVEY OF BACTERIAL FOLIAGE DISEASES IN ONIONS, THEIR VIRULENCE, EPIDEMIOLOGY AND MANAGEMENT

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ABSTRACT

SURVEY OF BACTERIAL FOLIAGE DISEASES IN ONIONS, THEIR VIRULENCE, EPIDEMIOLOGY AND MANAGEMENT

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Michigan has been one of major onion producing states, with an estimate production of more than 36 million kilograms annually. Bacterial diseases are major problems for onion growers both in fields and storages, with losses reached 100% for some fields in Michigan. During 2013 and 2014, symptomatic plants showing leaf blight with water-soak lesions were collected from six different counties in Michigan for isolation and identification of bacterial pathogens. Bacterial isolates were identified by using BIOLOG (Hayward, CA) and sequencing of universal 16s rDNA gene and diagnostic primers. A total of 414 isolates were obtained; *Pantoea agglomerans, P. anantis* and *Enterobacter cowanii* were most prevalent. Subsets of bacterial isolates were chosen for pathogenicity test on onion sets and bulbs, and copper hydroxide sensitivity testing. On onion sets, almost all of *P. ananatis* were pathogenic, while only approximately 50% of *P. agglomerans* and *E. cowanii* were pathogenic. When tested in onion bulbs, almost all of isolates were pathogenically positive. For copper hydroxide sensitivity test, 40% of *P. agglomerans,* and approximately 20% of *P. anantis* and *E. cowanii* isolates were tolerant to copper hydroxide at (200ug/ml). This study suggests that copper hydroxide might not be very effective to control these bacterial diseases in onions.

To understand the epidemiology of *P. agglomerans, P. ananatis* and *E. cowanii,* effects of temperature, relative humidity (RH) and plant ages on the disease development were investigated. The three bacteria significantly resulted in disease at temperatures ranging from 25 to 30^{o}C. Disease progressed rapidly on onion foliage under RH of 80 to 100%, but decline

approximately 30% or greater if the RH was reduced to ≤60%. Inoculating plants aged between 6 to 14 weeks old with bacterial pathogens indicated that onions become significantly (P <.0001) more susceptible as they aged.

Nine-teen long-day onion cultivars were evaluated in greenhouse condition for susceptibility to foliar blight incited by *P. agglomerans, P. ananatis* and *E. cowanii*. The area under the disease progress curve (AUDPC) data differed significantly among cultivars tested in each of the two trials (P<0.0001). Overall, cv. 'Sherman', 'Mandras', 'Moondance' and 'Milestone' displayed more frequently partial resistance to the three bacterial pathogens, whereas 'Highlander', 'Delgado', 'Patterson', 'Pulsar' and 'Red-defender' were often highly susceptible to the three tested bacterial pathogens causing leaf blight and rot in onions.

In Cambodia, sixteen short-day onion cultivars were also evaluated for adoptability and stress response under field condition in two different locations. Number of stand count, bulb diameter, bulb weight and stress rating were significantly different in both trials (P<0.01). Overall, cv. 'Texas Early Grano', 'Yellow Granex', and 'AVON1073' were phenomenal in almost all measuring parameters. Results indicated that select short-day onion cultivars may be suitable for the tropical agro-climate of Cambodia.

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LITERATURE REVIEW

ONION PRODUCTION

Onion is an important vegetable crop worldwide with approximately 5.3 million hectares producing more than 93 million tons in 2014 (FAO 2016). The U.S. ranks third worldwide behind China and India and produces approximately 3.06 billion kilograms each year on 50585 hectares (NOA 2017; FAO 2016). In the U.S., onions are grown in more than 20 states with nearly 80% of the production located in California, Washington, Oregon, Idaho and Nevada. In Michigan, onions are grown primarily in the western and south central regions of the state. In 2017, onions were produced on 1032 hectares in Michigan and produced approximately 37 million kilograms of bulbs (Schwartz and Bartolo 2013; NOA 2017) .

Onions are classified as short-day, intermediate and long-day types based on day-length responses. Short-day onions include Bermuda and Granex types that require approximately 12-13 h of light and are generally have a mild flavor and soft flesh. Intermediate-day onions require 13.5 to 14.5 h of light and are usually grown under mild temperatures in regions between the 32 to 38^{o} latitude. Their soft-flesh makes them suitable for the fresh market. Long day onions need more than 14.5 h of light and include yellow, white and red types that have moderate to long-term storage capability. When long day cultivars are grown in lower latitude, bulbs do not form (Shanmugasundaram and Kalb 2001). Cramer and Bartolo (2013) defined short-day onions needing 12 h of light, intermediate onions requiring 12-16 h of light, and long-day onions up to 16 h of light.

ONION DISEASE PROBLEMS

Diseases of onion, both in the field and in storage are a significant problem. More than 60 diseases affect onions in the U.S.; 40 of which caused by fungi and oomycetes, 14 by bacteria, 6 by nematodes, 3 by viruses, 1 by a yeast and 1 by a *Phytoplasma* (Schwartz and Mohan 2008). Important bacterial diseases include the following: *Xanthomonas* leaf blight incited by *Xanthomonas axonopodis* pv. *allii*; leaf streak and bulb rot incited by *Pseudomonas viridiflava*; soft rots incited by *Pectobacterium* spp., *Dickeya* (syn. Erwinia), *Pseudomonas* and *Enterobacter*; bacterial stalk and leaf necrosis incited by *Pantoea ananatis* (syn. *Erwinia ananas*) and *P. agglomerans* (syn. *Erwinia herbicola*); slippery and sour skin incited by *Burkholderia sp*.; and *Enterobacter* bulb decay incited by *Enterobacter cloacae* (Schwartz and Mohan 2008). In Michigan, bacterial diseases have been a limiting factor for onion growers in recent years. *P. agglomerans* and *P. ananatis*, were identified in Michigan in 2012 and cause leaf blight and water soaked lesions, causing significant yield loss (Hausbeck 2014). Symptoms caused by the two pathogens are often similar and include white streaks with water-soaked margins extending through the length of leaves (Gitaitis et al., 2002). *P. agglomerans* was first reported by Hattingh and Walters (1981) causing a stalk and leaf necrosis in onion and was later observed affecting garlic (Koch et al., 1996). This pathogen was reported on onions in Cuba, Israel, and South Africa (Hattingh and Walters 1981; Gent and Schwartz, 2008). In the U.S., *P. agglomerans* was first reported in 2006 as a causal pathogen of leaf blight and bulb rot of onions in Georgia (Edens et al. 2006). *P. ananatis* was first reported in Georgia in 1997, causing center rot in onions (Gitaitis and Gay 1997).

THE GENUS *PANTOEA* AND THEIR IMPACTS ON CROPS

In 1917, Winslow and co-workers described the genus of *Pantoea* as *Erwinia,* named after a phytobacteriologist Erwin F. Smith (Winslow et al. 1917) and placed in the *Enterobacteriaceae* family. *Pantoea* spp. are found on plant surfaces, in soil, water and are associated with opportunistic diseases affecting humans and animals (Gavini et al. 1989). There are at least fifteen described species in this genus and include the following: *P. agglomerans, P. ananatis, P. dispersa, P. stewarttii, P. citrea, P. punctata, P. terrea, P. septica, P. eucrina, P. brenneri, P. conspicua, P. cypripedii, P. eucalypti, P. vagans* and *P. anthophila* (Brady et al. 2010). Another species, *P. uredovora,* previously described as a synonym of *P. ananatis,* was not widely accepted and the name of *Erwinia uredovora* is commonly used in the literature (Young et al. 2004; CPC 2007). *E. uredovora* is sometimes listed as a pathovar of *P. ananatis,* namely, *P. ananatis* pv. *ananatis* and *P. ananatis* pv. *uredovora* (Young et al. 2004).

Pantoea spp. include plant pathogens that can cause disease on monocotyledonous and dicotyledonous crops, ranging from annual herbaceous to tree crops. Hosts include melon, pineapple, onion, rice, sudangrass, corn, orange, sugarcane, beet and cantaloupe. Symptoms caused by these bacteria vary, depending on *Pantoea* spp., strains, and their hosts and include fruit and bulb rots (Kageyama et al. 1992; Kido et al. 2008; Gitaitis and Gay 1997), galls, wilts, stem dieback or necrosis (Cother et al. 2004), leaf blight (Hattingh and Walters 1981), and leaf blotch and necrotic spots (Azad et al. 2000; Coutinho et al. 2002).

P. ananatis, P. stewarttii and *P. agglomerans* are considered among the most important plant pathogens within *Pantoea* spp., and are capable of causing significant loss in field crops. *P. ananatis* causes foliar blighting of 50% or greater on sudangrass (*Sorghum sudanense*) (Azad et

al. 2000). Losses up to 100% in onion were recorded in Georgia (Gitaitis and Gay 1997). *P. ananatis* outbreaks in onion were later reported in Colorado (Schwartz and Otto 2000) and Michigan (Hausbeck 2014). Severe infection was reported in corn resulting in leaf senescence and decreasing grain size and yield in Brazil (Paccola-Meirelles et al. 2001; Lana et al. 2012). In South Africa, infection by *Pantoea* spp. caused eucalyptus seedlings to fail and reduced wood yields (Coutinho et al. 2002). In Japan, *P. ananatis* was isolated from rotted melon fruit in 1998 (Kido et al. 2008). In rice, the incidence of palea browning incited by *P. ananatis* was a severe problem in Japan and was associated with increased temperatures (Tabei et al. 1988; Azegami 2013). *P. ananatis* has been reported to be seed-transmitted in onion (Walcott et al. 2002), rice, and possibly corn (Hasegawa et al. 2003).

P. stewarttii major hosts include all types of corn species. Outbreaks may occur where susceptible cultivars are grown and flea beetle epidemics occur. Severe losses were reported in Italy in the 1940s and 1980s (CPC 2007). In sweet corn, yield losses of approximately 0.8% occurs for every 1% of disease incidence (Freeman and Pataky 2001). Yield losses are minimal for resistant cultivars, but reached 40%-100% when susceptible hybrids were grown and the disease began before the 5-leaf stage (Pataky et al. 2001). Disease resistance incorporated into corn hybrids and effective seed treatments have greatly lessened the importance of this disease (Pataky et al. 1996).

P. agglomerans is considered a new plant pathogen. Significant losses were first reported in onion for seed production in South Africa in 1981 (Hattingh and Walters 1981). The disease loss on just one 12-ha seed field exceeded 30,000 USD. In the U.S., this pathogen was first reported infecting onions in Georgia (Edens et al. 2006), but it was relatively less important in onions as compared to *P. ananatis* (Gitaitis and Gay 1997). *P. agglomerans* was first reported

infecting onion in New York in 2010 (Beer et al. 2010) and in Pennsylvania two years later (Gugino and Pfeufer 2015; Pfeufer 2014). In 2013, onion growers in Michigan experienced significant bacterial outbreaks in the field and storage with *P. agglomerans* consistently isolated (Hausbeck 2014; Tho et al. 2015).

P. agglomerans and *P. ananatis* have been identified as beneficial as biological controls, food industry, and plant breeding. *P. agglomerans* was successfully tested as a biological control against fire blight (*Erwinia amylovora*) (Beer et al. 1984) and the post-harvest fruit pathogen *Penicillium expansum* (Torres et al. 2005). *P. ananatis* produces ice nucleation gene (*ina*) and has also been successfully tested and applied in the freezing of foods (Zasypkin and Lee 1999). To breed genetically modified rice that produces a yellow color on its endosperm (golden rice), breeders used phytoene desaturase from *P. ananatis* to introduce the beta-carotene biosynthesis pathway (Beyer et al. 2002).

EPIDEMIOLOGY OF DISEASES CAUSED BY *PANTOEA* SPECIES

Environmental Requirements. *Pantoea ananatis* and *P. agglomerans*, have been reported in onions. Detailed epidemiology of these species on various hosts remains unclear (Coutinho and Venter 2009). For P. ananatis on corn and eucalyptus, high humidity and moderate temperature conditions (20 to 25 oC) increase disease incidence and severity (Coutinho et al. 2002). For sudangrass, infection increased at 32 oC and high relatively humidity (Azad 2000). Schwartz and Mohan (2008) reported that P. ananatis was active at bulb formation, when relative humidity was high and temperatures ranged from 28 to 35 oC. For stem necrosis in rice in Australia, disease incidence reached its peak when the weather was hot, dry, and windy (Cother et al. 2004).

Transmission and Dispersal. *Pantoea ananatis* and *P. agglomerans* may enter plants through flowers, wounds, and wind and rain (Azad et al. 2000; Cother et al. 2004). The pathogens may also be transmitted by insect vectors and were found to be associated with the guts of different insects including brown hoppers (*Nilaparvata lugens*) (Fujimoto and Isomura 1996), mulberry pyralid (*Glyhodes pyloalis*) (Takahashi et al. 1995), and tobacco thrips in onions (*Frankliniella fusca*) (Gitaitis et al. 2003). *Pantoea ananatis* and *P. agglomerans* were found to be associated with different weed species, which may serve as reservoirs of inocula. In Georgia, Giatitis et al. (2002) determined that the pathogens were epiphytes on 25 asymptomatic weeds and crops including crabgrass, sicklepod, yellow nutsedge, Bermuda grass, cowpea, and soybeans. The pathogens were detected on seeds such of onion (Walcott et al. 2002), sudangrass and rice (Azad et al. 2000; Tabei et al. 1988).

IDENTIFICATION OF *PANTOEA* SPECIES

Pantoea ananatis and *P. agglomerans* are gram-negative facultative anaerobic, oxidase negative bacterium, rod shape, motile by peritrichous flagella and belong to the family *Enterobacteriaceae.* They are usually negative in utilizing and producing indole and oxidase, and for starch hydrolysis (Gavini et al. 1989; Schwartz and Mohan 2008).

Semi-selective media have been used to differentiate *Pantoea* spp. from others. Goszczynska et al. (2006) developed a semi-selective medium, named PA 20, to isolate *P. ananatis* from onion seeds. The medium had a pH of 8.0 and contained $NH_4H_2PO_4$, K_2HPO_4, magnesium sulphate, NaCl, d (+) arabitol, crystal violet, bromothymol blue and thallium nitrate (Goszczynska et al. 2006). Onion extract medium (OEM) is another semi-selective medium that was developed by (Zaid et al. 2012) to isolate bacteria associated with onion. This medium

distinguishes *P. ananatis, P. agglomerans, Burkholderia cepacia, Enterobacter cloacae, Pectobacterium carotovorum* subsp. *carotovorum, Xanthomonas axonopodis* pv. *axonopodis,* and several *Pseudomonas* spp. based on their colony colors and sizes.

BIOLOG (Hayward, CA) is commonly used to identify *Pantoea* spp. and is based on carbon utilization with other biochemical and chemical substances and can identify *P. agglomerans, P. cypripedii, P. dispersa* and *P. eucrina*. Serological tests can be used to detect *P. ananatis* and *P. agglomerans.* A polyclonal mouse anti-body was developed to detect *P. ananatis* infecting cantaloupe. However, this anti-body was also found to cross-react with *Erwinia herbicola* (*P. agglomerans*) (Bruton et al. 1991). Some companies have recently developed effective anti-bodies for detection of *P. agglomerans* (*Erwinia herbicola*) such as GeneTex (GeneTex Inc. Irvine, CA, USA) derived from rabbit polyclonal and from mouse monoclonal (Abcam Inc. Cambridge, UK).

For molecular identification, 16s rDNA is most commonly used. However, there could be challenges in differentiating among closely-related species. Sequencing the 16s-23s internally transcribed spacer (ITS) region, *gyrB* gene and multilocus sequence analysis (MLSA) are consistent techniques that differentiate *Pantoea* spp. (Brady et al. 2008; Delétoile et al. 2009). DNA-DNA hybridization can also be used to identify *Pantoea* up to species level (Brady et al. 2008; Brady et al. 2010).

Gitaitis et al. (2002) developed a pair of specific primers to identify *P. ananatis* and detects the pathogen from infected samples, bacterial cultures, and DNA. Another highly effective method is Fluorescent Amplified Fragment Length Polymorphisms (F-AFLPs). It was

developed by Brady et al. (2007) to identify plant-pathogenic and plant-associated species from the *Pantoea* genus.

DISEASE MANAGEMENT

Managing disease caused by *P. ananatis* has been discussed by Schwartz and Mohan (2008). Reducing or eliminating sources of primary inoculum is important (Gent and Schwartz 2008). *P. ananatis* has been reported as a seed-borne and seed-transmitted pathogen (Walcott et al. 2002 and Goszczynska et al. 2006). *P. agglomerans* has not yet been proved as a seed-transmitted pathogen in onions. However, it was found to be an important seed-transmitted pathogen on corn seeds in Mexico and severe infection of mother plants affect seed quality (Silva-Rojas et al. 2016). Therefore, it should be possible that *P. agglomerans* is also a seed-borne in onions. Hence, testing seeds for the presence of these two pathogens should be a routine practices. Information is not available regarding seed treatment technology to prevent this pathogen from contaminating the seed. Resistant onion cultivars have not been reported (CPC 2007).

P. ananatis and *P. agglomerans* were found to be transmitted by insects feeding on plants such as aphids (*Thrips tabaci*) (Dutta et al. 2014), brown hoppers (*Nilaparvata lugens*) (Watanabe et al., 1996), mulberry pyralid (*Glyhodes pyloalis*) (Takahashi et al., 1995) and tobacco thrips (*Frankliniella fusca*) (Gitaitis et al., 2003). *P. ananatis* were also found associating with 25 weed species in onion fields in Georgia, U.S. (Gitaitis et al. 2002). Grode et al. 2017 studied the relationship between onion thrips and *P.* ananatis. The results demonstrated that plant wounds created by thrip feeding creates entry points for the bacterium and facilitates central rot development (Grode et al. 2017). Therefore, limiting insects, weeds and volunteer

onions in the field could help reduce bacterial reservoirs and transmission. Crop rotation with species that do not support the epiphytic or pathogenic form of *P.* ananatis and *P. agglomerans* could reduce disease pressure (Gent and Schwartz 2008). In the U.S. it is recommended that onions are planted once every three to four years, depending on disease history. Generally, rotation crops for onions include corn, alfalfa, celery, potato, dry bean, sugar beets and other small grains (Bartolo et al. 2013). In Michigan, recommended rotation crops include carrot, celery and potato. Mint and small grains such as corn, sorghum and wheat should be included in every 3 to 5 years for soil organic matter inputs (Zandstra and Grafius 2018). However, corn and alfalfa can result in significant residues and are not recommended prior to planting onions due to debris problem in the seed-bed (Bartolo et al. 2013). Rotation with soybean and Bermuda grass should be avoided, as they could be hosts for *P. ananatis* (Gent and Schwartz 2008). Chemical control to limit thrips and maggot populations in the field should be implemented as they could create wounds for bacterial entry or serve as vectors (Gitaitis et al. 2003).

Cultural practices including irrigation and mulching were studied by Gitatitis et al. (2004) in Georgia. The incidence or severity of center rot was not affected by irrigation types including drip or overhead sprinkler. However, center rot caused by *P. ananatis* was delayed by 7 to 14 days when straw mulch or bared ground was used, compared to black plastic mulch. Straw mulch also reduced the levels of center rot, whereas black plastic increased disease incidence and hastened the onset of the epidemic. Gitaitis et al. (2004) theorized that this may be due to the black plastic causing increased soil temperature which favors disease development (Gitaitis et al. 2004). Proper application of fertilizer, especially nitrogen may reduce disease pressure. Onions require approximately 170 kg of nitrogen per hectare (Drost and Bartolo 2013). Soil testing is recommended prior to planting and if more than 40 ppm of nitrate-nitrogen is found, no

additional nitrogen is required (Drost and Bartolo 2013). When nitrogen fertilizer is needed, splitting the applications can avoid excessive nitrogen that may make plants more disease susceptible (Gent and Schwartz, 2005; Shanmugasundaram and Kalb, 2001).

P. ananatis was confirmed infecting onion leaves and moving into the bulbs causing bulb rot disease in storage (Carr et al. 2013). The incidence of postharvest rots may be reduced by harvesting bulbs at a proper stage of maturity. Other good management practices include avoiding wounding, curing bulbs with forced hot air at least for 72 h, and storing the bulbs under cooling conditions (Gent and Schwartz 2008).

Copper bactericides may reduce and delay the disease incidence at an early stage. Preventive copper application could be amended with ethylenebisdithiocarbamate (EBDC) fungicide such as mancozeb to reduce the extent of secondary dissemination of the bacteria and risks of copper resistance among the bacterial pathogen population (Gent and Schwartz 2008). In Michigan field trials in 2014, efficacy of four bactericides were tested; Kocide 3000 (copper hydroxide) significantly reduced bacterial disease severity as compared to untreated control. Similar to Kocide 3000, Kasugamicin also significantly reduced bacterial disease severity; but has not been registered for onion application. Cuprofix Ultra 40 and Nucop, on the other hand, did not significantly limit leaf blight severity as compared to untreated control (Wiriyajitsomboon et al. 2014). In Colorado, integration of acibenzolar-S-methyl and commercial biological control agents such as *Pantoea agglomerans* strain C9-1 and *Pseudomonas fluorescens* strain A506, with copper hydroxide could help manage Xanthomomas leaf blight (Gent and Schwartz 2005).

Copper resistant strains of *P. ananatis* have been observed in many onion growing areas, and pose a disease control challenge. Gitaitis (2006) reported copper-tolerant strains of *P. ananatis* in Georgia, where a majority (60-80%) of bacterial strains isolated from three different counties exhibited partial growth on copper-amended medium; but all strains were susceptible to a mixture of copper sulfate pentahydrate and maneb (Gitaitis, 2006). However, recently, maneb has no longer been registered for onions. The fatty acids cis-9-hexadecenoic acid /2-hydroxy-13-methyltetradecanoic acid, cis-9/ trans-12/ cis-7-octadecenoic acid and hexadecanoic acid were reported to be responsible for copper-tolerance of *P. ananatis* in onion and could be used to design a laboratory assay to identify copper-resistant bacteria using gas chromatography (Gitaitis 2006).

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CHAPTER I: ASSESSMENT OF BACTERIAL PATHOGENS ASSOCIATED WITH ONION FOLIAGE IN MICHIGAN AND THEIR COPPER SENSITIVITY

ABSTRACT

Plants with symptoms of bacterial foliar disease including blighted leaves with water-soaked lesions were collected in 2013 and 2014 from 17 fields in six major onion production counties in Michigan. Bacterial isolates were identified using BIOLOG and confirmed by sequencing the 16s rDNA gene. From a total of 414 isolates, ten bacterial species were identified. *Pantoea agglomerans* (42.5%), *Pantoea ananatis* (17.4%) and *Enterobacter cowanii* (7.5%) were most prevalent. Seven of the bacterial species were virulent on onion foliage and included *P. agglomerans, P. ananatis, E. cowanii, Dickeya chrysanthemi, Burkholderia ambifaria, Burkholderia cepacia* and *Pseudomonas fluorecens*. Inoculation of onion leaves with *Pantoea dispersa, Rahnella aquatilis,* and *Enterobacter cloacae* did not result in symptoms. When isolates of the bacterial species were injected into the onion bulbs, all producted symptoms. A subset of 197 isolates were tested for sensitivity to copper hydroxide. *P. agglomerans* (41%), *P. ananatis* (19%) and *E. cowanii* (22%) exhibited tolerance to 200ug/ml of copper hydroxide. Information regarding the bacterial species associated with symptomatic onion foliage in Michigan and the occurrence of copper tolerant isolates may improve disease management.

Keywords: *Onions, bacteria, foliage diseases, copper resistance*

INTRODUCTION

Onion (*Allium cepa* L.) is an economically important crop in the U.S. that contributed approximately $900 million annually from 2005 to 2010 on a maximum of nearly 62,000 ha (Schwartz and Bartolo 2013). In Michigan, onions are grown in the western and south-central regions and yielded approximately 40,000 tons valued at $11 million in 2016 (NOA, 2016). Onion cultivars suitable for Michigan are long-day types that are pungent and suitable for storage (Schwartz and Bartolo 2013; DARD 2015).

Bacterial pathogens may incite disease in the field and storage causing a significant problem for the U.S. onion industry (Schwartz and Mohan 2008). Foliar symptoms of bacterial infection include leaf blight, necrotic leaf tip, leaf streak, water-soaked lesions, soft rot, bleached tissue, and occasionally plant collapse (Schwartz and Mohan 2008). Annual losses due to disease are estimated to be 50% or more, depending on cultivar, region, pathogen species, and environmental conditions (Schwartz and Mohan 2008). Bacterial diseases include leaf blight (*Xanthomonas axonopodis* pv. *allii*); leaf streak and bulb rot (*Pseudomonas viridiflava*), soft rot (*Pectobacterium spp., Dickeya spp.* (syn. *Erwinia*), *Pseudomonas spp.* and *Enterobacter*), bacterial stalk and leaf necrosis (*Pantoea ananatis* and *P. agglomerans* (syn. *Erwinia herbicola*)), slippery and sour skin (*Burkholderia spp.*), and bulb decay (*Enterobacter cloacae*) (Schwartz and Mohan 2008).

In Michigan, different bacterial species have been reported infecting onion foliage, including *P. agglomerans* (Tho et al. 2015) and *P. ananatis* (Hausbeck 2014), whereas *Burkholderia cepacia* complex was reported infecting bulb (Jacob et al. 2008). Bacterial disease development on onion is favored by prolonged periods of rain, high relative humidity, and moderate to high temperatures (Schwartz and Mohan 2008). *P. agglomerans* previously named

as *Erwinia herbicola* or *Enterobacter agglomerans* (Gavini et al. 1989) is widely distributed in nature which could be isolated from plant, water, soil and animal specimens (Deletoile et al. 2009). It was firstly reported infected onions in South Africa in 1981 (Hattingh and Walters 1981) and in Georgia, U.S in 2006 (Edens et al. 2006). *P. ananatis*, primary inoculum for bacterial diseases on onion include weed species (Gitaitis et al. 2002), different plant debris, soil, irrigation water, volunteer plants (Ramette et al. 2005), insects (Gitaitis et al. 2003), and infested seed such as onion (Walcott et al. 2002), sudangrass (Azad et al. 2000) and rice (Cother et al. 2004). *B. cepacia* (formerly *Pseudomonas cepacia*) was firstly decribed as a pathogen causing sour skin and soft rot in onion in New York by W. H. Burkholder in 1950 (Burkholder, 1950). Ecological niches of *B. cepacia* include plant and human pathogens, biological control strains and biodegradation strains (Baldwin et al. 2005).

The identification and virulence of bacterial foliar pathogens affecting the crop is needed. Due to the prevalence of onion bacterial foliar diseases, Michigan growers have expressed interest in applying copper-based products even though field research has not demonstrated efficacy (Grode et al., Plant Disease submitted). Nischwitz et al. (2007) reported copper-tolerant *P. ananatis* strains from onion fields in Georgia (Nischwitz et al. 2007). The objectives of this study were to identify the bacterial species associated with onion foliar blight in Michigan, evaluate their virulence on seedlings and bulbs, and determine their sensitivity to copper.

MATERIALS AND METHODS

Sample collection, bacterial isolation, and biochemical identification. A total of 1080 onion samples displaying foliar bacterial symptoms were sampled from production fields in six

Michigan counties (Allegan, Ottawa, Ingham, Newaygo, Eaton and Calhoun) every two weeks beginning when plants were at the two-leaf stage (June) through bulb enlargement (August) in 2013 and 2014 (Table 1.1). Fourteen yellow and two red onion cultivars grown in 17 fields were included in the study. Each sampled field ranged in size from 2 to 5 ha. Symptomatic plants were collected, placed in an individual plastic bag for each field, labelled, placed in a cooler with ice, and transported to the laboratory for isolation. Each plant sample was rinsed under running tapwater, placed into aluninum containers, and kept in a cold room (at 5-7 ^{o}C) for 2-5 days until the plants were processed.

Onion extract medium (OEM) (Zaid et al. 2012) was prepared with chopped yellow onion bulbs (333g) that were autoclaved in 500ml of distilled water for 35 min at 121^{o}C, cooled at room temperature, and filtered to remove debris. The following ingredients were added to the filtered solution: 5.0 g of NaCl, 1 g of K_2HPO_4 (anhydrous), 3.8 g of KH_2PO_4 (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 L using distilled water. The medium was sterilized in an autoclave at 121^{o}C for 45 min.

Approximately 1 cm-square of symptomatic leaf tissue was surface sterilized with 70% ethanol for approximately 30 seconds, cleaned twice in sterile distilled water, blotted dried on sterile tissue papers and then crushed in micro-eppendorf tubes containing 0.1 ml of sterile distilled water with a sterile pestle. The resulting solution was plated on OEM agar, and incubated for 48 h in the dark at 30^{o}C. Single colonies were transferred to Nutrient Broth Yeast Agar (NBY) medium. Pure cultures were submitted to the Michigan State University Diagnostic Clinic for diagnosis using BIOLOG (Hayward, CA). The identified isolates were transferred to broth-glycerol cultures and kept in -80 ^{o}C for long-term storage.

Table 1.1 Summary of onion plant samples exhibiting symptoms of bacterial diseases in Michigan production fields from six counties that were cultured and identified using BIOLOG.

County	No. of fields sampled	Cultivars	No. plant samples	No. of identified isolates
Allegan	3	Redwing	25	7
		Sherman	28	4
		Keymaster	34	11
		Livingston	71	47
		Stanley	28	6
		Bradley	47	18
		Gunison	27	6
		Sub-total	***260***	***99***
Ottawa	3	Sherman	5	1
		Livingston	97	47
		Stanley	9	3
		Bradley	82	17
		Sub-total	***193***	***68***
Ingham	4	Redwing	12	1
		Delgado	26	12
		Hendrix	23	5
		Sedona	52	36
		Stanley	1	1
		Livingston	3	3
		Sub-total	117	58
Newaygo	3	Prince	6	2
		Stanley	24	0
		Bradley	105	63
		Sub-total	**135**	**65**
Eaton	2	Redwing	53	15
		Red-defender	42	18
		Highlander	2	2
		Sub-total	***97***	***35***
Calhuon	2	Patterson	95	20
		Scorpion	90	30
		Pulsar	33	20
		Highlander	53	8
		Brandt	11	11
		Sub-total	***282***	***89***
Total	17		**1080**	**414**

DNA Extraction. Genomic DNA extraction was accomplished using the Wizard Genomic DNA Purification Kit (Promega, Madison, U.S.) with the protocol for gram negative bacteria. Bacterial isolates were cultured overnight in 3.0 ml of NBY broth, incubated at room temperature on shaker set at 100 rpm. The culture broth (1.0 ml) of each bacterial isolate was pipetted into a 1.7 ml Eppendorf tube and centrifuged for 2 minutes at 13,000-16,000x g to collect the bacterial pellet. The supernatant was then discarded, and 600µl of the nuclei lysis solution was added to the tube and gently mixed using a pipette. The suspension was later incubated for 5 minutes at 80^{o}C and cooled to room temperature. RNase solution (3µl) was added, mixed, incubated at 37^{o}C for 15-60 minutes, and then cooled to room temperature. A protein precipitation solution (200µl) was added, the suspension vortexed, incubated on ice for 5 minutes, and centrifuged at 13,000-16,000 x *g* for 3 minutes. DNA precipitation and rehydration was accomplished by transferring the supernatant to a clean tube containing 600µl of isopropanol at room temperature. The suspension was mixed, centrifuged for 2 minutes, and the supernatant decanted. Ethanol (70%; 600µl) at room temperature was added, mixed, and centrifuged for 2 minutes. The ethanol was aspirated and air-dried for 10-15 minutes in an air flow hood. Finally, the DNA pellet was rehydrated in 100µl of rehydration solution and kept overnight at 4^{o}C. DNA samples were quantified by using NanoDrop-1000 Spetrophotometer (Thermo Scientific; Wilmington DE) and diluted to approximately 100 ng/ul.

PCR Identification. *P. ananatis* isolates could not be identified via BIOLOG as it was not available in the database, so an initial identification was accomplished using diagnostic primers, PanITS1 (5'-GTCTGATAGAAAGATAAAG-AC-3'), EC5 (5'-TGCCA GGGCATCCACCG-3'), using the protocol described in Gitaitis et al. (2002). The PCR-mixture was based on the Promega's standard application (Madison, WI, USA.). The 50 µl PCR-mixture contained 10.0 µl

of 5x Green GoTaq reaction buffer, 1.0 µl dNTP Mix (10mM each), 0.25 µl of DNA Taq polymerase, a 0.2-mM concentration of each primer, and 5 ml of a DNA suspension of the isolate, while the remaining volume was added by autoclaved ultra-filtered water. The PCR reaction tube was incubated in the thermal cycler at 95 ^{o}C for 1 min to warm, a denaturation of 5 min at 95 ^{o}C, annealing for 30 s at 52 ^{o}C, and the elongation phases were for 30 s at 72 ^{o}C. Typically, PCR runs were conducted for 30 cycles and samples were stored at 4 ^{o}C.

To confirm the species identified via BIOLOG or diagnostic primers as described above, 16S rDNA gene was amplified using the method described previously (Vaneechoutte et al. 2000; Baere et al. 2004) and using the universal primers (forward 5′-AGTTTGATCCTGGCTCAG-3′ and reverse 5′-TACCTTGTTACGACTTCGTCCCA-3′). The PCR reaction was also performed with Promega's standard procedure as described previously (Madison, WI, USA.). 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, 1 min at 72°C, and 30 cycles of 20 s at 94°C, 1 min at 50°C, 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 for *rpoB* gene with the primers and method described previously in (Brady et al. 2009; Brady et al. 2008) to confirm their identity.

Gel electrophoresis was conducted to check the presence of bands in 1% agarose gel, stained with 1:100 (v/v) of ethidium bromide. The DNA bands were viewed and photographed using BIO RAD Molecular imager® (Gel DocTM XR+ imaging system) connected to a computer.

To sequence the 16s rDNA and *rpoB* genes, post PCR products were cleaned-up to remove unincorporated nucleotides and residual primers to prevent interference with the

sequencing process by using the ExoSAP enzymatic protocol (BioProducts, Rockland, ME). The master mix of the cleaning enzyme was prepared by mixing 0.025 µl of Exonuclease I, 0.250 µl of Shrimp Alkaline Phosphatase, and 9.725 µl of Milli Q water. The cleaning enzyme (10 µl) was mixed to each PCR sample. Each reaction was incubated at 37^{o}C for 30 minutes and then 95^{o}C for 5 minutes in a PCR machine. Samples were stored at 4^{o}C until sequencing. 144 Representative isolates (144) were sequenced by Macrogen USA (Macrogen Corp. Rockville, MD) and the results were compared with all known species in the Gene Bank by using BLASTn.

Isolate Pathogenicity. A subset of isolates, consisting of 83 *P. agglomerans,* 51 *P. ananatis,* 24 *E. cowanii,* and 17 other (2 *Burkholderia ambifaria,* 1 *B. cepacia,* 2 *Pseudomonas fluorescens,* 8 *Pantoea dispersa,* 2 *Rahnella aquatilis,* and 1 *Enterobacter cloacae*) were selected to test for virulence on onion seedlings and bulbs. The bacterial inoculum was prepared by plating bacterial strains from long term storage on NBY medium and incubating overnight at approximately 30^{o}C. The bacterial suspension was prepared in sterile 15ml-falcon tubes by using sterile deionized water and adjusted spectrophotometrically to approximately 1.0 x 10^{8} CFU/ml. The same inoculum of each baterium was used for both seedling and bulb inoculations by splitting 2 ml of each bacterial suspension into individual 2.0 ml-effendorf tube for bulb inoculations; while the remaining bacterial suspension in each falcon tube was used for plant inoculations.

'Bradley' onion seedlings were used for virulence testing. Onion seeds (Bejo Seeds, Inc., Oceano, CA) were planted in 98-square-cell flats containing a soilless media (Hummert International, Earth City, MO), grown in the MSU Plant Sciences Research Greenhouses, and fertilized as needed with 200 ppm of 20-20-20 of Peters water soluble fertilizer (The Scotts Company, Marysville, OH). Two-month-old seedlings were removed from the plant trays; their roots were trimmed to approximately 1 cm, and rinsed under running water to remove the

soilless media. Each plant was disinfected with 5% sodium hypochlorite for 30 seconds in a plastic container (30 x 20 x 15 cm). Disinfected plants were rinsed three times in sterile water for 10 seconds and air dried in a laminar flow hood for 15-20 mins. The leaves were trimmed to 12 cm using sterile scissors dipped in bacterial inoculum prepared as described above. Plants treated with sterile water were used as a healthy control. Inoculated and control plants were placed into sterile test tubes with a cotton ball at the bottom, and covered with plastic caps. To promote high relative humidity in the tubes to facilitate bacterial infection, the cotton ball was wetted with 2.0 ml of sterile water. This virulence assay was conducted once. Three seedlings were used for each bacterial strain and the control. The test tubes were arranged in a complete randomized design in test tube racks and incubated at room temperature (22.0 – 24.0 ^{o}C) under continuous fluorescent light. Four days after inoculation, plants were visually assessed for disease severity using a scale from 1 to 5 as previously described (Schwartz 2013) where 1 = <1%, 2 = 1-10%, 3 = 11-25%, 4 = 26-50% and 5 = > 50% of leaves were collapsed (Figure 1.1).

'Bradley' onion bulbs obtained from a commercial grower's storage facility located in Grant, MI, were inoculated using the bacterial isolates and inocula as decribed above. The virulence assay was conducted as described by Schroeder, et al. (2010). Onion bulbs of similar size were selected and rinsed under running water to remove the soil. Bulbs were surface-sterilized with 5% of sodium hypochlorite for 2 mins and washed three times, 1 min each with sterile water. They were air-dried under laminar flow hood for 30 mins before inoculation. The bacterial suspension (0.5 ml) containing approximately 1.0 x 10^{8} CFU/ml) was injected into the shoulder of each onion bulb with a 12.7 mm-long hypodermic needle and 1-ml gauge syringe. Injection with sterile water was used as a control.

Following inoculation, the injection site was wiped 2-3 times with 70% of ethanol and marked with a marker, and then incubated in a clear plastic storage box (approx. 45L with lid) with wetted paper towel lining the inside to promote high relative humidity. The virulence assay for bulbs was conducted once and three onion bulbs were used for each isolated and arranged in a complete randomized design. Inoculated onion bulbs were incubated at room temperature (22.0 to 24.0 ^{o}C) for 2 wks under continuous light. After incubation, onion bulbs were cut in half at the injection site lengthwise and symptoms rated as described in (Schwartz 2013). The rating scale ranged from 1 to 5 whereas 1 = <1%, 2 = 1-10%, 3 = 11-25%, 4 = 26-50%, and 5 = > 50% of bulb damage (Figure 1.2). To confirm the pathogen, 10% of the symptomatic plants and bulbs were sampled and the resulting cultures grown on OEM medium to confirm the identity based on cultural characteristics (Zaid et al. 2012) and through BIOLOG.

Statistical analysis for virulent data. Analysis of variance (ANOVA) of virulence data was performed using SAS Version 9.4 (SAS Institute, Cary, NC). PROC GLIMMIX was used to compare virulence studies in onion seedlings and bulbs, with the degree of freedom according to "Satterthwaite" approximation as the number of isolate for each bacterial species were not equal. PARMS statements with (lowerb 0.0001) were also used for the analyses to overcome convergent issue, as there was only one bacterial isolate for some species. Letter of separations for means were performed by using student's *t* – test ($P < 0.05$). Comparison of bacterial virulence from different locations (counties) was also performed, but only with *P. agglomerans* and *P. ananatis* species, as the number of isolates for other species were limited.

1 = < 1% 2 = 1-10% 3 = 11-25% 4 = 26-50% 5 = > 50%

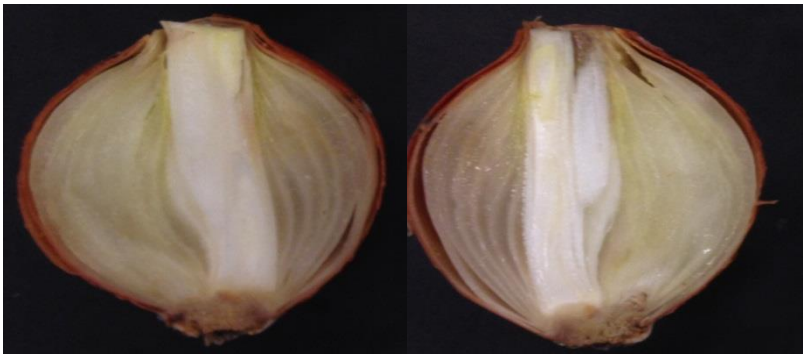
Figure 1.1 Plant damage rating scales. 1-5 scale of disease severity was used (where 1 = <1%, 2 = 1-10%, 3 = 11-25%, 4 = 26-50% and 5 = > 50% of leave collapsed).

1 = < 1%

2 = 1 - 10 %

3 = 11 - 25 %

4 = 26 - 50 %



5 = > 50 % of bulb damage

Figure 1.2 Bulb (lengthwise) damage rating scales. 1-5 scale of disease severity was used (where 1 = <1%, 2 = 1-10%, 3 = 11-25%, 4 = 26-50% and 5 = > 50% of bulb rot).

Copper-sensitivity of bacterial isolates. Assays were conducted with 197 bacterial isolates, including *P. agglomerans* (117), *P. ananatis* (58) and *E. cowanii* (22). Each isolate was recovered from -80^{o}C long-term storage, streaked onto NBY medium and incubated for 24h at 30^{o}C. Bacterial cells were collected using a loop, suspended in 1.0 ml sterile distilled water, and adjusted spectrophotometrically to approximately 1.0 x 10^{8} CFU/ml. Casitone yeast extract glycerol medium (CYE-G), a low nutrient medium with limited copper ion binding capacity (Zevenhuizen et al. 1979; Sundin et al. 1989; Andersen et al. 1991), containing 1.7 g/L casitone, 0.35g/L yeast extract, 2.0 ml glycerol and 15 g of technical agar (Dot Science, Inc. Burton, MI), was used for the assay. Filtered sterilized of copper hydroxide (Kocide 3000, Dupont Inc., Wilmington, DE) stock solution was added to the medium after autoclaveing to achieve concentrations of 50 ug, 100 ug, 150 ug and 200 ug/ml. Additional CYE-G medium containing no copper hydroxide was used as a control. A 10-µl droplet of bacterial suspension prepared as described above 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 48h at 30^{o}C and visible confluent growth of a bacterial strain on copper-amended plates was interpreted as tolerance for that copper concentration.

RESULTS

Bacterial leaf blight with water-soaked lesions was commonly observed from the onion plants sampled from Michigan fields in 2013 and 2014. Occasionally, an inner leaf appeared necrotic and shriveled (Figure 1.3). Disease symptoms were observed as early as at the two-leaf stage when plants were approximately 10.0 cm in height. More frequently, bacterial disease symptoms were observed 6 to 7 weeks after planting. Disease incidence progressed throughout the season and reached an estimated incidence of up to 80% or higher in some fields (*data not shown*).

A total of 414 bacterial isolates were obtained from symptomatic onion leaves. Based on BIOLOG identification and sequencing of the 16s rDNA, the majority of isolates were *P. agglomerans, P. anantis*, or *E. cowanii* with 176 (42.5%), 83 (17.4%) and 31 (7.5%) isolates, respectively. Other bacterial species detected included *P. fluorescens* (15), *P. dispersa* (10), *E. cloacae* (2), *B. ambifaria* (2), *D. chrysanthemi* (2) and *R. aquatilis* (2) (Table 1.2). In some instances, more than one species of bacteria was isolated from a single plant. *P. agglomerans, P. ananatis, E. cowanii* and *P. fluorescens* were found in all six counties (Table 1.2).

P. ananatis strains identified using PCR primers using PanITS1 and EC5 primers produced an amplicon of ~400bp, 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 the Gene Bank by using BLASTn, and the same identity to *P. anaantis* in the Gene Bank (97-99%) were also obtained. For other bacterial species, nucleotide sequencing of the 16s rDNA gene as compared to the Gene Bank were 97-99% identity, usually very similar to the identification through BIOLOG. Since *P. ananatis* is not available in the BIOLOG

database, all isolates (83) of *P. ananatis* were identified via BIOLOG as *Serratia oderifera*, a bacterium of the same family (*Enterobacteriaceae*) at 54-56% identity.

Forty seven (92%) *P. ananatis* isolates were virulent on seedlings with average disease severity = 3.88. Approximately 50% of the *P. agglomerans* and *E. cowanii* isolates were pathogenic (disease severity = 3.37 and 3.42, respectively). Disease symptoms were also observed on seedlings inoculated with *D. chrysanthemi, B. ambifaria, B. cepacia;* while inoculation with *P. fluorecens*. *P. dispersa, R. aquatilis,* and *E. cloacae* did not result in disease symptoms. Virulence varied significantly among bacterial spp. (P = 0.0075). *D. chrysanthemi* was similar in virulence (disease severity = 4) to *P. ananatis, E. cowanii, P. agglomerans,* and *B. ambifaria* (Table 1.3).

All bacterial species caused necrosis and bulb rot symptoms 2 wks post inoculation. Some isolates of *P. agglomerans, P. ananatis* and *E. cowanii* isolates did not cause disease symptoms. Virulence on onion bulbs was significant among bacterial isolates (P= 0.0105). Bulb rot severity of *B. cepacia* (3.67) and *B. ambifaria* (3.16) but was similar to *P. agglomerans, P. ananatis, E. cowanii*, and *R. aquatilis* (Table 1.3). *P. dispersa, R. aquatilis*, and *E. cloacae* were pathogenic on onion bulbs, but not on seedlings. *D. chrysanthemi* isolates were virulent on onion leaves, but caused little disease on onion bulbs. *B. cepacia* had low disease ratings on onion plants, but were virulent on onion bulbs.

Comparison of bacterial virulence on onion plants and bulbs in different locations were also conducted for *P. agglomerans* and *P. ananatis.* No significant differences were observed among locations for bacterial virulence on either onion plants or bulbs for *P. agglomerans*

(*P*=0.2692 and 0.8570, respectively) or (*P. ananatis: P*= 0.3283 and 0.2010, respectively) (*data not shown*).

Copper-sensitivity of bacterial isolates. A sub-set of 197 isolates, consisting of *P. agglomerans* (117), *P. ananatis* (58) and *E. cowanii* (22), from different locations were tested for copper hydroxide sensitivity. All isolates from the three species grew on CYE-G medium containing 50 and 100 ug/ml copper hydroxide. When the copper hydroxide concentration was increased up to 150ug/ml, 85 to 90% of *P. agglomerans* and *P. ananatis* isolates, respectively, produced confluent growth, while as all of *E. cowanii* isolates grew on the embedded medium. When CYE-G medium were embedded with 200ug/ml of copper hydroxide, 41% of 117 *P. agglomerans* were tolerant (Figure 1.5). Tolerant isolates of *P. agglomerans* were detected in all six counties in Michigan, but the majority (>50%) were collected from Allegan and Newaygo. For *P. ananatis*, 19% of the isolates were tolerant to 200ug/ml, and represented all six counties. Five *E. cowanii* isolates (22%) were copper hydroxide tolerant at 200ug/ml and originated from Calhoun, Eaton, and Allegan counties (Table 1.4).

Figure 1.3 Symptomatic onions in the field including **A**: Leaf blight with water-soaked lesions; **B**: Shriveled inner leaf; **C**: Leaf blight; **D**: Watersoaked tissue at the leaf base.

Table 1.2 Bacteria identified from onion leaf tissue when sampled in six different counties in Michigan in 2013 and 2014.

County	Allegan		Ottawa		Ingham		Newaygo		Eaton		Calhoun		Total
	2013	2014	2013	2014	2013	2014	2013	2014	2013	2014	2013	2014	
P. agglomerans	21	20	7	26	1	26	3	27	1	11	6	27	**176 (42.5%)**
P. ananatis	1	22	0	13	2	10	0	8	0	8	0	19	**83 (17.4%)**
E. cowanii	3	5	0	2	1	1	0	3	1	4	3	8	**31 (7.5%)**
P. fluorescens	1	2	1	1	0	1	0	4	0	2	0	3	**15 (3.6%)**
P. dispersa	1	3	0	1	0	0	0	1	0	2	0	2	**10 (1.2%)**
E. cloacae	0	0	0	0	0	0	0	2	0	0	0	0	**2 (0.5%)**
R. aquatilis	0	0	0	0	0	2	0	0	0	0	0	0	**2 (0.5%)**
B. ambifaria	0	0	0	0	0	0	0	0	0	0	1	1	**2 (0.5%)**
D. chrysanthemi	0	0	0	0	0	0	0	0	0	1	0	1	**2 (0.5%)**
Other species	0	0	0	1	0	0	0	2	0	1	1	1	**6 (1.4%)**
No ID	3	17	0	16	4	10	0	15	0	4	0	16	**85 (20.5%)**
Total	30	69	8	60	8	50	3	62	2	33	11	78	**414**

*Numbers in parenthesis are the percentage of each species as compared to the total number of isolate

Table 1.3 Virulence of bacterial species in onion seedlings and bulbs

	No. Seedlings			No. Bulbs	
Species	**N[x]**	**Pathogenic[y]**	**Virulent[z]**	**Pathogenic[y]**	**Virulent[z]**
D. chrysanthemi	1	1	4.00 a	1	1.67 bc
P. ananatis	51	47	3.88 a	49	3.07 ab
E. cowanii	24	12	3.42 a	21	2.96 ab
P. agglomerans	83	40	3.37 a	79	2.96 ab
B. ambifaria	2	2	3.00 ab	2	3.16 ab
B. cepacia	1	1	2.00 b	1	3.67 a
P. fluorescens	2	1	1.33 b	2	2.33 bc
P. dispersa	8	0	-	8	2.33 bc
R. aquatilis	2	0	-	1	2.66 abc
E. cloacae	1	0	-	1	2.33 bc

[x] Number of isolates tested for pathogenicity.

[y] Number of isolates were pathogenic.

[z] Virulence were determined by using 1-5 scale of disease severity was used (where 1 = <1%, 2 = 1-10%, 3 = 11-25%, 4 = 26-50% and 5 = > 50% of leave collapsed or bulb rot). Average virulence of each isolates was obtained from three replicated seedlings and bulbs. Means followed by the same letter are not significantly different by Fisher's protected least significant difference (LSD) at ($P \leq 0.05$). PARMS statement (with lower-boundary = 0.0001) was used to overcome data convergent issues as some bacterial species were represent by only one isolate.

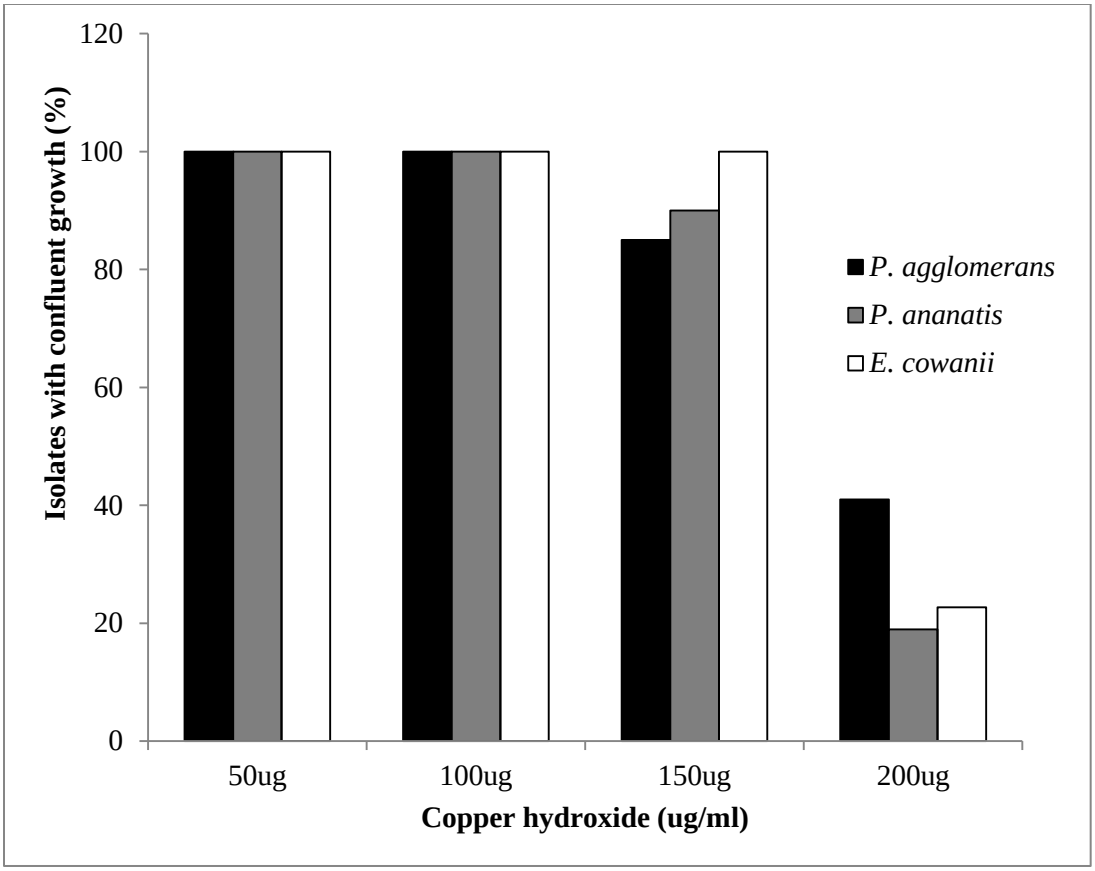


Figure 1.4 Frequency distribution of bacterial isolates with confluent growth on CYE-G medium containing 50-200ug/ml of copper hydroxide (Number of bacterial isolates tested, *P. agglomerans* (117), *P. ananatis* (58) and *E. cowanii* (22).

Table 1.4 Bacterial strains, *P. agglomerans*, *P. ananatis* and *E. cowanii* from Michigan counties tolerant to copper hydroxide at different concentrations embedded in CYE-G medium.

Location	Pathogens	No. of bacterial strains with positive growth (n=197)			
		50µg/ml	100µg/ml	150µg/ml	200µg/ml
Allegan	*P. agglomerans*	**33** (100)[a]	**32** (100)	**29** (87)	**17** (51)
	P. ananatis	**16** (100)	**16** (100)	**13** (81)	**3** (18)
	E. cowanii	**8** (100)	**8** (100)	**8** (100)	**1** (12)
Eaton	*P. agglomerans*	**13** (100)	**13** (100)	**11** (84)	**5** (38)
	P. ananatis	**6** (100)	**6** (100)	**6** (100)	**1** (16)
	E. cowanii	**2** (100)	**2** (100)	**2** (100)	**1** (50)
Ingham	*P. agglomerans*	**10** (100)	**10** (100)	**7** (70)	**3** (30)
	P. ananatis	**12** (100)	**12** (100)	**10** (83)	**1** (8)
	E. cowanii	**2** (100)	**2** (100)	**2** (100)	**0** (0)
Calhoun	*P. agglomerans*	**16** (100)	**16** (100)	**13** (81)	**4** (25)
	P. ananatis	**10** (100)	**10** (100)	**9** (90)	**1** (10)
	E. cowanii	**5** (100)	**5** (100)	**5** (100)	**3** (60)
Newaygo	*P. agglomerans*	**25** (100)	**25** (100)	**22** (88)	**13** (52)
	P. ananatis	**6** (100)	**6** (100)	**6** (100)	**2** (33)
	E. cowanii	**3** (100)	**3** (100)	**3** (100)	**0** (0)
Ottawa	*P. agglomerans*	**20** (100)	**20** (100)	**18** (90)	**6** (30)
	P. ananatis	**8** (100)	**8** (100)	**8** (100)	**3** (37)
	E. cowanii	**2** (100)	**2** (100)	**2** (100)	**0** (0)

[a]Number in parenthesis is the percentage of bacterial strains tolerant to copper hydroxide.

DISCUSSION

This study identified the bacterial species that are associated with foliar disease in the field and assessed their ability to cause disease on onion seedlings and bulbs. Their sensitivity to copper hydroxide was determined. *P. agglomerans, P. ananatis* and *E. cowanii* were most frequently isolated from the six Michigan counties that were sampled. Seven bacterial species were pathogenic on onion seedlings, while all ten tested bacterial species were pathogenic when inoculated in onion bulbs. However, except for *P. agglomerans, P. anantis* and *E. cowanii*, other number of other bacterial species were limited in our study – thereby further investigation would be necessary.

In the U.S., 14 bacterial species have been reported as common pathogens in onion (Schwartz and Mohan 2008). In Georgia, reports include *P. ananatis* (Gitaitis and Gay 1997), *P. agglomerans* (Edens et al. 2006), *Pseudomonas viridiflava* (Guillebeau 2003), *Pseudomonas* sp. (yellow bud disease) (Gitaitis et al. 2012), and *B. cepcacia* (sour skin) (Dutta et al. 2015). Major onion bacterial pathogens have been reported in Pennsylvania and include *P. agglomerans, P. ananatis, Pectobacterium carotovorum, Pseudomonas marginalis* and *B. gladioli*. Similar to our study, *P. agglomerans* was 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., Enterobacter cloacae* and *Rahnella spp.* (Beer et al. 2014). In our study, *Rahnella sp.* was also isolated and caused disease on onion bulbs, but not on the foliage. In Ontario, Canada, bacterial diseases in onions were reported to be slippery skin, sour skin, and soft rot caused by *Pseudomonas sp.* (syn: *Burkholderia*) and *Erwinia sp.*, respecitvely (syn: *Pectobacterium*) (OMAFRA 2009; Howard et al. 1994).

In Washington and Idaho, bacterial pathogens primarily cause bulb rot during storage and include *E. cloacae, B. cepacia,* and *B. gladioli* (Schroeder et al. 2010; Schroeder and du Toit 2010; B. K. Schroeder, *personal communication*). In Colorado, key onion bacterial pathogens include *B. cepacia, B. gladioli, P. carotovorum*, and *Xanthomonas axonopodis* pv. *allii* (Schwartz and Bartolo 1995). Onion leaf blight caused by *X. axonopodis* pv. *allii* is the most common and economically important foliar bacterial disease (Schwartz and Otto, 1998). Both *P. ananatis* and *P. agglomerans* are more common in Colorado, especially in areas where temperature and humidity are high. Disease caused by *P. ananatis* can reach 50 to 70% in regions where plants are damaged by rainfall and hail whereas areas with reduced rainfall and temperature have less disease (Schwartz and Otto, 2000; H. F. Schwartz, *personal communication*). The prevalence of *P. agglomerans* and *P. ananatis* in Michigan and other eastern states could be due to increased rainfall and humidity.

Previously, *P. ananatis* was found to be distributed by irrigation water, insects, and seeds (Gitaitis et al., 2003; Walcott et al. 2002). *P. ananatis* was first detected in onion in South Africa in 1981 (Hattingh and Walters 1981). In the U.S., *P. ananatis* and *P. agglomerans* were detected in Georgia in 1997 and 2006, respectively (Gitaitis and Gay 1997; Edens et al. 2006) and were believed to be introduced via infected seed from South Africa (Walcott et al. 2002).

E. cowanii was frequently isolated from infected onion plants from seven Michigan counties. This bacterium was first described by Inoue et al. (2000) as a new species in *Enterobacteriaceae* and a clinical pathogen. In plants, it was first found in association with plants as a pathogen of *Eucalyptus* that incited leaf blight and dieback in Uruguay in 2009 (Brady et al. 2009). The bacterium was reported as a pathogen on a native forest species (*Mabea fistulifera*) in Brazil in 2012 causing bacterial leaf spot (Furtado et al. 2012). Sequencing results

of their *rpoB* gene were compared in the Gene Bank by using BLAST method (National Center for Biotechnology Information [http://www.ncbi.nlm.nih.gov /BLASTn/]). Results indicated 99% identity to a *Enterobacter cowanii* strain with accession number (EU629168.1), a strain in a study of (Brady et al. 2009). Hypersensitivity response (HR) of 24 *E. cowanii* isolates on tobacco (cv. Virginia Gold) was also conducted; positive HR was consistently observed on tobacco leaves similar to that described in Brady et al. (2009).

A nonfluorescent *Pseudomonas sp*. was pathogenic and incited yellow bud disease on onions in Georgia (Gitaitis et al. 2012). Five species of lactic acid bacteria, *Lactococcus lactis, Lactococcus plantarum, Leuconostoc citreum, Leuconostoc mesenteroids, Leuconostoc pseudomesenteroids,* caused a leaf blight and bulb decay of onions in New York (Bonasera et al. 2017). In Puerto Rico, eleven bacterial genera were detected on onion foliage. Similar to our research, *Pantoea spp.* was most frequently isolated (Bellido et al. 2012).

In our study, *D. chrysanthemi, P. ananatis, P. agglomerans* and *E. cowanii* were highly virulent on onion seedlings. With the exception of *D. chrysanthemi,* these were the most prevalent species isolated from symptomatic onion plants in the field. These species were also virulent on onion bulbs, causing bulb rot at the site of inoculation, consistent to that described by Schwartz and Mohan (2008). *P. ananatis* infects onion leaves, moving through the neck and causing center rot in storage (Carr et al. 2013).

B. ambifaria, B. cepacia, and *E. cloacae* are commonly associated with bulb infection in storage (Schroeder et al. 2013; Schwartz and Mohan 2008) but were not commonly detected in our study. Jacobs et al. (2008) described that *Burkholderia* spp. are likely to be detected from the soil of the onion rhizosphere and roots.

Copper resistance among plant pathogenic bacteria has occurred worldwide (Voloudakis et al. 1993; Sundin et al. 1989; Martin et al. 2004; and Shenge et al. 2014). Gitaitis (2006) reported copper-tolerant strains of *P. ananatis* infecting onions in Georgia. Between 60-80% of bacterial strains isolated from different locations in Georgia exhibited partial growth on copper-amended medium (Gitaitis 2006). However, they were tested with copper sulfate pentahydrate (200ug/ml) embedded in nutrient agar (NA) medium, which consists approximately 25% of metallic copper. In our study, (200ug/ml) of copper hydroxide, consisting of 46.1% of metallic copper, and CYE-G medium (a low Cu^{2+} 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. A study conducted by (Nischwitz et al. 2007) used fatty acid methyl ester profiles to detect copper-tolerant strains of *P. ananatis*. Copper sulfate pentahydrate at (200ug/ml) was used in their study. Nischwitz et al. (2007) indicated that it is not clear whether copper-tolerant strains at this concentration were sufficient enough to be considered resistant to the recommended rate of copper sprayed in the field. Copper sprays were not effective in the field trials where the *P. ananatis* tolerant strains to copper sulfate at 200ug/ml were detected (Nischwitz et al., 2007). Our study suggests that using copper hydroxide may not limit bacterial pathogens in Michigan onion fields.

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APPENDIX

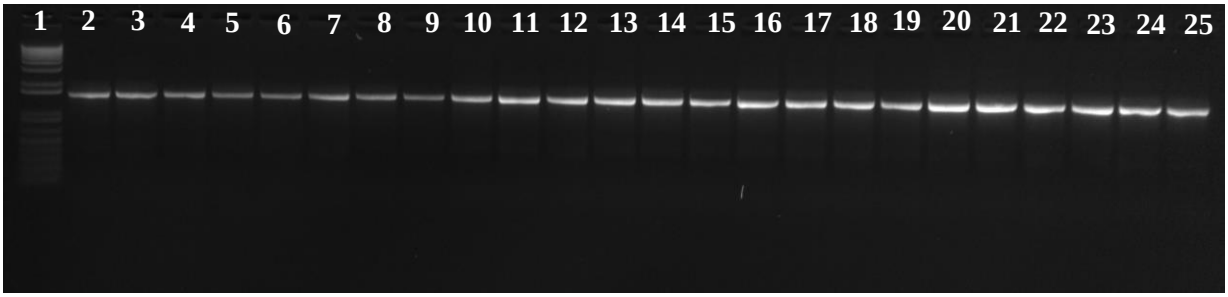

Figure A1. PCR fingerprint pattern of 16s rDNA genes by using universal primers previously described in (Vaneechoutte et al. 2000; Baere et al. 2004); Lane 1 ($1kb^{+}$ marker) and Lane 2-11 *P. agglomerans*; Lane 12-21: *P. ananatis*; Lane 22-25: *E. cowanii.* The product sizes are about 1500bp.

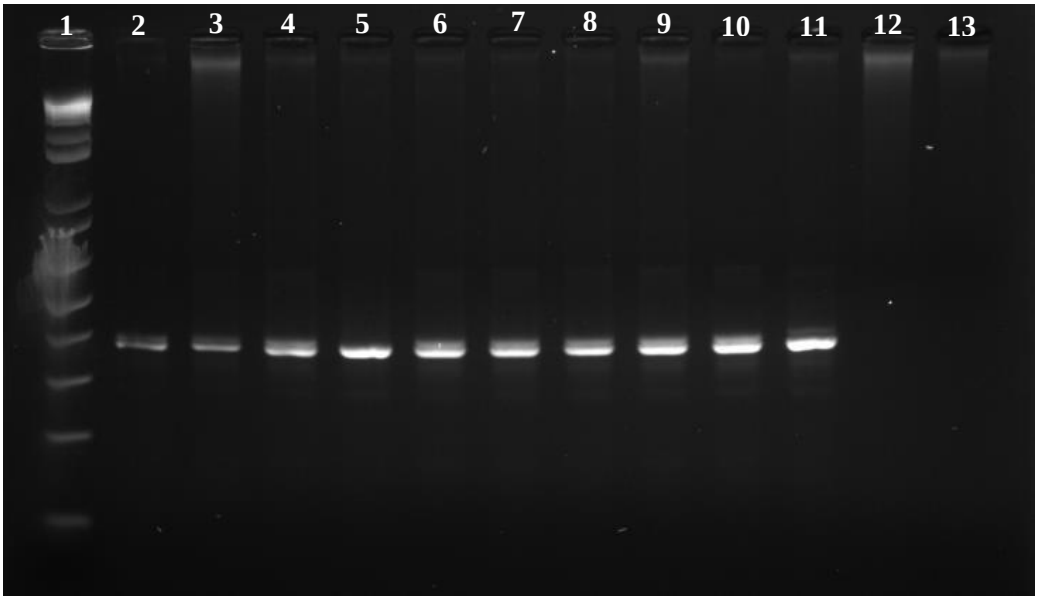

Figure A2. Identification of *P. ananatis* by using diagnostic primers PanITS1 and EC5 Lane 1: Marker; Lane 2-11: *P. ananatis*; Lane 12: *P. agglomerans*; Lane 13: *E. cowanii*

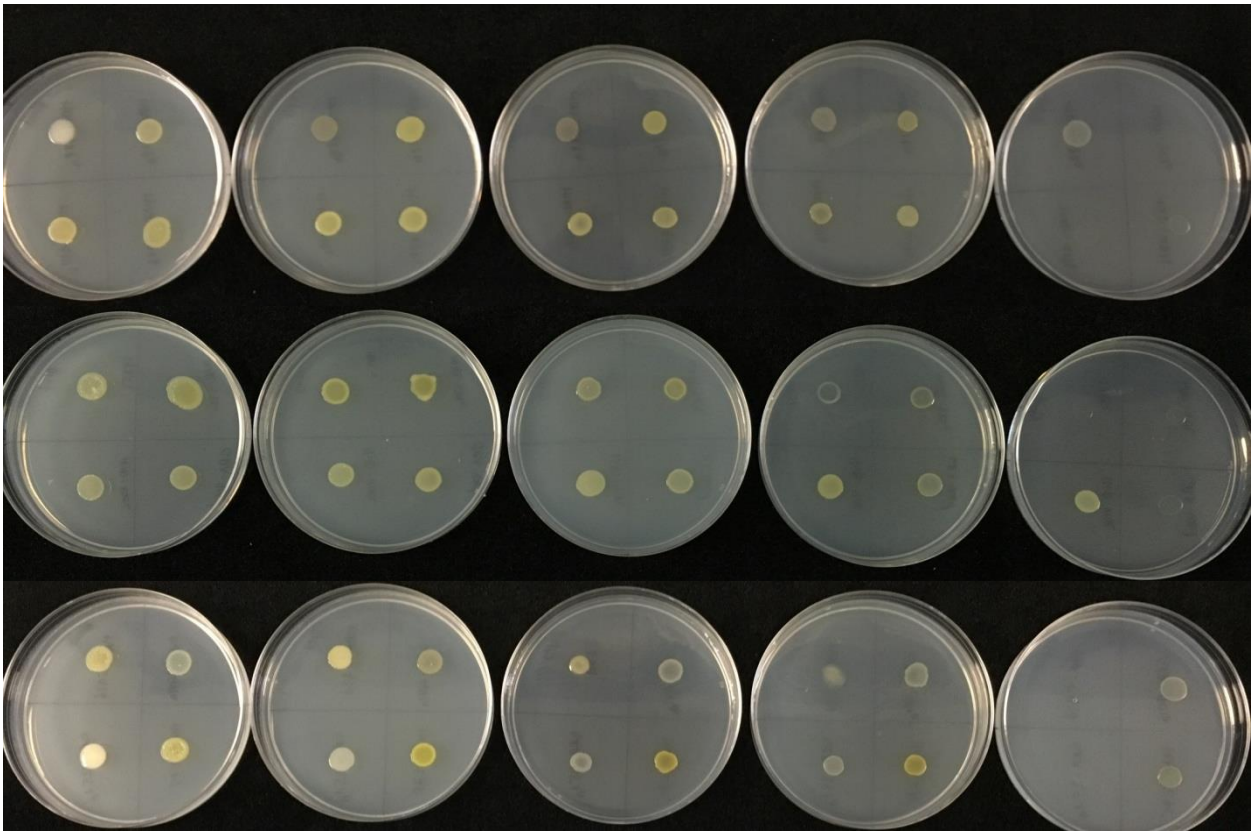

Figure A3. Copper hydroxide sensitivity tests: 10µl of approximately 10^8 CFU/ml of each bacterial isolates was pipetted on CYE-G medium embedded with 0, 50, 100, 150 and 200µg/ml of copper hydroxide (46.1% of metallic copper). The plates were incubated for 48h at 30°C and visible confluent growth was interpreted as tolerant. A total of 197 bacterial isolates were tested with three replicated plates each.

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CHAPTER II: EFFECTS OF TEMPERATURE, RELATIVE HUMIDITY AND PLANT AGE ON BACTERIAL DISEASE OF ONION PLANTS

ABSTRACT

The effect of temperature, relative humidity (RH) and onion plant age when inoculated with *Pantoea agglomerans, Pantoea ananatis* or *Enterobacter cowanii*, were examined in growth chamber and greenhouse experiments. These bacteria resulted in a significant level of disease at temperatures of 25 to 30^{o}C. Regressions between area under disease progress curve (AUDPC) and temperature were described by linear models and significant positive associations were detected for all bacteria (R^2=0.79, 0.70, and 0.77, at P <0.0001 for *P. agglomerans, P. ananatis* and *E. cowanii*, respectively). RH significantly influenced bacterial disease development (P <0.0001). Disease progressed rapidly on onion foliage from 80 to 100% RH but was limited at RH <60%. The association between RH and AUDPC described by linear regression was significant (P <.0001) and positive (R^2=0.45, 0.52, and 0.61) for *P. agglomerans*, *P. ananatis*, and *E. cowanii*, respectively. Plants aged between 6 to 14 weeks old and inoculated with bacterial pathogens were significantly (P <.0001) more susceptible to *P. agglomerans* (R^2=0.51), *P. ananatis* (R^2=0.53) and *E. cowanii* (R^2=0.54) as they aged. These results provide insight regarding the epidemiology of three bacterial pathogens of onions.

Keywords: *Pantoea agglomerans, P. ananatis, Enterobacter cowanii, temperature, humidity, age-related resistance*

INTRODUCTION

Michigan produces approximately 37 million kilograms of onion annually, ranking eleventh in the U.S. (NOA, 2016). Most of the state's onions are pungent long-day varieties with moderate to long-term storage capabilities (Schwartz 2013). Since 2011, bacterial diseases including *Pantoea agglomerans* and *Pantoea ananatis* have occurred in Michigan affecting onions in the field resulting in significant losses (Hausbeck, 2014). *P. agglomerans* (syn. *Erwinia herbicola*) was first reported in onion grown for seed production in South Africa in 1981 (Hattingh and Walters 1981). In the U.S., *P. agglomerans* was first reported as a pathogen causing leaf blight and rot diseases in onions in Georgia in 2004 (Edens et al., 2006), in New York in 2010 (Beer et al., 2010) and in Pennsylvania in 2014 (Gugino and Pfeufer, 2015; Pfeufer, 2014). In Michigan, *P. agglomerans* was first reported in 2013 as the causal agent of leaf blight and bulb rot in onion (Tho et al., 2015). *P. ananatis* was first reported in Georgia in 1997 and was associated with losses up to 100% (Gitaitis and Gay, 1997). Outbreaks of *P. ananatis* in onion were also reported in Colorado (Schwartz and Otto, 2000), New York (Carr et al., 2010), and Michigan (Hausbeck, 2014). *P. ananatis* could also cause center rot disease in storage which frequently associated with other secondary microbe that could liquidify storage bulbs and produce malodor. Walcott et al. (2002) reported that *P. ananatis* could be transmitted by seeds. *E. cowanii* was frequently isolated from diseased onion plants in Michigan in 2013 and 2014 (Tho et. al. unpublished data). On other plants, general symptoms caused by *E. cowanii* include leaf spot and blight (Brady et al., 2009; Furtado et al., 2012).

Schwartz et al. (2003) reported that *Pantoea* leaf blight is favored by prolonged periods of rain, high relative humidity, and warm temperatures (28 to 35^{o}C) during the bulbing stage (Schwartz et al., 2003). An outbreak of bacterial leaf blight and rot in Michigan in 2012 was also

believed associating with extraordinary warm temperature and prolonged periods of rainfall. Despite this anecdotal observation, there has not been any direct study on these aspects, especially on *P. agglomerans* and *E. cowanii*.

Age related resistance studies have been conducted with onion, including purple blotch incited by *Alternaria porri* (Miller, 1983), pink root incited by *Setophoma terrestris* (Wiriyajitsomboon, 2015), and Fusarium basal rot incited by *Fusarium oxysporum* f. sp. *cepae* (Cramer, 2000). The objective of this study was to determine the influence of temperature, relative humidity, and plant age on *P. agglomerans, P. ananatis*, and *E. cowanii* infection of onion.

MATERIALS AND METHODS

Bacterial inoculum. Isolates of *Pantoea agglomerans* (STO14), *Pantoea ananatis* (PA-49) and *Enterobacter cowanii* (PA-42) were obtained in 2014 from symptomatic onion leaves collected from commercial plantings located in the Michigan counties of Ingham, Allegan, and Eaton counties, respectively. These isolates were confirmed to be virulent on onion seedlings and bulbs. *P. agglomerans* and *E. cowanii* were identified by using BIOLOG (Hayward, CA) and sequencing of the 16s rDNA using the universal primers (forward 5′-AGTTTGATCCTGGCTCAG-3′ and reverse 5′-TACCTTGTTACGACTTCGTCCC A-3′) (Baere et al., 2004). *P. ananatis,* was identified using diagnostic primers, PanITS1 (5’-GTCTGATAGAAAGAT-AAAGAC-3’), EC5 (5’-TGCCAGGGCATCC ACCG-3’) (Gitaitis et al., 2002) and sequencing the 16s rDNA using the method and primers described above. The *E. cowanii* strain was also identified by using sequencing analysis of *rpoB* gene as described previously (Brady et al., 2008; Brady et al., 2009). The bacteria were grown overnight on

nutrient broth yeast extract agar (NBY) at approximately 30^{o} C in the dark. The bacterial suspensions were prepared using sterile deionized water and adjusted spectrophotometrically to approximately 10^{8} CFU/ml.

Effect of temperature. 'Bradley' onion seeds (Bejo Seeds, Inc., Oceano, CA) were planted in 98-cell flats (Hummert International, Earth City, MO) with SUREMIX perlite media (Michigan Grower Products, Inc., Galesburg, MI) and grown in raised plant benches in the MSU Plant Sciences Research Greenhouses for seven wks. 'Bradley' is widely grown in Michigan and bacterial disease symptoms have often associated with this cultivar. Temperatures in the greenhouse were 22 ± 4.27 ^{o}C. Plants were fertilized daily with 200 ppm of 20-20-20 of Peters water soluble fertilizer (The Scotts Company, Marysville, OH). Seedlings were transplanted into 10-cm-square pots and grown in the greenhouse for one wk before inoculation. Eight wk-old plants with 4 to 5 true leaves were inoculated using the method of Wright and Grant (1998) with the following modifications. Seedlings were cut back to 15 cm and bacterial inoculum (10^{8} CFU/ml) was sprayed on the foliage until runoff. Plants sprayed with distilled sterile water were used as a control. Each plant was covered with a clear plastic bag (20 x 10 x 45 cm) and 300 ml of water soluble fertilizer was added to the bottom of the bag to promote high relative humidity (RH) during incubation. A hand-made wire frame was put inside of the plastic bag to prevent direct contact of onion leaves with the bag.

Inoculated plants were placed in growth chambers (model SPC-7-2H (BioChamber Inc., Canada) and Convrion model: E7/2 (Controlled Environment Inc., Phembina, ND) set at 15^{o}C, 20 ^{o}C, 25 ^{o}C or 30 ^{o}C, with a 14-h photoperiod. A limited number of growth chambers were available so the experiment was conducted as an incomplete block by repeating the experiment several times until three replicates of each temperature were completed. Each run consisted of

two growth chambers and two temperatures. The experiment was a split plot experimental design with two factors (four temperatures as main plots, and three bacterial species and sterile water as sub-pots). Twenty onion plants (five for each level of sub-plot) were arranged in a complete randomized design; each plant was a sub-sample. A watchdog (Spectrum Technologies, Inc., Plainfield, IL) was placed inside the growth chamber to record the temperature and RH.

Effect of relative humidity. Plant and inoculum preparation were as described in the temperature study. Two growth chambers (model SPC-7-2H (BioChamber Inc., Canada) and Convrion model: E7/2 (Controlled Environment Inc., Phembina, ND) maintained at 25^{o}C with fluorescent lighting (14h/day) were used to test RH of 35, 60, 70, 80, and 100%. The experiment was arranged as a split plot design using two growth chambers, each set at a different RH. Five onion plants were inoculated individually with each of three bacteria and sterile water as a control. The twenty inoculated plants were arranged randomly in each growth chamber. Plants were inoculated using the method previously described but plastic bags were not used to cover the plants. A wire-frame was used to minimize contact of inoculated plants with each other. Each inoculated plant was placed directly onto a 13-cm saucer containing one-half strength of Hoagland solution liquid fertilizer. The experiment was conducted several times to achieve three replicates for each RH level. A watchdog (Spectrum Technologies, Inc., Plainfield, IL) was placed inside the growth chamber to record temperature and RH data.

Effect of plant age. 'Bradley' onions were directly seeded into approx. 10-cm-square pots (three seeds/pot) containing SUREMIX perlite media (Michigan Grower Products, Inc., Galesburg, MI). Plants were grown in the MSU Plant Sciences Research Greenhouses and fertilized daily with 200 ppm of 20-20-20 of Peters water soluble fertilizer (The Scotts Company, Marysville, OH). Four weeks after seeding, plants in each pot were thinned to one seedling and maintained in

a greenhouse to achieve 6-, 8-, 10-, 12- and 14-wk-old plants for inoculation. The inoculation was accomplished as described above. This experiment was a five-by-four factorial design (plant age x three bacteria and water control). Inoculated plants were arranged in a greenhouse in a randomized complete block design (RCBD) with eight replicate plants. The experiment was repeated.

Disease assessment and data analysis. Plants were assessed visually for disease severity every other day for 10 days following inoculation (DAI) using a rating of 1 to 5 (Schwartz 2013) to quantify leaf collapse as follows: 1 = <1%, 2 = 1-10%, 3 = 11-25%, 4 = 26-50%, 5 = > 50%. The area under the disease progress curve (AUDPC) (Jeger and Viljanen-Rollinson 2001) was calculated using Microsoft Excel. The AUDPC data were subjected to split-plot analyses of variance (ANOVA) for the temperature and RH studies, and randomized completed block design (RCBD) for the effect of plant age, by using PROC MIXED in SAS 9.4 (SAS Institute, Cary, NC). The normality assumption of each data set was investigated by observing studentized residual plots, residual vs. predicted mean plots and percentage of residual distribution histogram. Homogeneity assumptions were checked by using Levene's test ($P<0.05$) and comparing AIC values from both equal and unequal variance analyses. Means were separated using Fisher's protected least significant different (LSD) at ($P<0.05$). Regression analyses were also performed to quantify the relationship among the effects of temperature, RH and plant age versus AUDPC data by using PROC REG in SAS 9.4 (SAS Institute, Cary, NC). Residual plots were also observed in checking the pattern of relationship, before linear regressions were fitted for analyses.

RESULTS

Leaf blight symptoms including water-soaked lesions were observed on inoculated plants. At 25 and 30^{o}C, disease symptoms were observed two days after incubation; four days were required at 15 and 20^{o}C (*data not shown*). Temperature had a significant effect on the incubation period and disease severity regardless of bacterium. AUDPC data were significantly different when inoculated plants were incubated at different temperatures (P <.0001). Temperature-by-pathogen interaction was also significant (P = 0.0309). When plants were inoculated with *P. agglomerans*, AUDPC ranged from 21.07 ± 0.73 (means ± S.E) to 37.47 ± 0.70 for inoculated plants incubated at 15 and 30^{o}C, respectively. For inoculation with *P. ananatis*, means of AUDPC were between 22.27 ± 1.01 and 37.53 ± 0.66; whereas inoculation with *E. cowanii* ranged from 24.93 ± 0.88 to 39.80 for 15^{o}C and 30^{o}C incubation, respectively. Regardless of bacteria, temperatures of 25^{o}C and 30^{o}C were favorable for disease; AUDPC were significantly higher for these temperatures than for 15^{o}C and 20^{o}C (Table 2.1). Regressions between AUDPC and temperature were described by linear models and significant (P <0.0001) positive associations were detected for *P. agglomerans* R^2=0.79, *P. ananatis* R^2= 0.70 and *E. cowanii* R^2=0.77. These models were described by *AUDPC* = 1.45 + 1.21 *Temp* for *P. agglomerans; AUDPC* = 8.16 + 1.11 *Temp* for *P. ananatis* and *AUDPC* = 8.16 + 1.11 *Temp* for *E. cowanii*. The positive linear relationship across the bacterial pathogens suggests that increasing temperatures increases disease severity (Fig. 2).

Regardless of RH, most inoculated plants exhibited symptoms two days after incubation. Significant differences of AUDPC were detected for inoculated plants incubated at different RH levels and among the three bacterial pathogens (P<0.0001). A significant interaction was noted

between RH levels and the bacteria (P<0.0001). When plants were inoculated with *P. agglomerans,* AUDPC values ranged from 19.6 ± 0.75 to 29.6 ± 0.79, when incubated at 35 and 100% RH, respectively. AUDPC data when plants were incubated at 70 or 80% RH were similar. Inoculated plants incubated at 100% RH had the highest disease severity (Table 2.1). When plants were inoculated with *P. ananatis* and incubated at 100% RH, the AUDPC was significantly higher than that associated with other RH levels. When inoculated plants were incubated at 80% and 70%, AUDPC values were not significantly different from each other, but were significantly reduced as compared to the incubationat 100% RH. For RH levels at 60% and 35%, AUDPC values were not significantly different from each other, but were the lowest as compared to other RH levels. When plants were inoculated with *E. cowani* and incubated at 100% RH, the AUDPC was significantly higher than those for other RH levels. AUDPC data for incubation at 80 and 70% RH were 32.66 ± 1.32 and 27.80 ± 0.88, respectively; and differed significant differently according to Fisher's protected LSD (P<0.05). Incubation at 35 and 60% RH had the lowest AUDPC values, and were similar to each other according to Fisher's protected LSD (P<0.05) (Table 2.2).

The relationship between AUDPC vs. RH as described by linear regression equations were: $AUDPC$ = 12.45+ 0.15 RH. (R^2=0.45 at P <.0001), $AUDPC$= 11.52 + 0.18 RH. (R^2=0.52 at P <.0001), and $AUDPC$ = 12.24 + 0.24 RH. (R^2=0.61 at P <.0001) for *P. agglomerans, P. ananatis*, and *E. cowanii,* respectively. Based on these regression analyses, all slopes were significantly higher than zero, with R^2 between 0.45 and 0.61 (Fig. 2.3).

Regardless of plant age, symptoms were observed 2 days after inoculation. Overall, plant age had a significant effect on disease severity for the three bacterial pathogens (P<.0001); disease severity progressed more quickly as plants aged. Plant age-by-pathogen interaction was

also significant (P<.0001) (Fig. 2. 2). Six-week-old plants had significantly lower AUDPC values compared with older plants. When plants were 12 to 14 weeks-old when inoculated, the AUDPC was significantly higher than that of younger plants, but were not significantly different from each other. Relationship between AUDPC vs. plant age as described by linear regression equations were: *AUDPC* = 22.42 + 1.39 *Plant Age.* (R^2=0.51 at P <.0001), *AUDPC*= 22.49+ 1.40 *Plant Age.* (R^2=0.53 at P <.0001), and *AUDPC* = 26.72+ 1.13 *Plant Age.* (R^2=0.54 at P <.0001) for *P. agglomerans, P. ananatis*, and *E. cowanii*, respectively (Fig. 2.4).

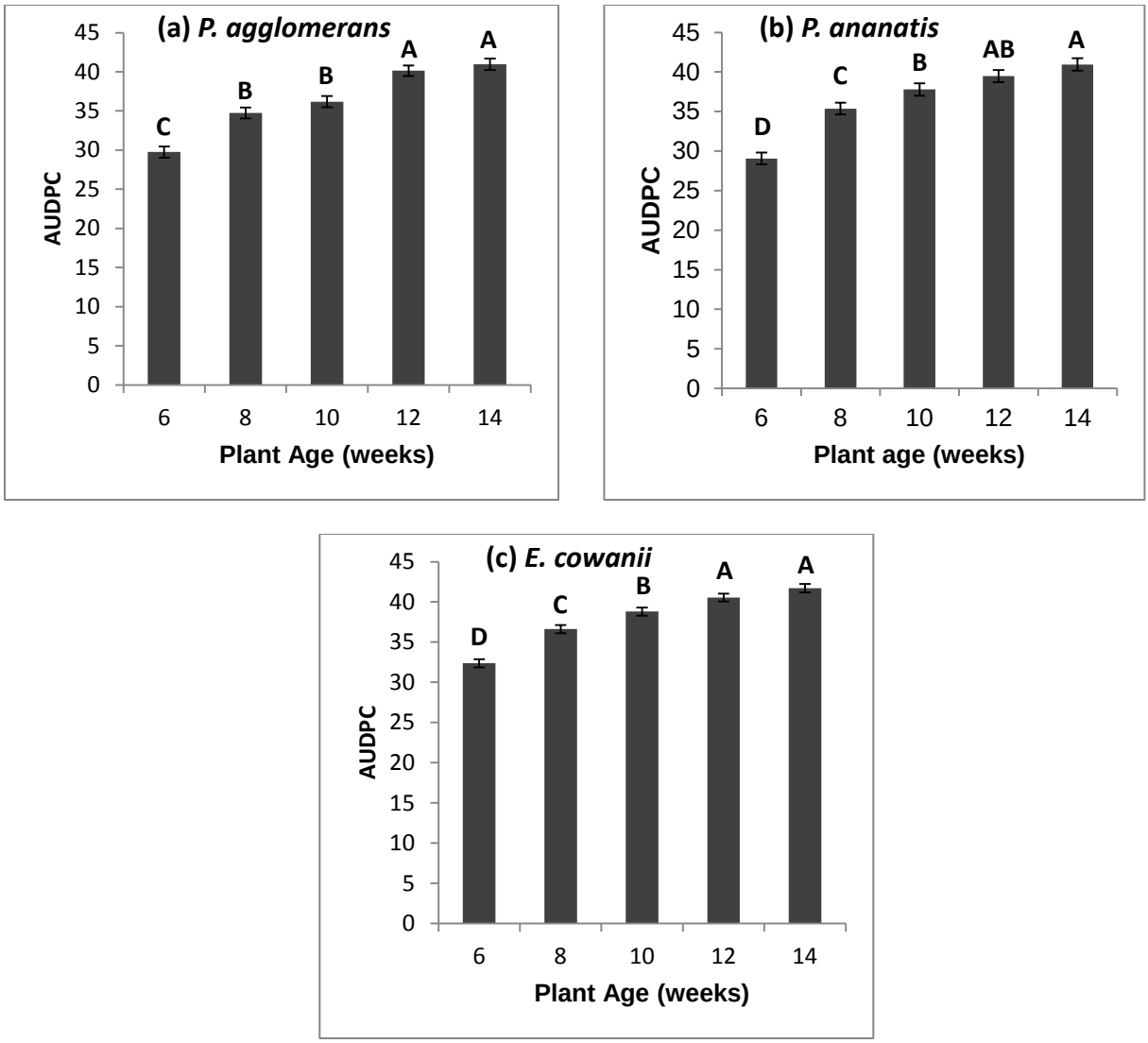

Figure 2.1 Effect of plant age on disease development when plants were inoculated with (a) *P. agglomerans*, (b) *P. ananatis* or (c) *E. cowanii*. Results are means of area under disease progress curve (AUDPC) with 16 replicated plants for each age. Data from repeated experiments were combined. Vertical bars represent the standard error. Means followed by the same letter are not significantly different based on Fisher's protected least significant difference (LSD) at ($P \leq 0.05$).

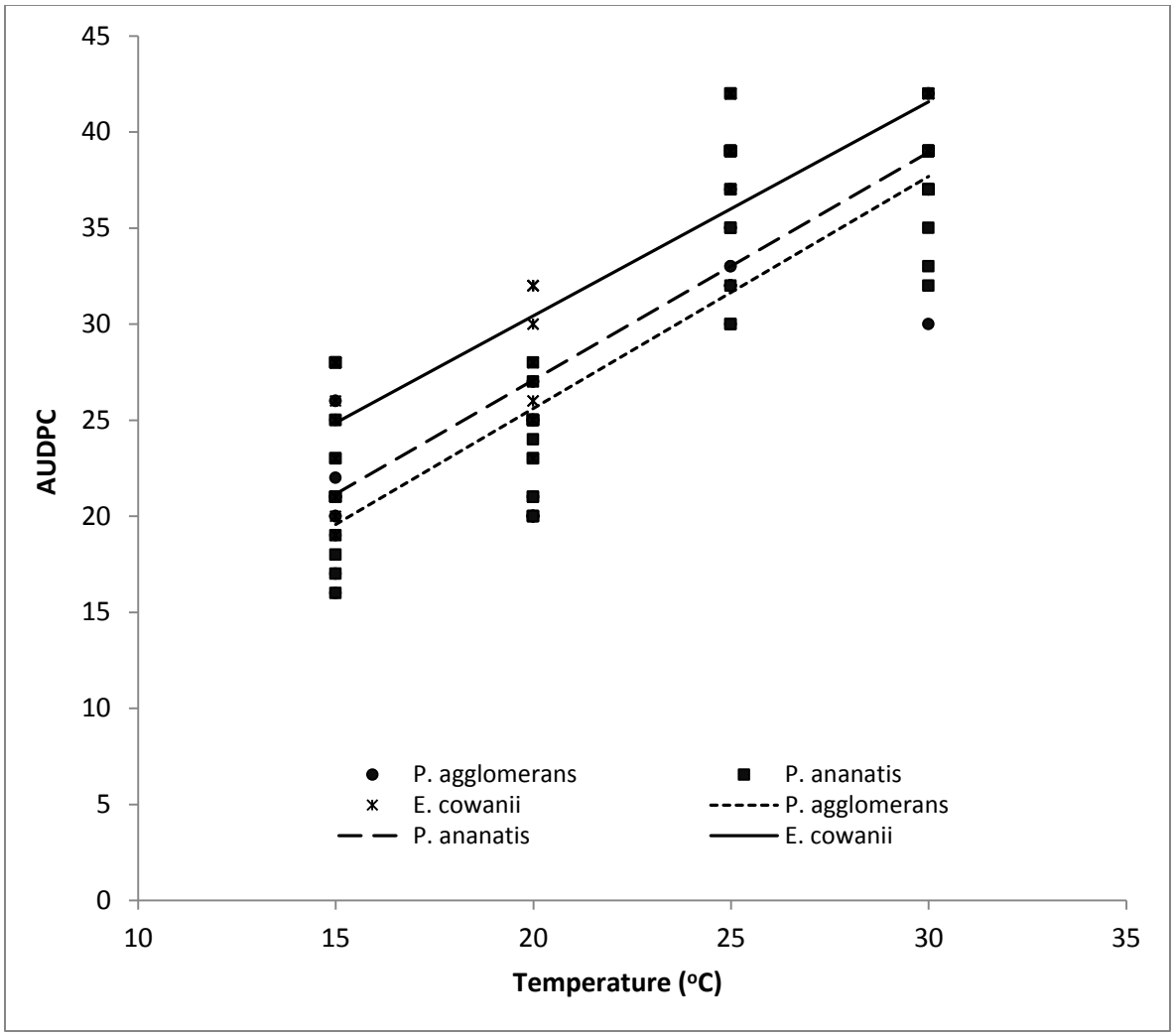

Figure 2.2 Relationship between AUDPC and incubation temperature as described by linear regression equations: *AUDPC* = 1.45 + 1.21 *Temp.* (R^2=0.80 at *P* <.0001), *AUDPC*= 3.38 + 1.18 *Temp.* (R^2=0.70 at *P* <.0001), and *AUDPC* = 8.16 + 1.11 *Temp.* (R^2=0.77 at *P* <.0001) for *P. agglomerans, P. ananatis* and *E. cowanii,* respectively. Each point represents the AUDPC value of each inoculated plant (subsample), calculated by rating the disease severity using a 1-5 scale (where 1 = <1% of leave collapsed, 2 = 1-10% of leave collapsed, 3 = 11-25% of leave collapsed, 4 = 26-50% of leave collapsed, 5 = > 50% of leave collapsed).

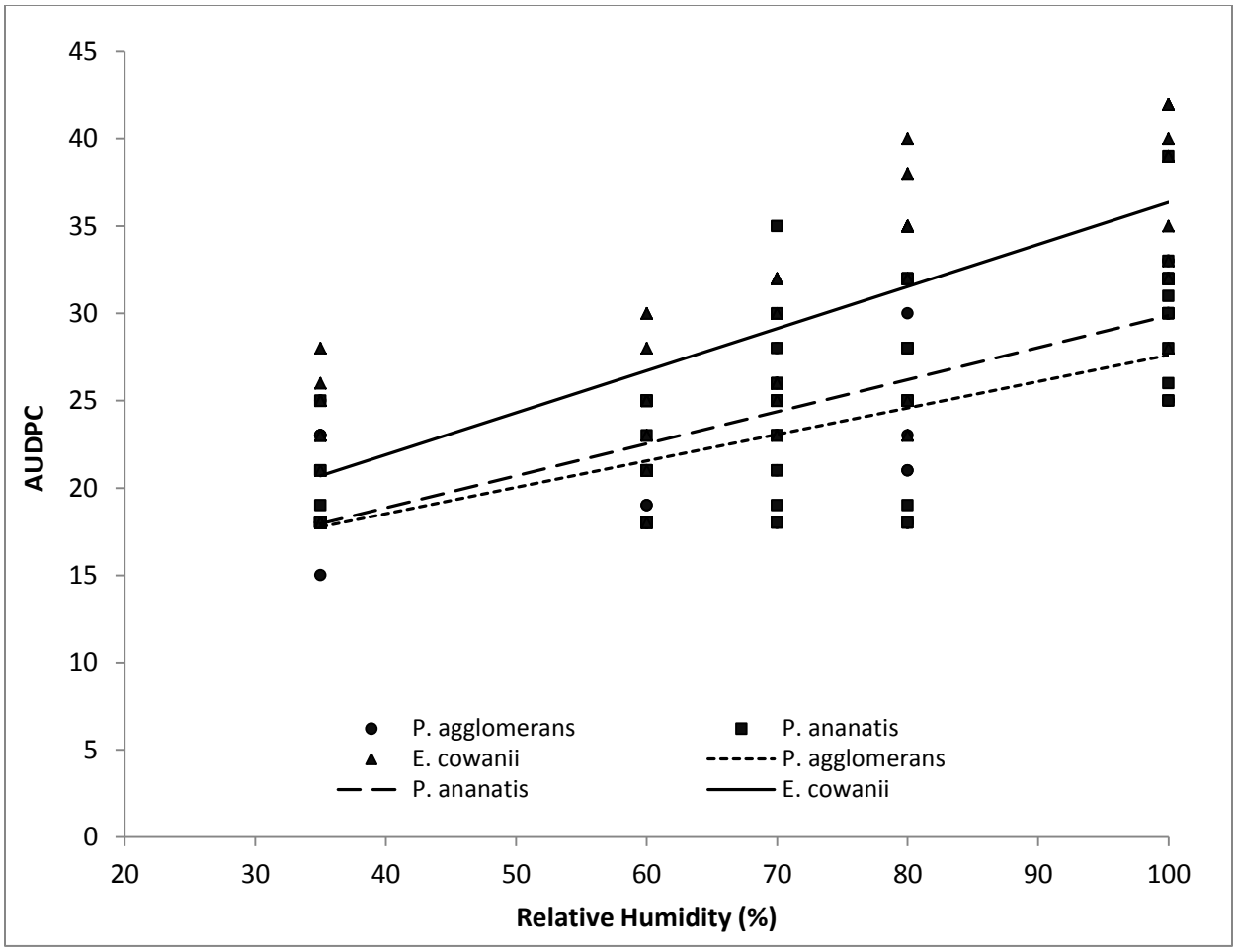

Figure 2.3 Relationship between AUDPC and relative humidity as described by linear regression equations: *AUDPC* = 12.45+ 0.15 *Humidity*. (R^2=0.45 at *P* <.0001), *AUDPC*= 11.52 + 0.18 *Humidity*. (R^2=0.52 at *P* <.0001), and *AUDPC* = 12.24 + 0.24 *Humidity*. (R^2=0.61 at *P* <.0001) for *P. agglomerans, P. ananatis* and *E. cowanii*, respectively. Each point represents the AUDPC value of each inoculated plant (subsample), calculated by rating the disease severity using a 1-5 scale of disease severity (where 1 = <1% of leave collapsed, 2 = 1-10% of leave collapsed, 3 = 11-25% of leave collapsed, 4 = 26-50% of leave collapsed, 5 = > 50% of leave collapsed).

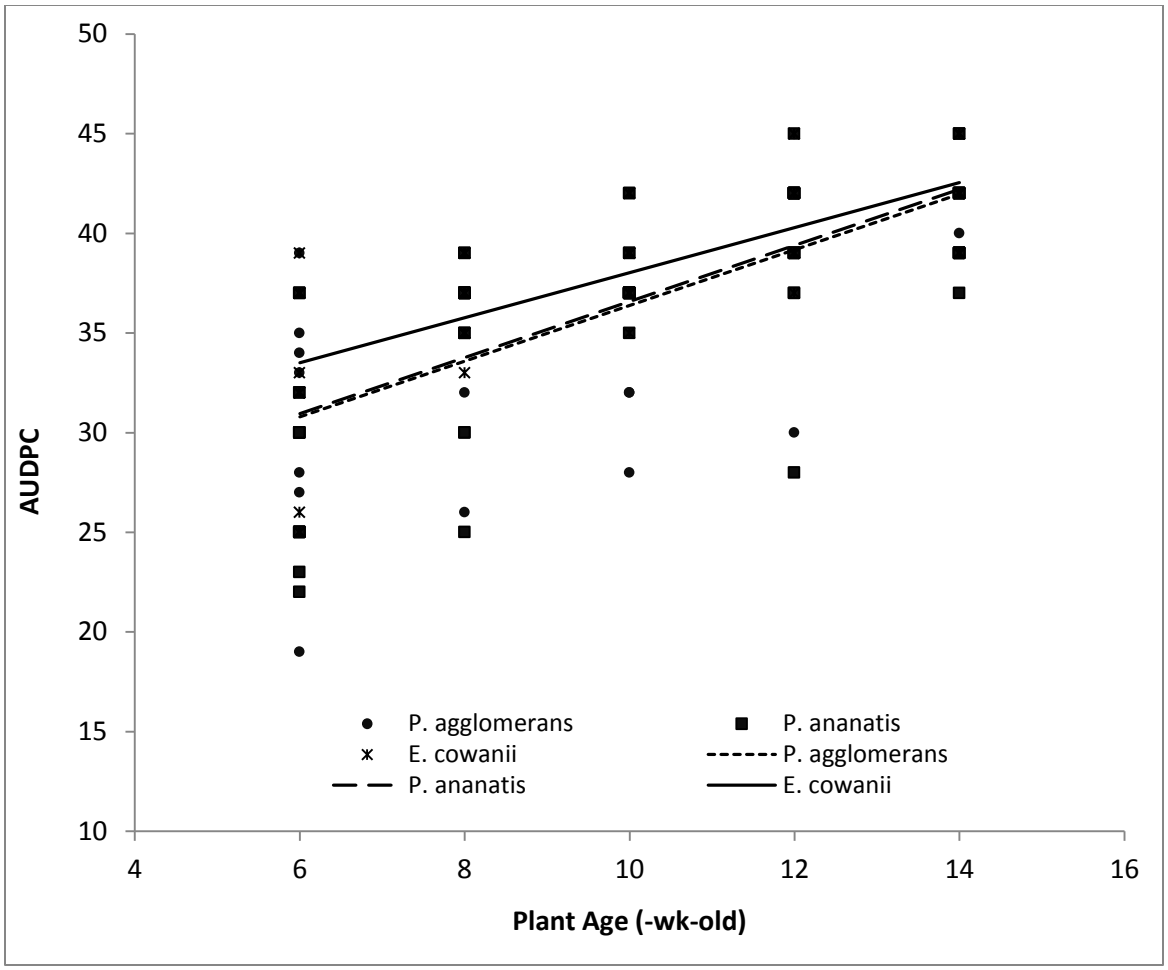

Figure 2.4 Relationship between AUDPC vs. plant age as described by linear regression equations: *AUDPC* = 22.42 + 1.39*Plant Age.* (R^2=0.51 at *P* <.0001), *AUDPC*= 22.49+ 1.40*Plant Age.* (R^2=0.53 at *P* <.0001), and *AUDPC* = 26.72+ 1.13 *Plant Age.* (R^2=0.54 at *P* <.0001) for *P. agglomerans, P. ananatis* and *E. cowanii,* respectively. Each point represents the AUDPC value of each inoculated plant, calculated by rating the disease severity in every other day for 10 days post inoculation. Each plant age and pathogen had 16 replicates, data of repeated experiments was combined. A disease severity scale was used (where 1 = <1% of leave collapsed, 2 = 1-10% of leave collapsed, 3 = 11-25% of leave collapsed, 4 = 26-50% of leave collapsed, 5 = > 50% of leave collapsed).

Table 2.1 Effect temperature on disease severity on onion plants when inoculated with *P. agglomerans, P. ananatis* or *E. cowanii*

Targeted Temp (^oC)[a]	Mean ±S.D[b]	AUDPC[c]		
		P. agglomerans	***P. ananatis***	***E. cowanii***
15	14.72 ± 0.14	21.07 ± 0.73 a	22.27 ± 1.01 a	24.93 ± 0.88 a
20	19.78 ± 0.11	22.40 ± 0.73 a	23.47 ± 0.66 a	28.53 ± 0.80 b
25	25.17 ± 0.12	33.60 ± 0.68 b	36.93 ± 0.98 b	39.60 ± 0.49 c
30	30.64 ± 0.27	37.47 ± 0.70 c	37.53 ± 0.66 b	39.80 ± 0.35 c

[a]Targeted temperatures set on growth chambers (^oC)

[b]Means of actual temperature obtained hourly from growth chamber ± standard deviation

[c]Means of area under disease progress curve (AUDPC) ± standard error (S.E). Means followed by the same letter in each column are not significantly different by Fisher's protected least significant different (LSD) ($P \leq 0.05$).

Table 2.2 Effect of relative humidity (RH) on disease severity of onion plants when inoculated with *P. agglomerans, P. ananatis* or *E. cowanii*

Targeted RH (%)[a]	Mean ±S.D[b]	AUDPC[c]		
		P. agglomerans	***P. ananatis***	***E. cowanii***
35	41.7 ± 1.80	19.6 ± 0.75 a	18.93 ±0.48 a	22.13 ± 0.88 a
60	60.4 ± 2.00	19.20 ± 0.51 a	21.0 ± 0.76 a	24.86 ± 0.79ab
70	71.2 ± 2.00	23.46 ± 0.83 b	24.2 ± 1.19 b	27.80 ± 0.88 b
80	80 ± 0.55	22.73 ± 1.08 b	26.33 ±1.27 b	32.66 ± 1.32 c
100	97.5 ± 1.70	29.6 ± 0.79 c	30.46 ± 0.98 c	37.06 ± 1.19 d

[b]Means of actual RH obtained hourly from growth chamber ± standard deviation

[c]Means of area under disease progress curve (AUDPC) ± standard error (S.E). Means followed by the same letter in each column are not significantly different by Fisher's protected least significant different (LSD) ($P \leq 0.05$).

DISCUSSION

Severity of bacterial disease on onion foliage was influenced by temperature, RH and plant age. Older plants in post bulb-growth stage, incubated between 25 and 30^{o}C at RH >80% had increased disease severity and AUDPC. Schwartz et al. noted a relationship between temperature and rainfall in the development of *Xanthomonas* and *Pantoea* leaf blights of onion in Colorado. The severity of disease associated with these pathogens increased dramatically at 28 to 35^{o}C and high cumulative rainfall during the bulbing stage (Schwartz et al., 2003). Paulraj and O'Garro indicated that temperatures of 25 to 30^{o}C and RH of 85 to 95% contributed to the outbreak of *Xanthomonas* leaf blight in onion in Barbados, Caribbean (Paulraj and O'Garra 1993). Effect of temperature and RH on bacterial soft rots in onions caused by *Dickeya, Erwinia, Pectobacterium* and *Pseudomonas* species have also been noted (Schroeder et al. 2013); warm temperatures and RH greater than 70% favor disease development in storage. For *Enterobacter* bulb decay incited by *E. cloacae*, disease development is exacerbated by curing temperature above 94^{o}F (~34^{o}C) (Schroeder and du Toit 2010). In this study, optimum temperatures for disease development were 25 to 30^{o}C. The temperature of 35^{o}C included in a study by Schwartz et al. (2003), was not included in our study since the growth chambers used in our study could not be elevated beyond 70%, and temperatures $\geq$ 35^{o}C in Michigan onion fields are not typical. Based on regression analyses for the effect of temperature and RH, our study agreed with (Schwartz et al., 2003) (as measured by growing degree day in their research) and (Salamanca 2013); when temperature and RH increases, disease develops faster.

'Bradley' onion used in our study is a long-day Spanish type with a relative maturity of approximately 118 days. Plant ages tested in our trial ranged from 6 to 14 wk, when plants had 3 to 4 true leaves and post-bulb growth stages (Schwartz 2013). Bacterial disease incidence in the

field increases in late June or early July, approximately 10 wks after planting (Schroeder et al., 2013). Bacterial isolates used in our study were highly virulent.

A preventive spray strategy by using copper-based bactericides on a 5 to 7-day interval have been used as a common effective bacterial disease management in onions (Schwartz et al., 2003 and M. K. Hausbeck, personal communication). Temperature between 25 and 30^{o}C, and high (>80%) RH could indicate vulnerability to *P. agglomerans, P. ananatis* and *E. cowanii*.

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CHAPTER III: RESPONSE OF LONG-DAY ONION CULTIVARS TO FOLIAR BACTERIAL BLIGHT

ABSTRACT

Long-day onion cultivars were evaluated in a research greenhouse for susceptibility to foliar blight incited by *Pantoea agglomerans, Pantoea ananatis*, or *Enterobacter cowanii*. The area under the disease progress curve (AUDPC) data differed significantly among cultivars tested in each of two trials ($P<0.0001$). In Trial 1, when onion cultivars inoculated with *P. agglomerans*, 'Sherman' was less diseased than many cultivars but similar to 'Bradley', 'Mandras', 'Moondance', 'Hamlet' and 'Red-defender'. For Trial 2, cvs. Highlander and Bradley were similar in their susceptibility. When plants were inoculated with *P. ananatis,* AUDPC values among tested onion cultivars were significantly different in both trials ($P=0.0177$ and $P = 0.0017$ for Trial 1 and 2, respectively). For both trials, 'Highlander' and 'Delgado' were more susceptible than 'Sherman'. AUDPC values among cultivars differed significantly among onions cultivars when inoculated with *E. cowanii* ($P = 0.0139$ for Trial 1 and $P < 0.0001$ for Trial 2). In Trial 1, 'Highlander' and 'Delgado' were significantly more diseased than 'Sherman'. However, in Trial 2, differences among these cultivars were not detected. Overall, cv. 'Sherman', 'Mandras' and 'Moondance' displayed more frequently partial resistance, whereas 'Highlander' and 'Delgado' were often more susceptible to the three tested bacterial pathogens causing leaf blight and rot in onions.

Keywords: *disease resistance, foliage bacterial disease, long-day onions*

INTRODUCTION

Michigan growers produced more than 37,000 metric tons of onions annually, valued at approximately $11 million (NOA, 2016). In recent years, onion growers in Michigan have observed foliar bacterial disease on their crop and *P. agglomerans* was consistently isolated (Hausbeck, 2014; Tho et al., 2015). Significant losses of onion incited by *P. agglomerans* have been reported in South Africa (Hattingh and Walters 1981) and Georgia (Edens et al., 2006). The pathogen has been confirmed in New York (Beer et al., 2010) and Pennsylvania (Gugino and Pfeufer, 2015; Pfeufer, 2014). *P. ananatis* was first reported causing leaf blight and center rot of onions in Georgia in 1997 resulting in field losses up to 100% (Gitaitis and Gay, 1997). Disease outbreaks incited by *P. ananatis* were also reported in Colorado (Schwartz and Otto, 2000), New York (Carr et al., 2010), and Michigan (Hausbeck, 2014). *Enterobacter cowanii* was frequently isolated from onion foliage with leaf blight symptoms in Michigan in 2013 and 2014 (K. E. Tho, unpublished data).

Historically, onion growers in Michigan have not needed to consider bacterial disease management strategies. With recent outbreaks of bacterial foliar blights, growers have sought integrated management options. Identifying onion cultivars that are less susceptible to bacterial disease could limit grower losses and guide onion breeders in selecting resistant onion germplasm for use in breeding programs. The objective of this study was to evaluate long-day onion cultivars suitable for Michigan's growing conditions for resistance to *P. agglomerans*, *P. ananatis*, and *E. cowanii*.

MATERIALS AND METHODS

Inoculum. Cultures of *P. agglomerans, P. ananatis*, and *E. cowanii* isolated from symptomatic onion leaves in Michigan, in 2014, were used for inoculum. Isolate virulence was previously confirmed on 'Bradley' onion plants and bulbs (data not presented). *P. agglomerans* and *E. cowanii* were identified by using BIOLOG (Hayward, CA) and sequencing of the 16s rDNA by using the universal primers (forward 5′-AGTTTGATCCTGGCTCAG-3′ and reverse 5′-TACCTTGTTACGACTTCGTCCCA-3′) (Baere et al. 2004). *P. ananatis* was identified using diagnostic primers, PanITS1 (5'-GTCTGATAGAAAGAT-AAAGAC-3'), EC5 (5'-TGCCA GGGCATCCACCG-3') (Gitaitis et al. 2002) and sequencing of the 16s rDNA with the method described above. The *E. cowanii* strain was also confirmed by sequencing of *rpoB* gene as described in (Brady et al., 2008; Brady et al., 2009). Bacterial strains were grown overnight on nutrient broth yeast extract agar (NBY) at approximately 30^{o} C in the dark. Inocula were prepared by using sterile deionized water and adjusted spectrophotometrically to approximately 10^{8} CFU/ml.

Cultivar evaluation. Seventeen yellow and two red long-day onion cultivars (Table 3.1) were seeded into 98-square-cell flats (Hummert International, Earth City, MO) with SUREMIX perlite and soilless media (Michigan Grower Products, Inc., Galesburg, MI). They were grown in a research greenhouse on the campus of Michigan State University in East Lansing, MI, for 7 wks and fertilized daily with 200 ppm of 20-20-20 of Peters water soluble fertilizer (The Scotts Company, Marysville, OH). Plants were transplanted into square pots (approx. 10 x10 cm) and grown in a greenhouse for 7 days before inoculation.

Inoculation and experimental design. Plants were inoculated using the method described by Wright and Grant (1998) with the following modification. Two-month old onion plants were cut to a height of 15 cm and sprayed individually until run-off with approximately 10^8 CFU/ml of a bacterial suspension of *P. agglomerans, P. ananatis,* or *E. cowanii*. Plants sprayed with distilled sterile water served as a control. Each plant was covered with a clear plastic bag (approx. 20 x 10 x 45 cm) and 300ml of 200 ppm of 20-20-20 of Peters water soluble fertilizer (The Scotts Company, Marysville, OH) was poured into the bottom of each plastic bag for plant nutrition and to promote high relative humidity. A hand-made wire frame was put inside each bag to prevent direct contact between the foliage and the plastic bag. Five single-plant replicates of each cultivar and bacterial species were arranged in a greenhouse in a randomized complete block design (RCBD). Five plants treated with sterile water were used as a control for each cultivar, in order to achieve a 19 x 4 factorial design (onion cultivar x pathogen/control). Four days after inoculation, each plastic bag was opened. Plants were irrigated by adding nutrient water as described above to the bottom of bags as needed.

Experiments were conducted on 19 September (Trial 1) and 20 December 2015 (Trial 2). A watchdog (Spectrum Technologies, Inc., Plainfield, IL) was placed inside the greenhouse. Hourly recorded temperature was 22 ± 4.2 ^{o}C (mean ± standard deviation) for Trial 1 and 22.07 ± 1.73 ^{o}C for Trial 2. Relative humidity was 53.50 ± 15.47% and 42.11 ± 11.13% for Trial 1 and Trial 2, respectively.

Plants were assessed visually for disease severity every other day for 10 days following inoculation (DAI) by using a 1 to 5 scale (Schwartz, 2013) to quantify leaf collapse, where 1 ≤ 1%, 2 = 1-10%, 3 = 11-25%, 4 = 26-50%, and 5 ≥ 50% (Figure 3.1). Area under the disease

progress curve (AUDPC) (Jeger and Viljanen-Rollinson, 2001) was calculated. Two wk after inoculation, fresh weight (g) of each plant was determined.

Data analysis. AUDPC and fresh weight data obtained from both trials were subjected to analyses of variance (ANOVA). Data were checked for normality by observing studentized residual versus predicted mean plots and percentage of residual distribution histogram. Levene's test was also conducted to determine homogeneity of each variable by using SAS 9.4 (SAS Institute, Cary, NC). Results of AUDPC data from the two trials were analyzed and summarized separately as significant interaction ($P = 0.0006$) between the two sets of trials was detected. ANOVA of AUDPC was conducted by using PROC MIXED with block as the random effect. Repeated measure grouped by pathogen and degree of freedom according to Kenward-Roger were used for both trials, as lack of homogeneity was detected in the cultivar-by-pathogen interaction effect by using Levene's test at ($P<0.05$). Means of AUDPC were sliced and summarized separately for each pathogen and were compared across different cultivars by using student's *t* - test at ($P<0.05$). For plant fresh weight data of each trial, ANOVA was also analyzed by using PROC MIXED in SAS, with block as the random effect. Normality and homogeneity assumptions were also tested using the same methods as described above. Means of plant fresh weight were compared within each cultivar by using Fisher's protect least significant different (LSD) and Student's *t* – test at ($P<0.05$) for Trial 1 and Trial 2, respectively.

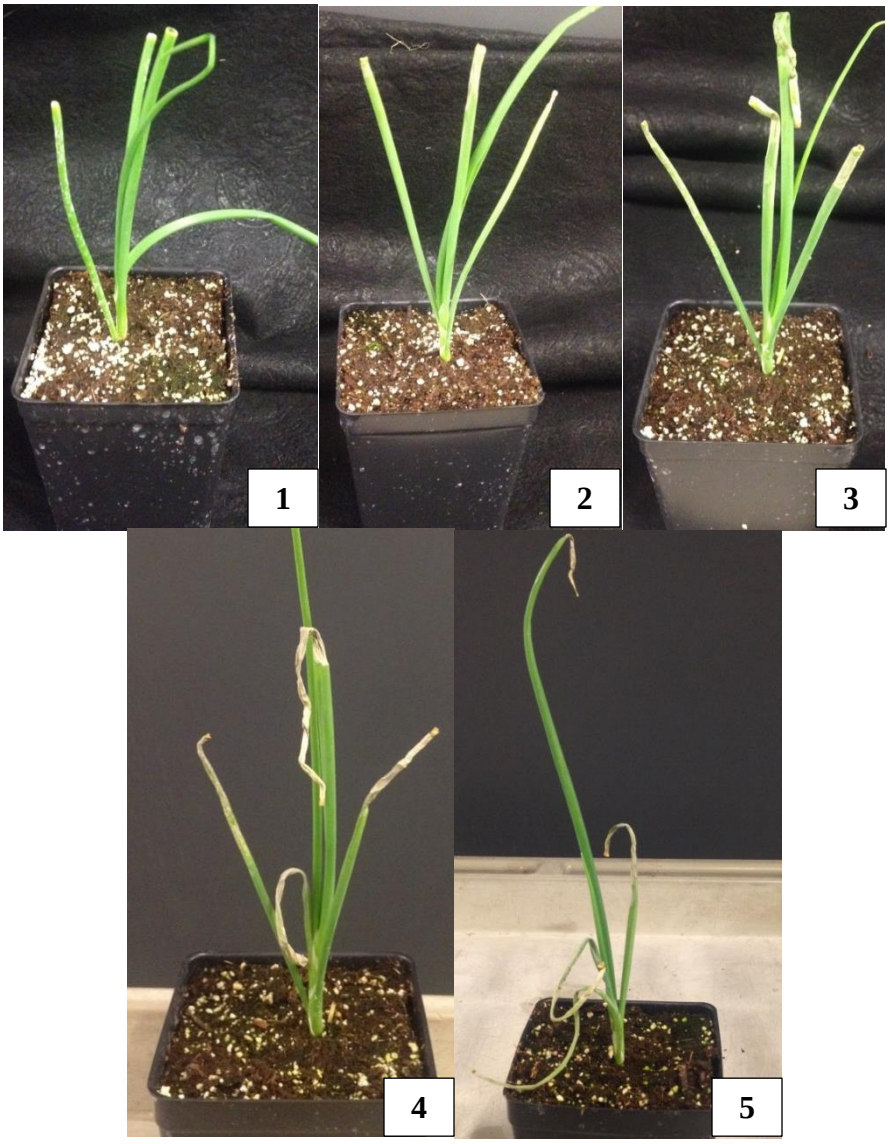

Figure 3.1 Disease symptoms observed on inoculated plants exhibiting ratings from 1 to 5 (where 1 ≤ 1%, 2 = 1-10%, 3 = 11-25%, 4 = 26-50% and 5 ≥ 50% of leave collapsed)

RESULTS

Leaf blight symptoms including water-soaked lesions were observed on inoculated plants. AUDPC values were significantly different among onion cultivars in each trial (P<0.0001). Significant differences were detected among the pathogens in both trials

(P<0.0001). Onion cultivar-by-pathogen interaction was significant (P< 0.01), suggesting that the onion cultivar response depended on the bacterial pathogen (*data not shown*).

For plants inoculated with *P. agglomerans,* the effect of cultivar on AUDPC were significant for both trials (P = 0.0015 and P = 0.0163 for Trial 1 and 2, respectively). In Trial 1, when onion cultivars were inoculated with *P. agglomerans,* 'Sherman' was less diseased than many cultivars but similar to 'Bradley', 'Mandras', 'Moondance', 'Hamlet' and 'Red-defender'. 'Bradley' and 'Highlander', two onion cultivars commonly grown in Michigan were significantly different in Trial 1; 'Highlander' was more diseased than 'Bradley'. In Trial 2, cvs. 'Highlander' and 'Bradley' were similar in their susceptibility. In both trials, 'Sherman' had less disease than 'Delgado' (Table 3.1).

When plants were inoculated with *Pantoea ananatis,* AUDPC values among tested onion cultivars were significantly different in both trials (P=0.0177 and P = 0.0017 for Trial 1 and 2, respectively). For both trials, 'Highlander' and 'Delgado' were more susceptible than 'Sherman' (Table 3.1).

AUDPC values among cultivars differed significantly among onions cultivars when inoculated with *E. cowanii* (P = 0.0139 for Trial 1 and P < 0.0001 for Trial 2). In Trial 1, 'Highlander' and 'Delgado' were significantly more diseased than 'Sherman'. However, in Trial 2, differences among these cultivars were not detected.

The effect of bacterial infection on plant fresh weight was significant at (P <.0001) for both trials. Cultivar-by-pathogen interaction effect on plant fresh weight were also significant (P= 0.0028 and 0.0076 for Trial 1 and 2, respectively). In Trial 1, most onion cultivars inoculated with a bacterial pathogen showed a reduction of plant fresh weight compared to the

control. Exceptions included ‘Moondance’ and ‘Braddock’ inoculated with *P. agglomerans* and ‘Safrane’ and ‘Patterson’ inoculated with *P. ananatis* (Table 3.2).

In Trial 2, plants inoculated with *E. cowanii* had fresh weight that was significantly reduced compared to the control, except for ‘Bradley’, ‘Frontier’ and ‘Vespucci’. When plants were inoculated with *P. agglomerans*, seven onion cultivars had significant reduction in plant fresh weight, including ‘Redwing’, ‘Red-defender’, ‘Mandras’, ‘Safrane’, ‘Milestone’, ‘Scorpion’, and ‘Moondance’. For plants inoculated with *P. ananatis*, the onion cultivars with a significant reduction in fresh weight compared to the control included ‘Red-defender’, ‘Redwing’, ‘Mandras’, ‘Moondance’, ‘Safrane’, ‘Milestone’ and ‘Talon’ (Table 3.3).

Table 3.1 Effects of different bacterial inoculation on AUDPC of long-day onion cultivars, tested in a greenhouse. Means followed by the same letters are not significantly different by using student's *t* – test at ($P \leq 0.05$).

Cultivar	Source[x]	*P. agglomerans*		*P. ananatis*		*E. cowanii*	
		Trial 1	Trial 2	Trial 1	Trial 2	Trial 1	Trial 2
		Mean[y]	Mean	Mean	Mean	Mean	Mean
Sherman	Bejo	26.5 e	24.8 f	31.2 b-e	23.4 e	34.3 cde	32.8 a-d
Red-defender	Harris	29.8 de	29.3 a-e	34.4 a-d	33.2 a	37.0 a-d	35.1 ab
Bradley	Bejo	29.8 de	29.4 a-e	30.7 cde	26.0 cde	36.2 b-e	30.0 cd
Mandras	Bejo	30.3 cde	30.1 a-d	28.5 e	25.9 de	34.9 b-e	30.1 cd
Harmlet	Seminis	30.8 cde	-	34.0 a-d	-	35.3 b-e	-
Moondance	Bejo	31.0 cde	28.2 b-f	30.9 cde	28.8 bcd	33.6 de	29.4 d
Pulsar	Nunhems	31.6 cd	30.1 a-d	35.9 ab	28.1 bcd	36.7 a-e	35.2 ab
Braddock	Bejo	35.2 abc	28.2 b-f	31.7 b-e	30.3 abc	36.6 a-e	33.9 abc
Safrane	Bejo	32.2 bcd	26.7 def	35.1 a-d	28.0 bcd	37.6 abc	32.9 a-d
Milestone	Takii	32.8 bcd	25.6 ef	33.2 a-e	23.3 e	33.3 e	23.9 e
Frontier	Takii	33.5 abc	26.8 def	33.6 a-d	26.7 cde	35.3 b-e	30.1 cd
Patterson	Bejo	34.3 abc	28.8 b-f	35.4 abc	29.4 a-d	40.0 a	32.4 a-d
Vespucci	Siegers	35.1 abc	28.1 b-f	30.4 de	30.0 a-d	34.3 cde	31.5 bcd
Highlander	Stokes	36.8 ab	27.4 c-f	36.7 a	28.9 a-d	38.0 ab	33.8 a-d
Delgado	Bejo	38.3 a	31.9 ab	37.2 a	31.7 ab	38.4 ab	34.8 ab
Talon	Bejo	-	31.4 abc	-	28.9 a-d	-	36.8 a
Scorpion	Crookham	-	26.4 def	-	27.6 b-e	-	29.6 cd
Redwing	Bejo	-	33.2 a	-	29.7 a-d	-	35.0 ab
Gunnison	Bejo	-	30.2 a-d	-	27.9 bcd	-	35.4 ab

[x] Seed companies: Bejo Seeds, Inc. (Oceano, CA); Siegers Seed Co. (Holland, MI); Harris Seeds Co. (Rochester, NY); Nunhems Seeds USA Inc. (Acampo, CA); Takii Seeds American, Inc (Salinas, CA); Seminis Vegetable Seeds, Inc (Oxnard, CA); Stokes Seeds, Co. (Buffalo, NY); Crookham Seeds Co. (Caldwell, ID).

[y] Means of AUDPC from five replicated plants, calculated by rating the disease severity in every other day for 10 days post inoculation; 1-5 disease scale was used (where 1 = <1%, 2 = 1-10%, 3 = 11-25%, 4 = 26-50% and 5 ≥ 50% of leave collapsed).

Table 3.2 Fresh weight of long-day onion cultivars when inoculated with *Pantoea agglomerans, Panotea ananatis, Erwinia cowanni*, or not inoculated in greenhouse Trial 1. Means followed by the same letters within each cultivar are not significantly different by Student's *t*-test at ($P \leq 0.05$).

Cultivar	**Source**[x]	**Trial 1**[y]			
		Water Control	*P. agglomerans*	*P. ananatis*	*E. cowanii*
Sherman	Bejo	5.466 a	3.500 b	2.808 b	1.212 c
Red-defender	Harris	10.51 a	6.218 b	5.106 b	3.070 c
Bradley	Bejo	7.756 a	3.836 b	4.266 b	1.672 c
Mandras	Bejo	7.696 a	3.910 b	3.904 b	2.344 b
Harmlet	Seminis	4.642 a	2.200 b	3.130 b	2.398 b
Moondance	Bejo	9.235 a	6.906 ab	6.348 b	4.542 b
Pulsar	Nunhems	5.304 a	2.416 b	2.046 b	2.284 b
Braddock	Bejo	4.969 a	3.582 ab	2.864 b	1.064 b
Safrane	Bejo	7.444 a	4.996 b	5.090 ab	3.390 c
Milestone	Takii	5.906 a	2.786 b	3.034 b	2.334 b
Frontier	Takii	5.656 a	3.774 b	3.548 b	2.026 b
Patterson	Bejo	6.184 a	4.132 b	5.286 ab	2.518 c
Vespucci	Siegers	4.916 a	2.104 b	2.690 b	1.330 b
Highlander	Stokes	9.350 a	4.948 bc	3.104 d	4.016 cd
Delgado	Bejo	8.026 a	3.436 b	4.562 b	2.426 c

[x] Seed companies: Bejo Seeds, Inc. (Oceano, CA); Siegers Seed Co. (Holland, MI); Harris Seeds Co. (Rochester, NY); Nunhems Seeds USA Inc. (Acampo, CA); Takii Seeds American, Inc (Salinas, CA); Seminis Vegetable Seeds, Inc (Oxnard, CA); Stokes Seeds, Co. (Buffalo, NY); Crookham Seeds Co. (Caldwell, ID).
[y] Means of plant fresh weight (g) from five replicated plants.

Table 3.3 Response of long-day onion cultivars when inoculated with bacterial pathogens in greenhouse Trial 2. Means followed by the same letters within each cultivar are not significantly different by Fisher's protected least significant difference (LSD) at ($P \leq 0.05$).

Cultivar	Source[x]	Trial 2[y]			
		Water Control	*P. agglomerans*	*P. ananatis*	*E. cowanii*
Sherman	Bejo	2.676 a	1.992 a	2.108 a	1.032 b
Red-defender	Harris	3.546 a	2.288 b	1.596 c	1.026 c
Bradley	Bejo	1.294 a	0.942 a	1.166 a	0.900 a
Mandras	Bejo	2.338 a	1.374 b	1.676 b	1.276 b
Moondance	Bejo	2.016 a	0.954 b	0.742 b	0.968 b
Pulsar	Nunhems	2.766 a	2.346 a	2.128 a	0.904 b
Braddock	Bejo	2.032 a	2.056 a	1.474 ab	0.802 b
Safrane	Bejo	2.468 a	1.682 b	1.594 b	0.668 c
Milestone	Takii	2.730 a	1.468 b	1.826 b	1.588 b
Frontier	Takii	1.716 a	1.578 a	1.496 a	1.496 a
Patterson	Bejo	2.194 a	1.762 ab	1.666 ab	1.182 b
Vespucci	Siegers	1.264 a	0.976 a	0.836 a	0.888 a
Highlander	Stokes	2.448 a	2.142 a	1.708 ab	1.046 b
Delgado	Bejo	1.924 a	1.348 a	1.294 a	0.800 b
Talon	Bejo	2.972 a	2.456 ab	1.824 b	0.942 c
Scorpion	Crookham	2.990 a	1.904 b	2.512 ab	0.998 c
Redwing	Bejo	3.644 a	2.532 b	1.896 bc	1.228 c
Gunnison	Bejo	2.136 a	1.844 a	1.662 ab	1.018 b

[x] Seed companies: Bejo Seeds, Inc. (Oceano, CA); Siegers Seed Co. (Holland, MI); Harris Seeds Co. (Rochester, NY); Nunhems Seeds USA Inc. (Acampo, CA); Takii Seeds American, Inc (Salinas, CA); Seminis Vegetable Seeds, Inc (Oxnard, CA); Stokes Seeds, Co. (Buffalo, NY); Crookham Seeds Co. (Caldwell, ID).
[y] Means of plant fresh weight (g) from five replicated plants.

DISCUSSION

The occurrence of bacterial foliar diseases has created significant risk for onion growers in Michigan. In this study, 19 long-day onion cultivars were evaluated for resistance to the bacterial pathogens, *P. agglomerans, P. ananatis* and *E. cowanii*, which are common in the state's onion fields. Based on repeated trials in a greenhouse, no cultivar was determined to be resistant. While 'Sherman', often appeared to be less susceptible than 'Delgado'and 'Highlander', results were not always consistent. White and Grant (1998) evaluated *Allium* germplasm for resistance to bacterial soft rot in onion plants caused by *Pseudomonas marginalis* and *Pseudomonas viridiflava* in a greenhouse. The onion genotypes (*A. cepa* L.) included in their trials were highly susceptible to bacterial infection. Other *Allium* spp. evaluated in their study, i.e. *A. sativum* showed promise as a source of resistance for breeding (Wright and Grant, 1998).

Enterobacter cowanii was first described by Inoue et al. (2000) as a new species in *Enterobacteriaceae*. Some strains are opportunistic pathogens in humans that are immunocompromised (Inoue et al., 2000; Wetzel et al., 2010)). *E. cowanii* was first reported as a plant pathogen on a native forest species (*Mabea fistulifera*) in Brazil in 2012 (Furtado et al., 2012). *P. agglomerans* is ubiquitous in nature and occurs in water and soil as a plant pathogen, endophyte, and epiphyte (Weinthal et al., 2007; and Manulis and Barash, 2003), and as an opportunistic pathogen for human (Delétoile et al., 2009). *P. agglomerans* and *P. ananatis* have been associated with the gut of brown hoppers (*Nilaparvata lugens*) (Watanabe et al., 1996), mulberry pyralid (*Glyhodes pyloalis*) (Takahashi et al., 1995) and tobacco thrips in onions (*Frankliniella fusca*) (Gitaitis et al., 2003). Additionally, *P. ananatis* has been found in various weed species that may serve as sources of inoculum (Gitaitis et al., 2002).

Onion cultivars were evaluated in storage for resistance to *Enterobacter cloacae* (Schroeder et al., 2010). 'Delgado', 'Pulsar', 'Talon', 'Frontier' and 'Gunnison', were included in the study by Schroeder et al., 2010 and demonstrated disease severity characterized as moderate to high; results were similar to our study, except onion plants at growing stage were evulated for our study. Red onion cultivars were found to be less susceptible to *E. cloacae* in storage for long-day onion cultivars (Schroeder et al., 2010) and for short-day onions against bacterial soft rot in storage (Ko et al., 2002). Results from our study suggest that red onion cultivars such as 'Redwing' and 'Red-defender' may be more susceptible than several yellow onion cultivars. This could suggest that susceptibility of onion cultivars against bacterial diseases could be different between foliage and storage conditions.

Methods of inoculation (Schroeder et al., 2010) and the strains (Galeano et al., 2014) significantly influence onion resistance assays. We used methods that Wright and Grant (1998) used to screen *Allium* germplasm against *Pseudomonas marginalis* and *Pseudomonas viridiflava* (Wright and Grant, 1998). A preliminary indicated that using this inoculation method provided reproducible and consistent disease symptoms beginning within two days post inoculation. Additional field studies could provide important information.

Long-day onions were evaluated for resistance to *Iris yellow spot virus* (IYSV) and thrips (*Thrips tabaci*) (Wohleb et al., 2012; Boateng et al., 2014; and Shaikh et al., 2014), *Rhizoctonia solani* (Sharma-poudyal et al., 2015), *Pratylenchus penetrans* and *Meloidogyne hapla* (Pang et al., 2009), and pink root disease (*Setophoma terrestris*) (Wiriyajitsomboon, 2015). Our study provides information that could be useful for growers regarding the response of long-day onions to three bacterial pathogens.

Cultivars less susceptible to bacterial disease could reduce grower risk and the need for bactericide sprays. Carr et al., (2013) reported that infection of onion leaves with *P. ananatis* could lead to central bulb rot in storage, as the bacterium could move down through neck into bulbs. Therefore, identifying less susceptible cultivars during planting stage in our study may help limit or slowdown foliar infection – thereby might reduce risks of bulb rot infection in storage. Management strategies for bacterial disease in onion includes crop rotation, application of bactericides, sanitation, and other cultural practices (Gent and Schwartz, 2005; Schwartz and Mohan, 2008), insect and weed control, and use of pathogen-free seed (Walcott et al., 2002; Gitaitis et al,. 2003). Screening *Allium* germplasm, including wild types, could identify potential sources of resistance against bacterial foliar diseases, which could be incorporated into long-day onions.

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CHAPTER IV: FIELD EVALUATION OF SHORT-DAY ONION CULTIVARS FOR ADAPTABILITY IN CAMBODIA AND STRESS RESPONSE

ABSTRACT

Sixteen short-day onion cultivars were evaluated for adaptability to Cambodia at two research stations. Cultivars were evaluated based on plant stand, bulb weight, bulb diameter. Symptoms of stress caused by biotic or abiotic agents were also noted. Number of plants, bulb diameter, bulb weight, and stress rating were significantly different in both trials (P<0.01). In Trial 1, onion 'Texas Early Grano' and 'AVON1073' had the highest stand count; both were significantly different from 'AVON1027', 'Chianti F1' and 'Yellow Granex F1'. 'White Caslte', 'Sweet Jalene', 'Sweet Uno', 'Mr. Buck' and 'Pirate F1' had the lowest stand count. In Trial 2, 'Yellow Granex' and 'AVON1073' had the highest stand count; while 'Sweet Uno', 'Pirate F1' and 'Sweet Harvest', the lowest. Based on bulb weight per meter-squared, 'Texas Early Grano' and 'Yellow Granex F1' had the best performance in both trials. 'Texas Early Grano', 'Yellow Granex' and 'Sweet Harvest' consistently produced the biggest bulbs in both trials. For stress severity ratings, 'Yellow Granex F1' and 'AVON1073' were consistently low; whereas 'Sweet Uno' and 'Pirate F1' were highly susceptible in both trials. Overall, 'Texas Early Grano', 'Yellow Granex', and 'AVON1073' were exceptional across all parameters. Results indicated that select short-day onion cultivars may be suitable for the tropical agro-climate of Cambodia.

Keywords: *Short-day onions, adaptability, biotic and abiotic stress, Cambodia*

INTRODUCTION

Onions (*Allium cepa* L.) are an important vegetable crop in Cambodia, where short-day cultivars are suitable (Shanmugasundaram and Kalb, 2001; Cramer and Bartolo, 2013). Both field and storage diseases have been problematic for onion growers in the U.S. (Schwartz, 2013; Schwartz and Mohan, 2008). Bacterial pathogens including *Pectobacterium carotovorum* subsp. *carotovorum, Dickeya* spp., *Xathomonas compestris, Pantoea ananatis, Pantoea agglomerans* and *Pseudomonas marginalis* pv. *marginalis* have been reported as major bacterial diseases on *Allium* spp. in the U.S. (CPC, 2007); similar information is not available for Cambodia. Bacterial pathogens have a wide host range (Ma et al., 2007), a diverse environmental niche (Delétoile et al., 2009), and may be seed-borne in onions (Walcott et al., 2002)

Foliar bacterial diseases present as leaf blight, water-soaked lesions, soft rot, and occasionally plant collapse (Schwartz and Mohan, 2008) and are favored by prolonged period of rains, high relative humidity and moderate to high temperature (Schwartz et al., 2003; Schroeder et al., 2013; Mishra et al., 2014). Bacterial diseases are managed by chemical pesticides, mainly copper-based bactericides or amended with an ethylenebisdithiocarbamate fungicide (e.g., mancozeb or maneb) (Gent and Schwartz, 2005).

Assessing the adaptability of short-day onion cultivars to Cambodian's conditions could help growers identify preferred cultivars. Researches have been conducted to evaluate short-day onion cultivars in some African and Asian countries (Lai et al. 1994; Msuya et al. 2005; Pal-Baliyan 2014; Abdelkader et al. 2014). Cultivar performance may vary under different agro-climatic conditions due to possible interaction between their genetic makeup and the environment (Jilani and Ghaffoor, 2003; Baliyan, 2014; Kimani et al., 1993). Evaluating the response to biotic or abiotic stresses among short-day type onion cultivars is important.

The objective of this study was to evaluate short-day onion cultivars for adaptability in Cambodia. Their response to biotic and abiotic stress was also of interest.

MATERIALS AND METHODS

One trial was established at the Royal University of Agriculture (RUA) Crop Research Station ($11^{o}30'47$" N, $104^{o}54'5$" E) in sandy-loam soil, 0.6% of organic matter (OM), pH 7.0, and cation exchange capacity (CEC) of 9.8 cmol/kg. A second trial was located at the Cambodian Agricultural Research and Development Institute (CARDI) Research Station ($11^{o}28'41$" N, $104^{o}48'30$" E) in silt-loam soil,1.34% OM, pH 5.87, and CEC of 19.8 cmol/kg. Sixteen short-day onion cultivars obtained from seed companies or GeneBank were planted at a seedbed at RUA's crop station on 9 January 2017. Seedlings were produced in 1 m x 9 m beds, using five rows per bed, with approximately 1 cm between seeds within a row.

Each cultivar was transplanted into four replicated plots arranged in randomized complete block designs on 25 and 27 February 2017 at RUA and CARDI, respectively. Each plot consisted of one 1 m x 4 m bed consisting of five rows of transplants spaced 10 x 10 cm. The total number of onion plants per plot was 165 or ~41 plants/m^{-2}. Rice straw was used as mulch at a 10-cm thickness. Inorganic fertilizer such as N, P_2O_5, and K_2O was used at 80, 90 and 40kg/ha, respectively, as basal fertilizers (broadcasting before planting), followed by two applications of side-dressing N fertilizer at 40kg/ha each (Shanmugasundaram and Kalb 2001). Hand-weeding was used as needed. Overhead irrigation was provided but was discontinued near harvest. Abamectin insecticide was used twice; other pesticides were not used. Onion bulbs were

harvested on 9 and 11 May at RUA and CARDI, respectively. Temperatures ranged from 21.6 to 36.0 ^{o}C, average relative humidity was 72.2%, and total rainfall was 613.1mm (Table 4.2).

Plant stand count and a stress rating were assessed two weeks prior to harvest. The entire plot was counted and the average number per m^2 was calculated. For the stress rating, the foliage in each plot was assessed visually using a rating system previously described for bacterial disease on onion (Schwartz 2013) where 1 = <1%, 2 = 1-10%, 3 = 11-25%, 4 = 26-50%, and 5 = > 50% of the leaves and plants collapsed. For bulb weight, all onions in each plot were harvested and the average of bulb weight per m^2 was calculated. Bulb diameter was calculated based on the average size of twenty onion bulbs randomly selected from each plot.

Pearson's correlation coefficients for bulb weight versus stand count, bulb diameter and stress rating were analyzed. Linear correlations of the same variables were also determined between the trials. Final stand count, bulb diameter, bulb weight and stress rating were subjected to analyses of variances (ANOVAs) by using Proc Mixed in SAS version 9.4 (SAS Institute, Cary, NC), with cultivar as the fixed and block (replicates) as the random effects in the model. Data from each trial were analyzed separately using F-tests in ANOVA with a significant level (P=0.05). Normality and homogeneity assumptions of each variable were tested by using residual and predicted residual distribution, and Levene's test at (P <0.05), respectively. In Trial 1, final stand count and bulb weight variable were squared-root transformed, while bulb diameter and the stress rating were power transformed by (1.5) and (2.0), respectively, as determined by using Cox-Box analyses in SAS. Models with the degree of freedom according to Kenward-Roger (Kr) were used for all variables in Trial 1, as lack of homogeneity was found by using Levene's tests (P=0.05) and comparing the model's fitness with AIC values. For Trial 2, cultivar means of all variables were directly compared by using Fisher's protected least significant

difference (LSD) at (P <0.05), as normality and homogeneity assumptions were conformed. Proc Glimmix was used to determine differences among the cultivars.

Figure 4.1 Stress symptoms observed in the onion cultivar field trials. **A.** Shriveling of the base causing collapse of onion leaves **B-C:** Leaf blighting with water-soaked tissue. **D:** Onion plant water-soaking, collapse and rot with malorder.

RESULTS

Seed germination among the cultivars varied from poor to very good. Two cultivars, 'Superex F1' (yellow) and 'AVON1074' (a heat tolerant red onion line brought from Mali, West Africa), did not germinate adequatley and were not included in the study. All cultivars had good to very good germination with the exception of 'AVON1027', 'Mr. Buck F1', 'Chianti F1', 'Rio Amarillo F1', and 'Sweet Jalene' which exhibited fair to medium germination.

The final plant stand among the onion cultivars included in the trials were significantly different (P <.0001). From an initial number of 41 plants /m^{-2}/cultivar at transplanting, the reduction (%) in plant stand count prior to harvesting, ranged from 33.4% to 88.3% (Trial 1) and 21.0% to 84.3% (Trial 2). 'Texas Early Grano' and 'AVON1073' had the highest stand count for Trial 1; but were statistically similar to 'AVON1027', 'Chianti F1' and 'Yellow Granex F1'. The onion cultivar with the lowest stand count was 'White Caslte' and was similar to 'Sweet Jalene', 'Sweet Uno', 'Mr. Buck', and 'Pirate F1' according to the student's *t*-test (P<0.05). In Trial 2, 'Yellow Granex F1' and 'AVON1073' had the highest stand count and were not significantly different from each other. 'Sweet Uno' had the lowest stand count, but was similar to 'Pirate' and 'Sweet Harvest' according to the LSD test (P< 0.05) (Table 4.1).

Bulb weight differed significantly among cultivars in both trials. In Trial 1, 'Texas Early Grano', 'Yellow Granex F1' consistently had the highest bulb weight/ m^2; both were significantly higher than other cultivars according to the Student's *t*-test (P< 0.05). The remaining cultivars tested in this trial, on the other hand, were not significantly different from one another. In Trial 2, 'Yellow Granex F1' and 'Texas Early Grano' still produced the highest

bulb weight/m^2; both were significantly different from each other. ‘Sweet Uno F1’ and ‘Red Creole’ produced the significantly least bulb weight according to LSD ($P < 0.05$) (Table 4.1).

Bulb sizes differed significantly among the cultivars in both trials ($P < 0.0001$ and $P < 0.0001$ for Trial 1 and 2). In Trial 1, ‘Texas Early Grano’, ‘Yellow Granex’ and ‘Sweet Harvest’ produced significantly larger bulbs than the other cultivars, except for ‘AVON1027’. In Trial 2, ‘Yellow Granex’ produced the largest bulbs, and was significantly different from the second and third largest bulb-producing cultivars, Sweet Harvest and Texas Early Grano. Stress rating was based on the degree of onion leaf blight with water-soaked lesion, leaf shriveling and soft rot with malodor. These symptoms were highly suspeciously recognized as bacterial disease infection (Schwartz and Mohan 2008) (Fig. 4.1). Significant differences were found for stress rating among different cultivars in both trials ($P < .0001$ and $P = 0.0044$ for Trials 1 and 2). In Trial 1, ‘Texas Early Grano’ and ‘AVON1073’ had a stress rating of 2.75, the lowest among the cultivars, but similar to ‘AVON1027’, ‘Chianti F1’, ‘Yellow Granex F1’, ‘Sweet Harvest’, and ‘Red Creole’. ‘Pirate’ and ‘Mr. Buck’ onions had the highest disease rating of 5, but was similar to other cultivars. In Trial 2, ‘Yellow Granex’ and ‘AVON1073’ were consistently less susceptible to stress but were similar to ‘Texas Early Grano’, ‘Sweet Harvest’, and ‘Pumba F1’(LSD $P < 0.05$). ‘Sweet Uno’ and ‘Pirate’ were consistently highly susceptible to stress in both trials (Table 4.1).

Correlation coefficients between bulb weight versus stand count, bulb size and stress rating of the two trials were analyzed. A highly positive correlation between bulb weight and stand count was detected in both trials ($r = 0.79$ and 0.70 at $P < 0.0001$ for Trial 1 and 2, respectively). Positive correlation were also noticed for bulb weight-by-bulb diameter ($r = 0.81$ and 0.82 at $P < 0.0001$ in Trial 1 and 2, respectively). Bulb weight-by-stress rating correlation,

on the other hand, were negative for both trials (r= -0.68 and -0.67 at $P < 0.0001$ in Trial 1 and 2, respectively). These suggest that onion cultivars with higher stand count and bigger bulb diameter tended to produce higher bulb weight/m^{-2} or yield. In contrast, bulb weight declined if onion cultivars tended to have higher stress severity rating. For example, 'Texas Early Grano' and 'Yellow Granex' had higher stand count and bulb diameter but lower in disease severity than other cultivars, tended to show higher bulb yield, and vice versa.

Moderate Pearson's correlation of stand count variables between Trial 1 and Trial 2 for the same tested cultivars were detected (r = 0.52 at P = 0.001), bulb weight correlation between both trials was (r = 0.51 at P = 0.001) and disease ratings correlation between both trials was (r = 0.47 at P = 0.003). Thus, cultivars with high stand count, bulb weight or stress severity in Trial 1 tended to also have high stand count, bulb weight or stress severity in Trial 2, and vice versa.

Table 4.1 Onion cultivars included in trials, source of seeds, bulb color, and days to maturity.

Cultivars	Companies/ Institutions[x]	Bulb Color	Days to Maturity
Texas Early Grano	Sustainable Seeds	Yellow	110
AVON1073	AVRDC	Yellow	115
AVON1027	AVRDC	Yellow	115
Chianti F1	Neseeds	Red	90-100
Yellow Granex F1	Park Seeds	Yellow	125
Sweet Harvest	Siegers Seeds	Yellow	100-110
Red Creole	Burpee Seeds	Red	110
AVON1028	AVRDC	Yellow	115
Pumba F1	Johnny Seeds	Yellow	140
Rio Amarillo F1	Neseeds	Yellow	120
Pirate F1	Bejo	Yellow	110-120
Sweet Uno (Enza)	Enza	Yellow	110
Mr. Buck F1	Neseeds	Yellow	110
Sweet Jalene	Siegers Seeds	Yellow	100-110
Sweet Uno F1	Siegers Seeds	Yellow	110
White castle	Johnny Seeds	White	150

[x]Seed companies or institution from which seeds were obtained: Bejo Seeds, Inc. (Oceano, CA); Asian Vegetable Research and Development Center (AVRDC, Shanhua, Taiwan); Neseeds, Inc. (East Hartford, CT); Park Seed Co. (Hodges, SC); Siegers Seed Co. (Holland, MI); Burpee Seeds Co. (Warminster, PA); Johnny Seeds Co. (Winslow, ME); Sustainable Seed Co. (Chico, CA); Enza Zaden Seeds Co. (Enkhuizen, Netherlands).

Table 4.2 Stand counts, bulb weight, bulb diameter and bacterial disease rating of 16 short-day type onion cultivars in two field trials.

		Stand count/m^2		Bulb weight (kg)/m^2		Bulb diameter (cm)		Disease rating	
		Trial 1[y]	Trial 2[v]	Trial 1[y]	Trial 2[v]	Trial 1[u]	Trial 2[v]	Trial 1[w]	Trial 2[v]
Texas Early Grano	Sustainable	27.31 a	26.63 bc	1.05 a	0.98 b	4.87 a	4.29 b	2.75 b	4.00 abc
AVON1073	AVRDC	23.18 ab	31.56 ab	0.35 bc	0.75 bc	3.18 c	3.41 bc	2.75 b	3.25 c
AVON1027	AVRDC	21.91 abc	-	0.41 bc	-	4.18 ab	-	3.67 b	-
Chianti F1	Neseeds	19.50 abc	-	0.49 abc	-	3.74 bc	-	3.25 b	-
Yellow Granex F1	Park	17.56 abc	32.38 a	0.64 ab	1.60 a	4.67 a	5.37 a	3.25 b	3.50 bc
Sweet Harvest	Siegers	15.68 bcd	20.38 de	0.66 abc	0.79 bc	4.77 a	4.87 bc	3.25 b	4.00 abc
Red Creole	Burpee	12.75 cde	25.38 dc	0.26 bc	0.30 de	3.67 bc	3.26 de	3.50 b	4.50 ab
AVON1028	AVRDC	11.93 c-f	-	0.18 c	-	2.90 c	-	4.50 a	-
Pumba F1	Johnny	11.68 c-g	29.19 abc	0.35 bc	0.89 b	3.67 bc	4.33 b	4.75 a	3.75 bc
Rio Amarillo F1	Neseeds	11.56 c-g	-	0.29 bc	-	3.13 c	-	4.50 a	-
Pirate F1	Bejo	7.43 d-h	20.31 e	0.19 c	0.63 bc	3.31 bc	4.53 bc	5.00 a	5.00 a
Sweet Uno (Enza)	Enza	7.00 e-h	19.38 e	0.21 c	0.55 cd	3.63 bc	4.34 cd	4.50 a	5.00 a
Mr. Buck F1	Neseeds	6.33 e-h	-	0.22 bc	-	3.58 bc	-	5.00 a	-
Sweet Jalene	Siegers	6.31 f-h	-	0.19 c	-	3.16 c	-	4.75 a	-
Sweet Uno F1	Siegers	5.50 gh	6.44 e	0.14 c	0.16 e	3.22 bc	3.27 e	4.75 a	5.00 a
White castle	Johnny	4.81 h	-	0.13 c	-	3.19 c	-	4.50 a	-

[v] Means followed by the same letters are not significantly different at ($P \leq 0.05$) based on Fisher's protected least significant difference (LSD) for that independent variable.

[y] Means (original scales) followed by the same letters are not significantly different at ($P \leq 0.05$) based on Student's *t* test for that independent variable (*data were squared-root transformed for analyses*).

[u] Means (original scales) followed by the same letters are not significantly different at ($P \leq 0.05$) based on Student's *t* test for that independent variable (*data were power transformed by **1.5 for analysis and back transformed to original scales for reporting*).

[w] Means (original scales) followed by the same letters are not significantly different at ($P \leq 0.05$) based on Student's *t* test for that independent variable (*data were power transformed by **2.0 for analyses*).

Table 4.3 Weather data for trials located in Phnom Penh, Cambodia from January to May 2017 (Data obtained from the Climate Office, Department of Meteorology[z]).

Month	Range of Temp (^{o}C)	Average Relative Humidity (%)	Total Rainfall (mm)
January	19.2 - 33.5	70	16.7
February	16.5 - 35.8	61	0.4
March	22.8 - 36.6	70	15.8
April	22.8 - 37.8	75	239.9
May	24 - 36.3	80.19	237
Average/Total	21.6 – 36.0[x]	72.2[x]	613.1[y]

[x] Average monthly temperature and relative humidity data

[y] Total monthly rainfall

[z] Pochentong Meteorology and Radar Station (distance is approximately 5 km from the studied areas)

DISCUSSION

Selecting the cultivars best suited for the environmental growing conditions impact onion profitability due to yield, bulb size, eating quality, and susceptibility to pests and diseases (Brewster 2006). Previously, studies evaluated adaptability and yield performance of short-day type onion cultivars in tropical regions of some Asian and African countries. Sixty onion cultivars were tested at the Asian Vegetable Research and Development Center (AVRDC) in Taiwan (Lai et al. 1994), where 'Granex YPRR' and 'Granex 429' were among the top producing cultivars. A study in Tanzania reported that 'Texas Grano 438' and 'Granex 429' produced the highest marketable yield and bulb size among 21 tested cultivars in two consecutive years (Msuya et al. 2005). Six short-day onion cultivars were tested in Botswana where 'Texas Grano' produced the highest total yield. However, the bulb quality was low and only 60% of the yield was marketable (Pal-Baliyan 2014). In our study, 'Texas Grano' and 'Granex' types were also top yielding cultivars in Cambodia. Thus, 'Texas Grano' and 'Granex' types appeared to be suitable for onion growers in Cambodia. Our results differed from the study conducted by Abdelkader et al. (2014), who tested eight cultivars in Tunisia and found 'Early Yellow Texas Grano' was not productive. Tunisia is located in the temperate region of northern Africa where short-day onion cultivars may be less suitable than intermediate-day cultivars (Cramer and Bartolo 2013, Shanmugasundaram and Kalb 2001).

In Taiwan, Ko et al. (2002) investigated the storage variability of twelve short-day onion cultivars against bacterial soft rot in storage under warm temperature and high humidity conditions. Storage losses were high among 'Texas Grano' and 'Genex' type cultivars, ranging from 60.8 to 80.2%. Less susceptible cultivars were 'Red Pinoy' and 'Serrana', whereas the white onion 'Torrens White' and 'Early Supreme', were highly susceptible in three years of

consecutive trials (Ko et al., 2002). Red onions were also less susceptible to bulb rot in storage, caused by *Enterobacter cloacae,* than yellow and white onions (Schroeder et al. 2010). Our results indicate that white and sweet onions were susceptible to stress, but not for the case of red onion cultivars. However, our study differed from (Ko et al., 2002) and (Schroeder et al. 2010) as onion cultivars were tested at growing stage and field conditions.

Allium germplasm was evaluated for susceptibility to foliar bacterial soft rot caused by *Pseudomonas marginalis* and *Pseudomonas viridiflava* in a greenhouse. Onions (*A. cepa* L.) were very susceptible to the two bacterial pathogens. However, other *Allium* species showed potential sources of bacterial disease resistance for onion breeders to obtain and transfer genetic resistance to commercial *A. cepa* L. species (Wright and Grant 1998).

Results for adaptability and resistance to stress of short-day onion cultivars tested in this study provide useful information for onion production in Cambodia. 'Texas Early Grano', 'Yellow Granex', and 'AVON1073' could be options in areas with wet and humid conditions that favor abiotic stress.

Onion seed is not been commercially available in Cambodia or in neighboring countries. Most of the seed used in this study was provided by companies and gene-banks. Some cultivars showed poor germination rates.

Environmental parameters could also have significant effects on cultivars' performance. Based on the meteorological data during our trials (Table 4.2), rainfall and humidity were very high during late bulb enlargement and bulb ripening stages (April and May). Consequently, these conditions could have advanced abiotic stress in the field. These stresses predominantly appeared as leaf blight with water-soaked lesion, leaf shriveling and soft rot were highly suspecious as bacterial disease infection in onions (Schwartz and Mohan 2008) (Fig. 4.1). Nevertheless, since

these suspected bacterial infection occurred very quickly in the fields after prolong rainfalls and some limiting facilities in plant pathogenic bacterial identification in Cambodia; future research on identification of bacterial pathogen species and their population in onions is very essential for the development of disease and crop management strategies.

This study illustrates how different cultivars perform in Cambodia. Additionally, bacterial diseases in onions have not been reported in Cambodia. Field and/or leaf inoculation assays to evaluate short-day onion germplasms with bacterial suspension under field conditions or in a controlled environment, might provide additional information. Repeated cultivar trials in different soil types and agro-climates in Cambodia are also needed.

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PROSPECTS FOR FUTURE WORKS

Although the results of this study provided some useful information on the bacterial species diversity in onions, their epidemiology and management, some research questions are yet to be determined. To further enhance our knowledge on bacterial pathogens infecting onions and their interaction with onion plants, prospects for future works could be the followings.

From the chapter I of this research, three more aspects are recommended for future works. First, except for *P. agglomerans, P. anantis* and *E.* cowanii, numbers of isolates of other bacterial species were very limited. Further bacterial collection is recommended to clearly determine their involvement in onion diseases. Molecular characterization of *P. agglomerans* and *P. ananatis*, the top two important bacterial pathogens, should be investigated to determine their source of origins and interaction with virulence and epidemiology aspects. Third, copper hydroxide tolerance was observed within the bacterial population, would suggested that copper spray alone might not be effective. Exploring the efficacy of other disease management strategies could be very useful.

Reseach results from the chapter II also suggested two more research hypotheses. First, would the results be alike with our research if different bacterial concentration and inoculation practices were imployed? Second, for age-related resistance, would tissue structure, water and sugar contents in plant tissues, and biochemical defence properties interact with plant ages and their ability to reduce the disease pressure?

Two more research questions were also generated from the chapter III of this research. First, how different onion cultivars would interact with the bacterial pathogens when inoculation

was done at field condition? Second, how less aggressive inoculation or different inoculation methods affect on results of the cultivar trials?

For the chapter IV of our cultivar trials in Cambodia, repeated trials in different soil types and agro-climates in Cambodia are needed to determine the consistency of cultivars' responses. Most of biotic/ abiotic stresses observed during the two trials were highly suspected to be caused by bacterial pathogens. Their identification and assessment are recommended to help improve onion management strategies. Finally, studying on the effects of different disease management and agricultural pratices are susgested to further investage for future optimization of onion production practices in Cambodia.