FACTORS AFFECTING BACTERIAL SURVIVAL AND FIELD MANAGEMENT OF DIVERSE *PANTOEA* SPECIES IN THE *ALLIUM*-POACEAE PATHOSYSTEM

by

SANTOSH KOIRALA

(Under the Direction of Bhabesh Dutta)

ABSTRACT

Center rot of onion is a bacterial disease caused by diverse plant-pathogenic *Pantoea* species that leads to substantial losses in both field and in storage. *Pantoea stewartii* subspecies *indologenes* (*Psi*), known to cause disease in millets, was recently identified as a causal agent of center rot in onion. The *Psi* isolates have not been well-characterized and their transmission risk to onions in cropping systems where millets precede onion planting is less understood. In this study, we characterized 17 *Psi* strains isolated from various hosts and classified them into two distinct pathovars based on their distinctive pathogenicity profiles. We also evaluated the survival of onion-pathogenic *Psi* in corn and millet residues and assessed its potential transmission risk to onions. Although the pathogen was present in soil at the time of onion transplanting, the risk of transmission to the crop was low. We further investigated the role of exopolysaccharide production, flagellar motility, quorum sensing and pathogenicity-related genes in the survival of onion-pathogenic *Psi*. Exopolysaccharide production seemed to play a minor role in survival in pearl millet residue, while the other targeted genetic factors showed no contribution to survival in millet residue nor in bare soil. Additionally, we explored strategies to

incorporate biocontrol agents (BCAs) into bactericide spray programs for center rot management. Our aim to enhance the efficacy of BCAs by favoring early colonization of onion foliage had limited effect. Onion plots pre-treated with peroxygen before BCA application exhibited similar disease levels in both foliage and bulbs as compared to those treated with BCAs alone. Findings from our study to integrate *Bacillus* species with copper to manage center rot indicated that, under low disease pressure, rotation interval between *Bacillus* species and copper could be extended to 10-14 days without compromising disease control efficacy. However, tank-mix applications of *Bacillus* with copper offered no additional benefits, hence, rotation would be preferrable than tank-mixing these products. Overall, our findings elucidated taxonomic standing of *Psi*, its survival on crop residues and emphasized the importance of integrated-management program with BCAs and copper-based bactericides for effective center rot management.

INDEX WORDS: *Pantoea stewartii*, onion, center rot, biocontrol agents, organic, metagenomics, peroxide, copper, disease management, Vidalia onions, crop residue, bacterial survival, cover crops

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INTRODUCTION AND LITERATURE REVIEW

Onion (*Allium cepa* L.) belongs to the family *Amaryllidaceae* of the order Asparagales (Hanelt 1990). It is the most important crop in the genus *Allium* (Shigyo et al., 2018) that comprises at least 26 other economically important species, including bunching onion (*A. fistulosum),* chive (*A. schoenoprasum*), leek (*A. ampeloprasum* var. *porrum*) and garlic (*A. sativum*) (Schwartz and Mohan, 2007). Onion has been a part of the human diet for over 4000 years and is considered one of the oldest cultivated vegetables (Rabinowitch and Currah, 2002). It was likely domesticated in southwest or central Asia (Brewster 2008; Rubatzky and Yamaguchi 1997; Rabinowitch and Brewster, 1990). Onion is widely cultivated throughout the world for its food and medicinal value (Labate et al., 2007; Sellappan and Akoh, 2002).

Onion is a biennial crop producing bulbs in the first growing season and flowers in the second season (Rabinowitch and Brewster, 1990). They are cool-season vegetables cultivated in a wide range of climates and soil types (Rubatzky and Yamaguchi, 1997). Means of onion propagation include seeds, transplants and sets (small bulbs) (Rabinowitch and Brewster, 1990).

The United States is one of the major producers of onions in the world. The U.S. planted an estimated 136,500 hectares of onion in 2024, worth approximately $1.76 billion (USDA, 2024). In the same year in Georgia, USA, the value of utilized production for the sweet onion crop was $159.9 million from 11,800 ha (USDA, 2024). The Vidalia onion is the official state

crop of Georgia. They are produced in only 20 legally specified counties in southeastern Georgia (Boyhan and Torrance, 2002). Vidalia onions are short-day Granex-type onions producing yellow bulbs and are widely recognized for their low-pungency and sweet flavor (Howard 2002). Onions in Georgia are a winter crop transplanted in main fields in mid-November to late December or early January and harvested in the following spring (Howard 2002). Efforts to expand the Vidalia sweet onion market include conventional growers adopting organic production practices (Johnson et al., 2017; Boyhan et al., 2006).

Onions are susceptible to a wide range of bacterial and fungal diseases (Dutta and Gitaitis, 2020; Belo et al., 2023). Center rot of onion accounts for huge yield losses in the field and postharvest onions in the Vidalia region (Gitaitis et al., 2003; Walcott et al., 2003). The first outbreak of center rot in the U.S. was reported in Georgia in 1997, when losses of up to 100% were recorded in some fields (Walcott et al., 2002). It has since become a persistent threat for the Vidalia onion growers (Dutta et al., 2014).

Brief history of the center rot

Bacterial blight of onion, eventually referred to as the center rot, was first observed in an onion seed production field of Little Karoo, South Africa, in 1977-1978 (Hattingh and Walters, 1981). A bacterial pathogen was isolated from the diseased stalks of onion and identified as *Pantoea agglomerans* (formerly *Erwinia herbicola*). Another causal agent, *P. ananatis* (formerly *Erwinia ananas*), was reported from diseased onion tissues during the 1997 center rot epidemic on Vidalia onions (Gitaitis and Gay, 1997). Since its first outbreak, *P. ananatis* has consistently threatened the onion production industry in Georgia (Dutta et al., 2014). In the U.S., *P. agglomerans* was first reported from diseased samples isolated from the foliage of Vidalia sweet

onions in 2006 (Edens et al., 2006). Similarly, in 2011, a novel species of *Pantoea* causing center rot was isolated from infected plants and seeds of onions and was designated as *P. allii* (Brady et al., 2011). More recently, Stumpf et al. (2018) added *P. stewartii* subsp. *indologenes* as the fourth member of the *Pantoea* species that can cause center rot of onion.

Biology and Symptomatology

The genus *Pantoea* belongs to the *Erwinaceae* family of the order Enterobacteriales (Adeolu et al., 2016). They are Gram-negative, rod-shaped, motile bacteria with peritrichous flagella (Breed et al., 1957), which do not form spores (Adeolu et al., 2016). The members of the *Pantoea* species share similar colony morphology, such as smooth and translucent colonies with entire margins. The colonies are moist, yellow-pigmented, circular, dense, and convex in appearance (Mergaert et al., 1993).

Foliar symptoms of center rot appear first on the few infected leaf blades at the center of the onion plant. It is characterized by white streaking with water-soaked margins (Gitaitis et al., 2002). Severely infected leaves become blighted, and eventually, bleaching and wilting may be observed (Schwartz and Otto, 2000; Dutta et al., 2014). Similarly, seed stalks may be bleached and rotted in the fields (Gitaitis and Gay, 1997). The disease can progress from the infected leaves to the corresponding interior scales of the bulb (Carr et al. 2013). The infected scales are firm and intact; thus, no disease symptoms may be visible externally; however, colonization by secondary microbes may lead to maceration and liquefaction of the diseased tissues, producing a foul odor (Carr et al. 2013; Gitaitis et al. 2002; Gitaitis et al. 2003).

Host range and geographical distribution

P. ananatis causes disease on several economically important crops worldwide. Brown-rot of pineapple fruitlets (*Ananas comosus*) caused by *P. ananatis* was reported to be fairly widely distributed in the Philippines in 1928 (Serrano 1928). The same study reported that punctured sugarcane (*Saccharum officinarum*) inoculated with this pathogen can show symptoms of red streaking. *P. ananatis* was later reported from Mexican pineapples in 1950 (Smith and Ramsey, 1950). Strains of *P. ananatis* were isolated from infected honeydew melons (*Cucumis melo* L.) showing firm, brown lesions that originated in California, Ecuador, Guatemala, and Venezuela (Wells et al., 1987).

Reports of center rot caused by *P. ananatis* and *P. agglomerans* have been recorded in several onion-growing states of the U.S., including Georgia, Colorado, New York, Michigan, and Arizona (Gitaitis and Gay 1997; Schwartz and Otto, 2000; Carr et al., 2010; Tho et al., 2015; Hu 2019). Center rot has also been reported from Morrocco, Korea, and Taiwan (Achbani et al., 2016; Kim et al., 2012; Wang et al., 2018).

P. ananatis was isolated from water-soaked lesions of cantaloupe (*Cucumis melo* var, *cantalupensis*) in Texas (Bruton et al., 1991), leaf blotches of sudangrass (*Sorghum bicolor* ssp. *drummondii*) in California (Azad et al., 2000), leaf blights and dieback symptoms of *Eucalyptus* in South Africa (Coutinho et al., 2002), palea browning symptoms of rice (*Oryza sativa*) in Italy (Cortesi and Pizzatti, 2007), and grain discolorations and leaf blights of rice in China, India, and Russia (Yan et al., 2010; Mondal et al., 2011; Egorova et al., 2015). In maize (*Zea mays*), *P. ananatis* was reported to cause brown stalk rot in South Africa (Goszczynska et al., 2007) and leaf spots in Brazil, Argentina, Mexico, Poland (Paccola-Meirelles et al., 2001; Alippi and Lopez, 2010; Pérez-y-Terrón et al., 2009, Krawczyk et al., 2010). *P. ananatis* causes disease in

sorghum, strawberry (*Fragaria* × *ananassa*) and mango (*Magnifera indica*) as reported from Brazil, Canada and Spain, respectively (Cota et al., 2010; Bajpai et al., 2020; Gutierrez-Barranquero et al., 2019). In Japan and Poland, it was reported from diseased netted melon and wheat (*Triticum*), respectively (Kido et al., 2008; Krawczyk et al., 2020).

On the other hand, *P. stewartii* subsp. *indologenes* (*Psi*) causes leafspot of foxtail millet (*Setaria italica*) and pearl millet (*Pennisetum glaucum*), rot of pineapple and an individual strain has been associated with cluster bean (*Cyamopsis tetragonoloba*) (Mergaert et al., 1993). *Psi* was also reported to cause leaf blight in several members of the Poaceae family, such as rice in Malaysia (Azizi et al., 2019), leaf blight of lucky bamboo (*Dracaena sandriana*) and millet (*Pennisetum glaucum*) in China and India, respectively (Zhang et al., 2020, Mushineni et al., 2021).

Epidemiology

Over the past few decades, several aspects of *P. ananatis* survival and mode of transmission in onion have been elucidated. The bacterium is seed-borne and seed-transmitted and has been detected on naturally infested onion seeds using immunomagnetic separation and polymerase chain reaction (IMS-PCR) (Walcott et al., 2002). The authors suggested that infested seeds alone may not cause a center rot epidemic in the Vidalia region, as onion seed production is not a common practice in Georgia.

P. ananatis can survive epiphytically on weeds such as carpetweed, common cocklebar, crabgrass, Florida beggarweed, Florida pusley, sicklepod, verbena and yellow nutsedge that are widespread in the Vidalia onion region (Gitaitis et al., 2002). Several monocot and dicot plants, such as cowpea, soybean and millet, can also harbor the pathogen epiphytically (Gitaitis et al.

2002). In Florida pusley, low levels of the pathogen inoculum can survive under a 12 h:12 h (wet and dry) moisture regime or a continuous wet regime. Prolonged leaf wetness can contribute to the epiphytic survival of the pathogen and a temperature of 21.1 °C compared to 15.5 °C could be optimum for the pathogen survival (Dutta et al., 2017). Weeds commonly found in the fields during and after the onion-growing season could serve as a local source of inoculum for the subsequent growing seasons. Consequently, onion seedlings in the seedbed could be exposed to the bacteria from the weeds. The pathogen could remain dormant on transplants during colder months and the disease re-emerges under favorable conditions (Gitaitis et al., 2002).

Alternately, insect hosts could vector the bacteria from weeds to healthy onions, promoting disease spread in onion fields. Thrips can harbor *P. ananatis* and *P. agglomerans* in their guts and vector transmission can play a critical role in the disease spread in Vidalia onions. *P. ananatis* isolated from the gut of tobacco thrips were pathogenic on healthy onion plants in a greenhouse trial (Gitaitis et al., 2003). Moreover, in the same study, the infected tobacco thrips transmitted the disease to 52% of the onion plants challenged. Similarly, Dutta et al. (2014) showed that *P. ananatis* and *P. agglomerans* reside in the gut of onion thrips and likely transmit the disease through feces. In their experiment, fecal rinsates from the infested thrips were contaminated with the pathogen. Moreover, onion plants mechanically inoculated with fecal rinsates from the thrips developed center rot symptoms, whereas inoculation with salivary secretions failed to produce any disease symptoms in onions. The same group of researchers later established that onion thrips can transmit *P. ananatis* through feces in a persistent, non-circulative manner without affecting thrips fecundity (Dutta et al., 2016). Thrips are considered the most damaging insect to Vidalia onions (Riley et al., 2014), and the wounds from thrips feeding are likely to facilitate pathogen invasion and disease development in onion leaves (Riley

et al., 2014; Grode et al., 2017). Moreover, Stumpf et al. (2021) reported that thrips activity can reduce the efficacy of copper-based protective chemicals such as Kocide against center rot.

Bacterial progression from the foliage to the subsequent scale on the bulb has been demonstrated experimentally (Carr et al., 2013). Interestingly, the authors also implied that the age of the infected leaf might influence the symptom development in the bulb, as disease progression towards the bulb from the older leaves is quicker than from the younger ones. Susceptibility of the bulb to center rot also depends upon the growth stage at which the onion is exposed to the pathogen. Onions infected at the first leaf senescence stage are more vulnerable to bulb infection as compared to the onions exposed to the pathogen during bulb initiation and bulb swelling (Stumpf et al., 2017). Similarly, Stumpf et al. (2017) reported that the susceptibility to center rot could vary among the sweet onion cultivars. The use of highly susceptible onion cultivars and the introduction of highly virulent strains in the onion production site could also contribute to center rot epidemics (Gitaitis et al., 2002).

Management

Onion varieties with complete resistance to center rot are not commercially available (Dutta and Gitaitis, 2020). Currently, a multifaceted approach to center rot management in the field is recommended that involves the use of healthy disease-free seeds, management of weeds and insect vectors in the field, improved cultural practices and chemical control (Dutta and Gitaitis, 2020). As the disease is seed-borne as well as seed-transmitted, planting certified pathogen-free onion seeds might help to reduce the amount of initial inoculum (Walcott et al. 2003). As thrips can spread the pathogen, a proper thrips management strategy may reduce pathogen spread (Stumpf et al. 2021). Similarly, good field sanitation, including weed removal,

may help reduce the buildup of pathogen inoculum (Gitaitis et al. 2002). The Vidalia region of Georgia experiences hot and humid weather in the late spring that can favor disease development in the field. Therefore, growing early maturing varieties could be an option to avoid climatic conditions that are optimum for pathogen and vector prevalence in the fields (Agarwal et al. 2019).

Similarly, preparing clean seedbeds and removing weeds from the main onion fields and their periphery before transplanting could reduce the inocula from weeds (Gitaitis et al. 2002). Dry conditions are shown to be unfavorable for pathogen survival in weeds (Dutta et al., 2017), therefore, the use of sub-surface or drip irrigation instead of overhead irrigation might reduce epiphytic populations of the pathogen. Similarly, straw mulching could be another option to lower weed population and delay center rot, as straw mulching causes a setback of center rot incidence by a week or two as opposed to black plastic mulch (Gitaitis et al., 2004).

Onion growers primarily rely on foliar protectants to control bacterial diseases in the field. Copper-containing bactericides and an ethylene-bis-dithiocarbamate fungicide (EBDC), for example, mancozeb, maneb, and dithane, are mixed and applied preventatively at 7-10-day intervals during the onion growing season (Gent and Schwartz, 2005; Harrison et al., 2008; Pfeufer and Gugino, 2018; Dutta and Gitaitis, 2020). Another copper-based chemical, Kocide, was found to reduce center rot in the absence of thrips, however, the presence of thrips reduced the efficacy of Kocide (Stumpf et al., 2021). Heavy reliance on copper-based bactericides is not a sustainable management option as copper can accumulate in the environment, and *Pantoea* species strains with copper tolerance have been reported from onion fields (Nischwitz et al., 2007; Wang et al., 2018; Tho et al., 2019).

Insect vectors are an additional component of the traditional disease triangle (susceptible host, environment, and pathogen) (Stevens 1960; Francl 2001). Tobacco thrips and onion thrips, in the case of the onion-center rot pathosystem, further complicate the disease management strategy as they facilitate movement of pathogens within and from outside the field (Gitaitis et al., 2003; Dutta et al., 2014, 2016). Reduced levels of thrips incidence in onions have been associated with lower disease incidence caused by *Pantoea* spp. (Grode et al., 2019). Chemical treatments like malathion, cypermethrin, lambda cyhalothrin, and methomyl are recommended for thrips control in onion (Harrison et al., 2008). Effective thrips control is a challenge for onion growers as thrips can reside between the fissures of younger leaves in the neck region of onion plants and evade pesticides (Brewster 2008). Brewster (2008) suggested that a certain level of resistance to thrips could be offered by onion cultivars with innermost leaves bred to have a wider angle of divergence.

Challenges to center rot management in organic Vidalia onions

Multiple sources of inocula, including infested seeds, weeds and thrips, have been reported for center rot in onion. Weeds of at least 25 different species found in the Vidalia onion production site can support epiphytic populations of *P. ananatis* (Gitaitis et al., 2003). One of these weeds, Florida pusley, is widely distributed in the Vidalia onion production zone. A study using mean temperatures prevailing in March and May in the Vidalia region showed that even low inoculum levels of *P. ananatis* can survive in Florida pusley (Dutta et al., 2017). Similarly, poor weed management in the field is likely to increase disease incidence in stored bulbs (Johnson et al., 2012). Therefore, the management of weeds is one of the crucial aspects of controlling the center rot of onion. However, weed control is the greatest challenge facing

organic growers (Boyhan 2010; Johnson et al., 2012). Hand weeding is a primary method of weed control in organic Vidalia onions, however, cost and labor management are difficult issues faced by the growers (Johnson et al., 2012). Tine weeders have provided some weed control, but only a few alternative remedial options are available if this method fails (Johnson et al., 2012).

Solarization and clove oil herbicidal treatments are shown to play a marginal role in weed control and do not play any role in center rot incidence on stored bulbs (Johnson et al., 2012). In general, herbicides made from essential oils do not provide any residual weed control and do not consistently control weeds (Johnson and Davis, 2014a). Additionally, pelargonic acid, a fatty acid with herbicidal properties, and clove oil adjuvants had similar efficacy as clove oil and were inconsistent in controlling weeds (Johnson et al., 2014a; Johnson et al., 2014b). Similarly, natural mulches are reported to be much less effective for weed control in organic onion (Boyhan et al., 2006). While straw mulches could delay center rot incidence by a week or two, they can negatively impact yield and delay harvest (Gitaitis et al., 2004).

Onion thrips and tobacco thrips are known to transmit center rot through feces and their feeding can promote *P. ananatis* invasion in onions (Gitaitis et al., 2003; Dutta et al., 2014; Grode et al., 2017). In the Vidalia region, populations of thrips increase with higher temperatures during the late onion production season and pose a higher risk of pathogen transmission (Sparks et al., 2011). Therefore, effective thrips control is an important aspect of center rot management in organic Vidalia onions. Thrips management in organic onions primarily relies on the use of insecticides. Organic growers are only allowed to use insecticides approved by the Organic Materials Review Institute (OMRI) and have limited choices of insecticides as opposed to conventional growers (Iglesias et al., 2021).

Only a few bio-insecticides have been assessed for thrips control in organic Vidalia onions. Use of neem oil was found to be ineffective in controlling thrips (Gitaitis et al., 2000). On the other hand, spinosad and pyrethrums derived from natural sources are considered effective thrips control options (Boyhan 2010). However, the use of Spinosad should be optimized as onion thrips with a high level of resistance to Spinosad have already been reported (Lebedev et al., 2013).

Similarly, seeds as a source of inoculum can impact center rot incidence in organic onions. The clear impact of infected seeds in center rot epidemics has not been studied in detail, nevertheless, it could contribute to the center rot epidemiology (Walcott et al., 2003). The use of disease-free seeds could ensure a reduced source of initial inoculum, however, there are challenges to producing disease-free onion seeds as the infected mother plant may not show obvious symptoms of center rot (Walcott et al., 2003).

Summer crops and pathogen survival in the Vidalia onion production system

Double cropping, which refers to the production of two different crops in the same year, is widely practiced by onion growers in the Vidalia region of GA (Watson 2016). Besides onions, growers have cover crops and high-value crops in the field during summer. This practice is driven mainly by its potential benefits to soil, weed control and other financial benefits. Onions are typically transplanted in December in Georgia, USA, and harvested in the late spring from April – May (Boyhan and Torrance, 2002; Harrison et al., 2008). After onion harvest, some growers plant cover crops such as Sunn hemp (*Crotalaria juncea*), cowpea (*Vigna unguiculate*), sorghum (*Sorghum bicolor*) and pearl millet, and cash crops such as corn (*Zea mays*), peanut (*Arachis hypogaea*), cotton (*Gossypium* spp.) and soybean (*Glycine max*) (Pollock-Moore 2014;

Cover crops, n.d., Chrys Tyson; personal communication). Cover crops are vital in an organic production system as they enrich the soil with organic matter, decrease evapotranspiration, inhibit weeds, improve soil quality and fertility, reduce soil erosion, and promote the growth of subsequent crops (Hendrickson and Stute, 2012; Schwartz 2013).

Field experiments studying the effect of double-cropping on the incidence of sour skin caused by *Burkholderia cepacia* suggested that the incidence of sour skin is lower in onions planted after pearl millet than those planted after corn (Gitaitis et al., 2005; Nischwitz et al., 2007; Watson, 2016). Watson (2016), using a bacterial growth assay, suggested that root exudates of pearl millet reduce the growth of *B. cepacia* as compared to that of corn. However, the authors found a decline in the effectiveness of pearl millet in reducing the *B. cepacia* population over time.

Besides pathogenic strains, epiphytic and symbiotic strains of *P. ananatis* have been described in several hosts, including corn (Coutinho and Venter, 2009). Moreover, *P. ananatis* can survive in corn debris (Sauer et al., 2015). On the other hand, *Psi* is pathogenic on pearl millet and foxtail millet, which are grown in the summer in the Vidalia region. The susceptibility or tolerance of the crops to pathogens is not the selection criterion for the choice of summer crops by onion growers in Georgia (Watson 2016). Organic producers in Georgia have limited knowledge of the role of high-residue systems in insect and disease occurrence (Reberg-Horton et al., 2012). Studies elucidating the survival ability of *Pantoea* spp. on the foliage and debris of different hosts and the factors affecting the survival and transmission of center rot are of crucial importance for Vidalia onion growers.

Factors affecting pathogenicity, virulence and survival of *Pantoea* species

Several studies on genetic determinants of pathogenicity and virulence in *P. ananatis* have elucidated the mechanisms and factors involved in onion pathogenicity. Type II, III and IV secretion systems are associated with many phytopathogenic bacteria (De Maayer et al., 2014), which play a major role in pathogenicity on hosts. The Type II secretion system is used by several pathogens of *Enterobacteriaceae*, causing soft rot symptoms, to deliver cell wall-degrading enzymes (Charkowski et al., 2012). Similarly, most gram-negative plant pathogens use type III and type IV secretion systems to translocate proteins that can suppress host immunity and facilitate nutrient acquisition from the hosts (Holeva et al., 2004; Zechner et al., 2012; Chang et al., 2014). Interestingly, *P. ananatis* does not possess any of these three secretion systems (De Maayer et al., 2014). However, the Type-III secretion system (T3SS) is present in *Psi* and acts as a primary virulence factor for pathogenicity on the foliage of pearl millet (Zhao et al., 2023).

Based on the whole genome comparison of onion-pathogenic and onion non-pathogenic strains of *P. ananatis*, a 16.5 to 20 kb region of DNA (termed HiVir) has been identified that is necessary to cause center rot symptom in onion leaves and bulbs (Asselin et al., 2018). The authors reported that the gene cluster is located on a chromosome and was likely acquired during a horizontal gene transfer event. Deletion of phosphoenolpyruvate mutase (*pepM*), the first gene of the HiVir cluster, renders *P. ananatis* non-pathogenic and reduces its population in onion leaves. Asselin et al. (2018) presumed that the HiVir locus produces phosphonate toxin based on the presence of the *pepM* gene involved in phosphonate biosynthesis. Later, it was confirmed that at least three different phosphonates were produced by the HiVir locus and ‘pantaphos’ was designated for the phosphonate that had a clear role in causing center rot symptoms in onion

bulbs (Polidore et al., 2021). Pantaphos is a phosphonate natural product similar in structure to citrate, isocitrate, aconitate, isopropylmaleate and maleate. It has a phytotoxic effect similar to that of glyphosate (a commercial herbicide) and phosphinothricin and is also cytotoxic to human cells (Polidore et al., 2021). Recent studies have associated HiVir pantaphos biosynthetic clusters with onion pathogenicity in *P. agglomerans* strains and *P. allii* strains (Shin et al., 2025a; Shin et al., 2025b).

Similarly, an OVRA (onion virulence region-A) gene cluster (later designated as '*alt*') has been identified in the plasmid of *P. ananatis* that promotes onion tissue-colonizing ability of pathogenic strains by conferring tolerance to allicin (a thiosulfinate with antimicrobial property released by damaged allium tissues) (Stice et al., 2018; Stice et al., 2020). Moreover, screening of 252 strains of different *Pantoea* spp. showed that the '*alt*' cluster is highly prevalent in strains isolated from onions (Stice et al., 2021).

Interestingly, strains without HiVir and/or *alt* cluster can be pathogenic on onion, which suggests the involvement of other pathogenicity factors in *P. ananatis*. Numerous novel genes associated with onion pathogenicity were identified in *P. ananatis* during a pan-genome-wide study and horizontal gene transfer events were implicated to play a significant role in the distribution of genes that enabled strain diversification, niche adaptation, and acquisition of onion pathogenic and virulence genes (Agarwal et al., 2021).

Moreover, the study of *P. stewartii* subsp. *indologenes* genomes revealed a gene cluster with eleven genes that are linked with onion pathogenicity (Agarwal et al., 2021). The *pepM* gene of only one *Psi* strain (PNA 03-3) shared similarity with the *pepM* gene of the 'HiVir' cluster reported in *P. ananatis*. The other three onion-pathogenic *Psi* strains, including PNA 14-12, have a *pepM* homolog in the novel gene cluster, different from that of 'HiVir'. Zhao et al.

(2023) later characterized the distinct phosphonate biosynthetic cluster as “halophos” and showed its importance in causing necrotic lesions in leaves and red onion scale.

In *P. stewartii subsp. stewartii* (*Pss*) the exopolysaccharide (EPS) stewartan, is essential for Stewart’s wilt symptom development in corn (Dolph et al., 1998; Beck von and Farrand, 1995; Herrera et al., 2008), and the formation of multicellular community structure (Herrera 2008) that aids bacterial survival in nature. Similarly, flagellar motility helps bacteria seek out favorable surroundings and helps in bacterial aggregation, which is required during biofilm formation and colonization of the hosts (Herrera et al., 2008; Ramsey and Whiteley, 2004; Roper 2011). On the other hand, the T3SS in *Psi*, a pathogenicity determinant of millet, could be essential for extended survival in crop residue. The pathogenic strain of *Psi* was shown to reach a significantly higher population as compared to the non-pathogenic strain lacking the *hrcC* gene, a key component of the T3SS (Zhao et al., 2023). Moreover, quorum sensing (QS), a cell-density-dependent communication system in bacteria, may contribute to pathogen survivability under adverse conditions. QS can regulate several genes, including EPS production and motility, that ultimately contribute to bacterial adhesion on surfaces, biofilm development, motility and host colonization (Koutsoudis et al., 2006; Herrera et al., 2008; Doblas-Ibanez et al., 2019; von Bodman et al., 1998). A similar role of QS in EPS biosynthesis, biofilm formation has been described in *P. ananatis* (Morohoshi et al., 2007).

Justification

Several species within the genus *Pantoea,* namely, *P. ananatis, P. agglomerans* and *P. allii* can cause center rot of onion. A few isolates of *P. stewartii* subsp. *indologenes* were recently identified to cause center rot symptoms in onion leaves and bulbs. *Psi* has previously been associated with bacterial blight and leafspot of millets. These observations indicate that different isolates within *Psi* may exhibit host specificity, potentially representing different pathovars. However, comprehensive host-range assays involving diverse *Allium* species and the Poaceae family are lacking to make such claims. Additionally, genotypic and phenotypic characterization of these strains is essential to better understand the role of *Psi* in center rot-onion pathosystem.

Cover crops and cash crops such as millets and corn are commonly cultivated during the summer following onion harvest in Vidalia, Georgia, USA. Considering the host range of *Psi*, which includes millets, its potential to survive in crop residue during the summer raises concerns for onion growers. The risk of *Psi* transmission to onions in the cropping systems where summer crops precede onion planting is largely unknown. Therefore, it is also important to investigate the role of summer crop residue in *Psi* survival and spread in onion fields and evaluate the risk of its transmission to winter-grown onions. Studies aimed at identifying various sources of pathogen inoculum in the field are critical to developing effective cultural intervention strategies, including crop rotation practices and residue management techniques.

In addition to identifying pathogen inoculum sources, understanding the biological mechanisms that support *Psi* survival is equally important. While various studies have identified factors that aid *Pantoea stewartii* colonization of the hosts and its persistence in nature, the specific role of these factors in *Psi* survivability on summer crops and in bare soil remains poorly

understood. Understanding the genetic factors affecting pathogen survival could provide valuable insights for devising targeted management strategies and limiting inoculum buildup in onion fields.

Onion growers face consistent threats from center rot, largely due to the limited availability of effective management options. Currently, no onion cultivar resistant to center rot is commercially available. Gowers primarily rely on protective applications of copper-based bactericides before disease onset to manage center rot. The same necessity that drives the heavy use of copper is inadvertently leading to the development of copper tolerance in bacteria. In response, growers' testimonials, alongside various reports, indicate a surging interest in incorporating beneficial microbes into spray programs. Despite this interest, the efficacy of BCAs to manage center rot remains poorly understood. Specifically, studies to enhance their efficacy against center rot are lacking.

Moreover, BCAs could potentially be applied in combination with copper to reduce the overall copper usage in disease management. However, the optimum intervals for alternating copper and BCAs have not been established yet. Similarly, the efficacy of tank-mix applications of copper and BCAs over their standalone applications remains largely understudied. Optimizing the timing and method of BCAs and copper application and effectively incorporating them into spray programs may equip growers with improved and durable center rot management techniques.

Objectives

Four major objectives have been proposed to test the hypotheses mentioned above;

1. Characterization of *P. stewartii* subsp. *indologenes* (*Psi*) and re-evaluation of its taxonomical standing.
2. Evaluating options to increase the efficacy of biocontrol agents for the management of *Pantoea* spp. under field conditions.
3. Integrating biocontrol agents with copper for center rot management in onion.
4. Survivability of *Pantoea stewartii* subspecies *indologenes* in crop residue and its transmission risk to onions in Poaceae-*Allium* cropping systems.

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CHAPTER 2

IDENTIFICATION OF TWO NOVEL PATHOVARS OF *PANTOEA STEWARTII* SUBSP. *INDOLOGENES* AFFECTING *ALLIUM* SP. AND MILLETS

Koirala, S., Zhao, M., Agarwal, G., Gitaitis, R., Stice, S., Kvitko, B., & Dutta, B. 2021. Identification of two novel pathovars of *Pantoea stewartii* subsp. *indologenes* affecting *Allium* sp. and millets. Phytopathology, 111(9), 1509-1519.

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Abstract

Pantoea stewartii subsp. *indologenes* is a causative agent of leafspot of foxtail millet and pearl millet; however, novel strains were recently identified that are pathogenic on onion. We phenotypically and genotypically characterized seventeen *Pantoea stewartii* subsp. *indologenes* strains from onion and other sources (pearl millet, foxtail millet, guar pulse, verbena and corn). Based on the host range evaluation, we propose two pathovars *P. stewartii* subsp. *indologenes* pv. *cepacicola* pv. nov. and *P. stewartii* subsp. *indologenes* pv. *setariae* pv. nov. *Pantoea stewartii* subsp. *indologenes* pv. *cepacicola* pv. nov. causes symptoms on *Allium* species (leek, onion, chive and Japanese bunching onion) and also on foxtail millet, pearl millet and oat. However, *P. stewartii* subsp. *indologenes* pv. *setariae* pv. nov can only infect the members of *Poaceae* (foxtail millet, pearl millet and oat). We also propose that the type strain of *P. stewartii* subsp. *indologenes* (LMG 2632^{T}) should be designated as a pathotype strain of *P. stewartii* subsp. *indologenes* pv. *setariae* and recommend that the strain 'PNA 14-12' be designated as the pathotype strain of *P. stewartii* subsp. *indologenes* pv. *cepacicola*. The digital DNA-DNA hybridization, average nucleotide identity and multi-locus sequence analysis study showed that the two pathovars are genotypically closely related. Our study also showed that *P. stewartii* subsp. *indologenes* pathovars and *P. stewartii* subsp. *stewartii* share high genotypic relatedness and cannot be differentiated by dDDH and ANI values. Although, the newly proposed pathovars are not clearly distinguishable by their fatty acid and methyl esterase profiles, and substrate utilization patterns, a fatty acid (unknown with retention time: 10.9525) and few metabolites (3-methyl glucose, Na butyrate and fusidic acid) can be potentially used to distinguish them. We also report the distribution of previously known pathogenicity (HiVir, *hrcC*) and virulence (*alt*)

factors of *Pantoea* sp. in the new pathovars. The impact of these new pathovars in the center rot pathosystem of onion is yet to be determined.

Introduction

Mergaert et al. (1993) reclassified *Eriwinia stewartii* as *Pantoea stewartii* and proposed two subspecies *P. stewartii* subsp. *stewartii* and *P. stewartii* subsp. *indologenes*. The two subspecies considerably differed in their electrophoretic protein profiles, fatty acid composition, and biochemical properties. The DNA-DNA hybridization value for the type strain of *P. stewartii* subsp. *stewartii* (LMG 2715^{T}) and that of *P. stewartii* subsp. *indologenes* (LMG 2632^{T}) was 79%. However, unlike *P. stewartii* subsp. *stewartii, P. stewartii* subsp. *indologenes* can produce indole, hydrolyze esculin, grow on cis-aconitate and form acid from cellobiose, maltose, lactose, arbutin, salicin and D-arabitol (Mergaert et al. 1993). Moreover, *P. stewartii* subsp. *stewartii* lacks the fatty acids 2-hydroxytetradecanoic acid (14:0 2OH) and cyclo-heptadecanoic acid (17:0 cyclo) that are present in *P. stewartii* subsp. *indologenes* (Mergaert et al. 1993). *Pantoea stewartii* subsp. *stewartii* causes Stewart's bacterial wilt of corn (*Zea mays* L.) and is transmitted by the corn flea beetle (*Chaetocnema pulicaria*) (Wensing et al. 2010). Although, the pathogen can be seed borne in corn, it is not a major contributor to disease epidemics in the field (Block et al. 1998). Interestingly, *P. stewartii* subsp. *indologenes* is non-pathogenic on corn (De Maayer et al. 2017) but has been associated with leafspot of foxtail (*Setaria italica*) and pearl millet (*Pennisetum glaucum*), pineapple (*Ananas comosus*) rot (Mergaert et al. 1993), and recently with leaf blight of rice (*Oryza sativa*), and leaf blight and wilt of *Dracaena sanderiana* (Azizi et al. 2019; Zhang et al. 2020).

Gehring et al. (2014) developed single-nucleotide polymorphism (SNP)-based primer pairs from *galE* and *recA* genes that are specific to *P. stewartii* subsp. *indologenes*. Using these primers along with a host range test, Stumpf et al. (2018) identified two novel strains of *P. stewartii* subsp. *indologenes* (PNA 03-3 and PNA 14-12) that could cause foliar and bulb symptoms similar to that observed in center rot of onion (Gitaitis et al. 1997). The authors later proposed the addition of this subspecies as a member of the center rot complex in addition to other known *Pantoea* sp., namely, *P. ananatis*, *P. agglomerans*, and *P. allii* (Gitaitis et al. 2002; Edens et al. 2006; Brady et al. 2011). These novel strains (PNA 03-3 and PNA 14-12) were also pathogenic on pearl millet; however, the type strain of *P. stewartii* subsp. *indologenes* (LMG 2632^{T}) was not pathogenic on onion foliage or bulbs but was able to cause foliar symptoms on pearl millet. These observations indicate that these two strains may belong to a new pathovar of *P. stewartii* subsp. *indologenes*, which can infect both onion and pearl millet. Thus, the type strain of *P. stewartii* subsp. *indologenes* (LMG 2632^{T}) requires a new pathovar designation. However, at that time, a detailed host-range assay with plants of different *Allium* and *Poaceae* species was lacking to make such claims.

The International Society for Plant Pathology (ISPP) defines a pathovar as "a strain or set of strains with the same or similar characteristics, differentiated at infra-subspecific level from other strains of the same species or subspecies on the basis of distinctive pathogenicity to one or more plant hosts" (Dye et al., 1980; Young et al., 2001). In this manuscript, we provide evidence for the two newly proposed novel pathovars, *P. stewartii* subsp. *indologenes* pv. *cepacicola* pv. nov. and *P. stewartii* subsp. *indologenes* pv. *setariae* pv. nov. The proposal was based on detailed host range tests on cultivated *Allium* sp. in the family *Amaryllidaceae*, and cultivated members in the *Poaceae*, molecular and biochemical characterization that included whole

genome based average nucleotide identity (ANI), digital DNA-DNA hybridization analysis (dDDH), fatty acid composition, and carbon substrate utilization and chemical sensitivity assay. We also report the distribution of previously known loci (HiVir and *alt*) in *Pantoea* sp. responsible for pathogenicity and virulence in onion (Asselin et al. 2018; Stice et al. 2020), and a *hrcC* gene, a component of a Type III secretion system present in *P. stewartii* subsp. *stewartii* (Merighi et al. 2006), in *P. stewartii* subsp. *indologenes* pv. *cepacicola* and *P. stewartii* subsp. *indologenes* pv. *setariae*.

Materials and Methods

Screening of UGA CPES culture collection for *Pantoea stewartii* subsp. *indologenes*

The University of Georgia Coastal Plain Experiment Station (UGA CPES) has a repository of *P. ananatis* strains (*n* =217) that were identified previously based on the primer set by Gitaitis et al. (2002). This primer set cannot differentiate between *P. ananatis* and *P. stewartii* subsp. *indologenes* and hence, a thorough screen of the culture collection was performed to identify *P. stewartii* subsp. *indologenes* strains that were misidentified and curated as *P. ananatis*. The *P. ananatis* strains screened for *P. stewartii* subsp. *indologenes* is listed in the Supplementary Table 2.1. These strains were screened with the primer pair 3614galE/3614galEc (Table 2.1), which was reported as specific for *P. stewartii* subsp. *indologenes* based on the single nucleotide polymorphism of *galE* gene (Gehring et al. 2014). Briefly, the bacterial strains stored in 15% aqueous glycerol solution at -80°C were streaked on nutrient agar (NA) plates. For DNA amplification, a 25 µl reaction mixture was used that consisted of 0.25 µl of 5 units/µl HotStar Taq DNA Polymerase (Qiagen, Valencia, CA), 1 µl each of 10 µM 3614galE and 3614galEc primers, 0.4 µl of 10 mM dNTP, 2 µl of 10X PCR Buffer (Qiagen), and 18.35 µl nuclease-free

water. For DNA template, a single bacterial colony grown on 48 h NA was suspended in 60 µl of nuclease-free water, heated for 4.5 min at 95°C, cooled for 1 min, and then 2 µl of the bacterial suspension was used for amplification. PCR amplification was carried out as described previously (Gehring et al. 2014; Stumpf et al. 2018). Nuclease-free water was used as a negative control. PCR products were separated by electrophoresis on 1% agarose gels stained with GelGreen nucleic acid stain in 1X Tris-Borate-EDTA buffer for 1 h at 90 V. The amplicons were observed on a BIO-RAD Gel Doc XR+ imaging system (Bio-rad, CA, USA).

DNA extraction, purification, and sequencing

A single colony of each strain from a 48 h-old culture on NA was transferred to 3 ml of nutrient broth and was grown overnight in a rotary shaker (MaxQ 4450, Thermo Scientific; Waltham, MA) at 200 rpm at 28°C. A total of 16 strains were sequenced with 11 *P. stewartii* subsp. *indologenes* strains from GA (5 strains from onion and 6 strains from sources other than onion), and five *P. stewartii* subsp. *indologenes* strains from the National Collection of Plant Pathogenic Bacteria (NCPPB, UK); NCPPB 2275, NCPPB 1877, NCPPB 2282, NCPPB 1562, NCPPB 2281. Total genomic DNA of *P. stewartii* subsp. *indologenes* strains (Table 2.2) was extracted from overnight cultures using the E.Z.N.A® Bacterial DNA kit (Omega Biotek, Norcross, GA) according to the manufacturer's instructions. DNA was quantified using a NanoDrop Lite spectrophotometer (ThermoFisher Scientific, Madison, WI) and 100 µl of >60 ng/µl genomic DNA was sent to Novogene (Novogene corporations Inc, Sacramento, CA) for whole-genome sequencing using an Illumina platform. The bacterial DNA was fragmented and end repairing and A-tailing were performed that resulted in end-repaired 5'-phophorylated and 3'-dA-tailed DNA fragments. Adapters of DNA fragments with a 3'-dTMP overhang wereligated to 3'-dA-tailed molecules followed by post-ligation cleanup to remove unligated adapters and/or

adapter-dimer molecules. Library amplification was performed using a high-fidelity, low-bias PCR assay to ensure the amplification of library fragments with appropriate adapters on the ends. Library preparation and PCR amplification involved dual indexing (introducing indexes into both library adapters) to avert mixed clusters on the flow which are the primary source of error during multiplex sequencing. DNA libraries of the strains were pooled and sequenced on Illumina Nextseq500 using a high output run. Sequences with 150 bp paired end reads were obtained for all the strains.

Read data filtering and genome assembly

The raw fastq files obtained from Illumina sequencing platform were run on a FastQC to assess the data quality. Low quality bases were filtered out and the read data were trimmed using Trimmomatic (v 0.36) in paired end mode (Bolger et al. 2014) for read containing primer/adaptor sequences. Moreover, all 5' and 3' stretches of ambiguous 'N' nucleotides were clipped. Trimming was repeated once to ensure high quality reads. The trimmed data were re-assessed using FastQC and used for genome assembly.

Processed reads were assembled using SPAdes (V3.11.1) (flags--isolate--cov-cutoff auto) (Nurk et al. 2013) and filtered to a minium contig size of 500 bp. The quality of the assembly was verified using Quast (Gurevich et al. 2013). The contigs of each assembly were reordered according to the complete genome of *P. stewartii* strain ZJ-FGZX1 (chromosome, NZ_CP049115.1; plasmid unamed1, NZ_CP049116.1; plasmid unnamed 2, NZ_CP049117.1) using Mauve Contig Mover (Rissman et al. 2009). Assembly files were submitted to NCBI database under the bio-project PRJNA676043 with accession numbers SAMN16866615 to SAMN16866629.

Pairwise digital DNA-DNA hybridization (dDDH) values and average nucleotide identity (ANI)

The pairwise digital DNA-DNA hybridization (dDDH) values were estimated using the recommended settings of the genome-to-genome distance calculator (GGDC) 2.1 (Meier-Kolthoff et al., 2013). The genome sequence data were uploaded to the Type (Strain) Genome Server (https://tygs.dsmz.de) and values calculated using formula d4 were recorded. ANI values based on MUMmer (ANIm) were calculated using Pyani v0.2.10 (Pritchard et al. 2016). The whole-genome sequence of other type strains [*P. stewartii* subsp. *indologenes* LMG 2632^T (NZ_JPKO00000000.1); *P. stewartii* subsp. *stewartii* LMG 2715^T; GCA_008801695.1 *P. ananatis* LMG 2665^T (NZ_JMJJ00000000); *P. allii* LMG 24248^T (NZ_NTMH00000000)] were utilized for the analysis.

Multi-locus sequence analysis

An *in silico* multi locus sequence analysis (MLSA) was performed using partial nucleotide (nt) sequences of *atpD* (657 nt), *gyrB* (578 nt), *infB* (612 nt), and *rpoB* (409 nt) genes previously used by Brady et al. (2008). For *in silico* MLSA, 16 *P. stewartii* subsp. *indologenes* strains (sequenced in this project) were used along with the strains whose draft genomes were publicly available; *P. stewartii* subsp. *indologenes* strains (ZJ-FGZX1: NZ_CP049115, LMG 2632^T: NZ_JPKO00000000.1), *P. ananatis* LMG 2665^T (NZ_JMJJ00000000), *P. allii* LMG 24248^T (NZ_NTMH00000000), *P. stewartii* subsp. *stewartii* strains (LMG 2715^T: GCA_008801695.1 DC283: NZ_CP017581) and *P. agglomerans* LMG 1286^T (NZ_FYAZ00000000). The gene fragments were extracted from draft genomes using BLASTn with the partial sequences reported by Stumpf et al. 2018 for *P. ananatis* (PNA 97-1) as the query sequences, then imported, concatenated, and analyzed in Geneious Prime® (v2019.2.3). The concatenated sequences (2,256

nt) were aligned using MAFFT (v 7.294b) (Katoh and Standley, 2013). The alignment was used to construct a phylogenetic tree using maximum-likelihood (ML) method by PHYML using the estimated best model with 1,000 bootstrap replicates.

Pathogenicity and host-range test on *Allium* species (onion, leek, chive, and Japanese bunching onion) and *Poaceae* species (oat, pearl millet, foxtail millet and corn)

Inoculum preparation

For inoculum preparation, single colony of each strain was transferred from a 48 h-old NA culture to 3 ml of nutrient broth. The broth was shaken for 12 h at 200 rpm in a rotary shaker (MaxQ 4450) at 28°C. A 1.6 ml of bacterial culture was centrifuged at 8000 rpm for 2 min (Centrifuge 5430, Eppendorf, Boston, MA). The supernatant was discarded and the bacterial pellet was re-suspended in 0.01 M phosphate buffer saline (PBS). The inoculum was adjusted using a spectrophotometer (Bio Photometer, Eppendorf, Boston, MA) to an optical density of 0.3 at OD_{600nm} (~1×10^8 CFU/ml). For the phenotypic and genotypic characterization of *P. stewartii* subsp. *indologenes*, 11 strains isolated from onion and non-onion sources, five NCPPB strains and the type strain of *P. stewartii* subsp. *indologenes* LMG 2632^T (Table 2.2) were used.

(i) Pathogenicity on *Allium* species

A host range study with *P. stewartii* subsp. *indologenes* strains from onion (n = 5) and other sources (n = 12) (Table 2.2) was conducted on four *Allium* species, onion (*Allium cepa*; cv. Sweet harvest), chive (*Allium schoenoprasum*; cv. Dolores), leek (*Allium porrum*; cv. King Richard), and Japanese bunching onion (*Allium fistulosum* cv. Guardsman). *Pantoea ananatis* strains [(PNA 97-1 (onion), PANS 02-7 (chive), PANS 99-11 (leek), and PNA 99-14 (Japanese bunching onion)] that are known to cause foliar symptoms based on a preliminary study were used as respective positive controls for each *Allium* species. Seedlings were established in plastic

pots (T.O. plastics, Clearwater, MN) with dimension of 9 × 9 × 9 cm (length × breadth × height) containing a commercial potting mix (Sta-green, Rome, GA). The seedlings were maintained under a greenhouse condition at 25-28°C and 70-90% relative humidity with a light:dark cycle of 12:12 h. Osmocote smart-release plant food (The Scotts Company, Marysville, Ohio) was used for periodic fertilization. For all the *Allium* sp. two-months old plants were inoculated after cutting the central leaf 2 cm from the apex with a sterile pair of scissors. Using a micropipette, a 10 µl drop of a 1×10^8 CFU/ml bacterial suspension (1×10^6 CFU/leaf) was deposited at the cut end of the leaf. Seedlings inoculated with PBS as described above were used as negative controls. Three replications per strain per host were used for one experiment and the experiment was repeated once. The seedlings were observed daily for symptom development until 7 days post-inoculation (DPI) and were compared with the foliar symptoms displayed by the positive controls for each *Allium* species. At 7 DPI, lesion length of each inoculated leaf was measured and recorded. Strain aggressiveness was determined based on the mean foliar lesion length. Strains causing a mean lesion length <1.5 cm were considered less aggressive, 1.51-2.5 cm moderately aggressive, and >2.5 cm highly aggressive on onion, Japanese bunching onion, and chive. For leek, strains causing a lesion length <0.6 cm were considered less aggressive, 0.61-1.2 cm moderately aggressive, and >1.2 cm highly aggressive. In the absence of lesion development, strains were considered as non-pathogenic. To confirm if the symptoms were caused by *P. stewartii* subsp. *indologenes*, bacteria were isolated from the region adjoining the symptomatic and healthy tissue on NA medium and incubated for 48 h at 28°C. Yellow-pigmented colonies were observed on plates and the identities were confirmed using the subspecies-specific primer set mentioned above (Gehring et al. 2014).

(ii) Pathogenicity on the species of *Poaceae* members

Pantoea stewartii subsp. *indologenes* strains from onion (n = 5) and non-onion sources (n = 12) were used in this study. Seedlings of oat (*Avena sativa* cv. Coaker 227), foxtail millet (*Setaria italica* cv. Golden German), pearl millet (*Pennisetum glaucum* cv. TifGrain 102) and corn (*Zea mays* cv. Seneca) were grown under similar conditions in the greenhouse as the *Allium* species described above. Inocula were prepared as described above. As a control for foliar symptoms, a known foliar pathogenic strain for foxtail millet and pearl millet [*P. stewartii* subsp. *indologenes* type strain (LMG 2632^T)], oat [*Pseudomonas coronafaciens* (PCF 93-2)] and corn [*P. stewartii* subsp. *stewartii* LMG 2715^T] was used. Seedlings were inoculated using a syringe, after pricking the leaf with the sterile needle, with 0.3 ml of 1×10^7 CFU/ml (approx. 1×10^6 CFU/leaf) inoculum on the abaxial surface. Eight weeks old healthy-appearing leaves of oat, pearl millet, foxtail millet and corn were used for the inoculation. Plants inoculated with PBS were used as negative controls. Inoculated leaves were observed daily for symptom development. Three replications were performed per strain per host for one experiment and two independent experiments were conducted. Final disease ratings (incidence and severity) were taken at 21 DPI for oat, and 14 DPI for pearl millet, foxtail millet and corn. The disease severity scale was based on the mean lesion length on the inoculated leaves. Strains causing a mean lesion length 0.51-1 cm was considered less aggressive, 1.1-1.5 cm as moderately aggressive, and lesions >1.5 cm as highly aggressive on pearl millet, foxtail millet, oat and corn. Strains that did not cause necrotic lesions or displayed dry lesion with length less than 0.5 cm were considered as non-pathogenic as these inoculation lesions (<0.5 cm) were also observed in the PBS-inoculated leaves. For further confirmation, isolations on NA medium were attempted from hosts that did not display symptoms or displayed small dried lesions (<0.5 cm). In addition, confirmation of leaves

displaying symptoms from each host was made by bacterial isolation on NA medium followed by identification using *P. stewartii* subsp. *indologenes*-specific PCR assay (Gehring et al., 2014). To achieve this, symptomatic leaves (*Allium* sp. and Poacea hosts) were randomly selected and bacterial isolations were made on NA medium. After 48 h of incubation, yellow colored colonies were isolated, which were later confirmed using *P. stewartii* subsp. *indologenes*-specific PCR assay (Gehring et al., 2014).

(iii) Red onion scale necrosis (RSN) assay

Red onions (cv. Red Barrett) were surface sterilized with 70% ethanol and the outer scale was sliced into rectangles approximately 3 cm × 4 cm. Each scale was placed in a sterile petri dish containing two layers of sterile paper towels (5 cm × 5 cm) pre-moistened with sterile water. Onion scales were wounded at the center of the scale with a sterile needle and inoculated with a bacterial suspension (10 µl) at 1×10^6 CFU/ml. Inocula of *P. stewartii* subsp. *indologenes* strains [$n = 17$; onion ($n = 5$) and other sources ($n = 12$), Table 2.2] were prepared as described above. A red-scale necrotic strain of *P. ananatis* (PNA 97-1) was used as a positive control. The petri dishes were kept on an aluminium tray (46 × 25 × 10 cm) and covered with a plastic lid. The onion scales were incubated at room temperature for 7 days in the dark and the area of pigment clearing with necrotic lesions was measured at 7 DPI. Strains unable to clear the red anthocyanin pigment or cause scale pitting were considered as non-pathogenic. Strains causing pitting with <1 cm^2 scale clearing were considered as less aggressive, strains clearing 1-2 cm^2 of red scale were considered moderately aggressive and those causing ≥ 2 cm^2 scale clearing were considered highly aggressive. Three replications were performed for each strain and in total two experiments were conducted. Scales inoculated with PBS were used as negative control.

(iv) Whole onion-bulb inoculation assay

Onion bulbs (cv. Century) were surface sterilized with 70% ethanol after removal of tunic layers. Each bulb was placed on an individual plate containing two layers of paper towel pre-moistened with sterile water. Onion bulbs were inoculated longitudinally at the shoulder with a syringe and a sterile needle containing a volume of 400 µl (1×10^8 CFU/ml) (Schroeder et al. 2010). Special attention was given to the uniformity of depth of inoculation into each bulb, which was ascertained by placing a thin rubber stopper in the needle. Following inoculation, bulbs were incubated at 25°C in an aluminium tray. After 12 days of incubation, bulbs were sliced vertically alongside the inoculation site and the weight of the whole bulb and symptomatic scales with necrotic lesions (and visual rot) was measured and recorded. Relative weight of rotten tissue was determined as follows; (weight of rotten tissue/weight of the entire bulb) × 100. Strains that caused relative rotten tissue weight of ≤4% were considered as less aggressive, 4-8% as moderately aggressive and strains that resulted in ≥8% of relative rotten tissue weight were considered as highly aggressive. Strains unable to cause any disease symptom on the scales were considered as non-pathogenic. Bulbs inoculated with *P. ananatis* strain (PNA 97-1) was used as a positive control and bulbs inoculated with PBS served as a negative control. Three replications were performed for each strain and total two independent experiments were performed.

Screening of *P. stewartii* subsp. *indologenes* pv. *cepacicola* and *P. stewartii* subsp. *indologenes* pv. *setariae* strains for HiVir and *alt* gene clusters

Primer pairs HiVir2_pF/HiVir2_pR and AltF/AltR (Table 2.1) were used to amplify HiVir (Asselin et al. 2018) and *alt* (Stice et al., 2020) gene clusters, respectively. PCR reaction mixture contained 25 µl total volume and consisted of 2 µl DNA template, 0.4 µl of 10 mM dNTP, 2 µl of 10X PCR Buffer (Qiagen), 0.25 µl of 5 units/µl HotStar Taq DNA Polymerase (Qiagen), 1 µl

each of 10 µM primers, and 18.35 µl nuclease-free water. The PCR cycling conditions for both gene clusters include initial denaturation at 95°C for 5 min, 35 cycles at 98°C for 10 s, 47°C for 30 s, and 72°C for 30 s, followed by final elongation at 72°C for 8 min. *Pantoea ananatis* strain (PNA 97-1) was used as a positive control and nuclease-free water as a negative control. The strains of *P. stewartii* subsp. *indologenes* pv. *cepacicola* (PNA 03-3, PNA 14-12, PNA 14-11, PNA 14-9) and *P. stewartii* subsp. *indologenes* pv. *setariae* (LMG 2632^T, PNA 15-2, PANS 07-4, PANS 07-6, PANS 07-10, PANS 07-12, PANS 07-14, PANS 99-15, NCPPB 1562, NCPPB 1877, NCPPB 2275, NCPPB 2281, NCPPB 2282) were used in this study.

Screening for *hrcC* gene in *P. stewartii* subsp. *indologenes* pv. *cepacicola* and *P. stewartii* subsp. *indologenes* pv. *setariae* strains

The primer set PsiHrcCF/PsiHrcCR (Table 2.1) was designed using Primer3web (version 4.1.0, http://bioinfo.ut.ee/primer3/) to amplify 553-nt of *hrcC* gene (locus tag *C7433_10520*) of *P. stewartii* subsp. *indologenes* PNA 03-3 (Accession number GCA_003201175.1). PCR was carried out in a total reaction volume of 25 µl using 2 µl DNA template, 0.4 µl of 10 mM dNTP, 2 µl of 10X PCR Buffer (Qiagen), 0.25 µl of 5 units/µl HotStar Taq DNA Polymerase (Qiagen), 1 µl each of 10 µM primers, and 18.35 µl nuclease-free water. PCR was performed under the conditions described by Naum et al. (2009). *Pantoea ananatis* (PNA 97-1) was used as a negative control. The strains of *P. stewartii* subsp. *indologenes* pv. *cepacicola* (PNA 03-3, PNA 14-12, PNA 14-11, PNA 14-9) and *P. stewartii* subsp. *indologenes* pv. *setariae* (LMG 2632^T, PNA 15-2, PANS 07-4, PANS 07-6, PANS 07-10, PANS 07-12, PANS 07-14, PANS 99-15, NCPPB 1562, NCPPB 1877, NCPPB 2275, NCPPB 2281, NCPPB 2282) were used in this study.

Fatty acid composition analysis in *P. stewartii* subsp. *indologenes* pv. *cepacicola* and *P. stewartii* subsp. *indologenes* pv. *setariae* strains

Fatty acid methyl esterase (FAME) analysis was conducted and differences in fatty acid composition were evaluated for the *P. stewartii* subsp. *indologenes* strains (PNA 03-3, PNA 14-12, PNA 14-11, PNA 14-9, PNA 15-2, PANS 07-4, PANS 07-6, PANS 07-10, PANS 07-12, PANS 07-14, PANS 99-15, NCPPB 1562, NCPPB 1877, NCPPB 2275, NCPPB 2281, NCPPB 2282) along with the type strain LMG 2632^T and *P. stewartii* subsp. *stewartii* (LMG 2715^T). Strains were cultured on tryptic soy broth agar for 24 h at 28°C, and whole-cell fatty acids were saponified, methylated, and extracted as described previously by Miller and Berger (1985). FAME analysis was conducted using the Microbial Identification System, Sherlock version 3.10 (MIDI).

Substrate utilization by *P. stewartii* subsp. *indologenes* pv. *cepacicola* and *P. stewartii* subsp. *indologenes* pv. *setariae* strains

Substrate utilization and chemical sensitivity patterns were tested for all the strains of *P. stewartii* subsp. *indologenes* (PNA 03-3, PNA 14-12, PNA 14-11, PNA 14-9, PNA 15-2, PANS 07-4, PANS 07-6, PANS 07-10, PANS 07-12, PANS 07-14, PANS 99-15, NCPPB 1562, NCPPB 1877, NCPPB 2275, NCPPB 2281, NCPPB 2282) along with the type strain LMG 2632^T and *P. stewartii* subsp. *stewartii* (LMG 2715^T) using BiOLOG GENIII microplates (BiOLOG, Hayward, CA). The inoculum was prepared by selecting a single bacterial colony with an inoculation loop from 24 h-old NA plate and gently rubbing the loop against the bottom of the tube containing inoculation fluid (IF). The transmittance of the bacterial suspension was adjusted to 95% (~0.0223A at 600 nm) and the resulting suspension was poured into a multichannel pipette reservoir. Using an eight-channel, repeating pipette (BiOLOG, Hayward,

CA) fitted with 1,500 µl-capacity sterile tips, 100 µl of the suspension were dispensed into each well of a microplate which was then covered with a lid. The microplates were incubated at 30°C for 24 h and color changes in the wells were recorded according to the manufacturer's instructions. Later, the qualitative data for utilization of each substrate for each of the strains were recorded and compared.

Results

Identification of *P. stewartii* subsp. *indologenes* from UGA CPES culture collection

Out of 217 *P. ananatis* strains screened, 11 strains were amplified with primer-pair (3614galE/3614galEc) specific for *P. stewartii* subsp. *indologenes* (Supplementary Table 2.1). Among the 11 strains, five strains were isolated from either onion bulb or foliage, five strains were isolated from the members of *Poaceae* family [i.e, pearl millet (*Pennisetum americanum*), foxtail millet (*Setaria italica*), corn (*Zea mays*), and crabgrass (*Digitaria sanguinalis*)] and one strain was isolated from verbena (*Verbena officinalis*). The six strains from the members of *Poaceae* and verbena were isolated from the foliar surface of aymptomatic plants. All 11 strains were isolated in Georgia, USA between 1999-2015.

Pairwise digital DNA-DNA hybridization (dDDH) values and average nucleotide identity (ANI)

The pairwise digital DDH and ANI values were calculated for all 16 *P. stewartii* subsp. *indologenes* test strains and compared with the type strains of other *Pantoea* sp. (Table 2.3). The type strains of both *P. stewartii* subsp. *indologenes* (LMG 2632^T) and *P. stewartii* subsp. *stewartii* (LMG 2715^T) shared dDDH value greater than 91% and ANI values greater than 99% with all 16 test strains. However, the dDDH and ANI values shared by the 16 test strains with

both *P. ananatis* LMG 2665^T and *P. allii* LMG 24248^T were <28 % and ≤86 %, respectively, which were below the species cut-off values of 70% for dDDH and 95~96 % for ANI (Konstantinidis & Tiedje. 2005, Chun et al. 2018).

Multi-locus sequence analysis

A maximum likelihood tree of *P. stewartii* subsp. *indologenes* strains along with other *Pantoea* strains was constructed using concatenated nucleotide sequences of four housekeeping genes (*atpD* (657), *gyrB* (578), *infB* (612), and *rpoB* (409). All 16 test strains clustered with the strains of *P. stewartii* subsp. *indologenes* (LMG 2632^T and ZJ-FGZX1) and *P. stewartii* subsp. *stewartii* (LMG 2715^T and DC283), and formed a distinct clade separate from *P. ananatis* LMG 2665^T, *P. allii* LMG 24248^T, and *P. agglomerans* LMG 1286^T with a bootstrap value of 100 (Figure 2.1).

Pathogenicity and host-range test on *Allium* species (onion, leek, chive, and Japanese bunching onion) and *Poaceae* species (pearl millet, foxtail millet, and oat)

(i) Pathogenicity on *Allium* species

Out of the total seventeen *P. stewartii* subsp. *indologenes* strains, four strains (PNA 03-3, PNA 14-12, PNA 14-11, and PNA 14-9) were able to cause foliar lesions on onion with varying levels of aggressiveness at 7 DPI. The strain PNA 14-12 was highly aggressive on onion with an average lesion length of 3.07 cm. The other three pathogenic strains were moderately aggressive on onion with average lesion lengths of <2.5 cm but >1.5 cm (Table 2.4). None of the other strains including the type strain (LMG 2632^T) produced any necrosis on onion seedlings (Supplementary Figure 2.1). Plants inoculated with PBS buffer displayed no symptom at 7 DPI.

On leek, the four onion-pathogenic *P. stewartii* subsp. *indologenes* strains (PNA 03-3, PNA 14-12, PNA 14-11, and PNA 14-9) produced typical foliar necrotic symptoms at 7 DPI. The level of aggressiveness varied among the strains: PNA 03-3 was highly aggressive, PNA

14-12 moderately aggressive, and PNA 14-9 and PNA 14-11 less aggressive. Other test strains produced no symptoms on leek. Similarly, Japanese bunching onion and chive seedlings inoculated with the four onion-pathogenic strains showed characteristic foliage-necrosis symptoms at 7 DPI (Supplementary Figure 2.1). These strains were graded as less-to-moderately aggressive on both Japanese bunching onion and chive. PCR amplification of randomly selected symptomatic samples using the subspecies-specific primer set confirmed the isolates recovered were *P. stewartii* subsp. *indologenes*.

(ii) Pathogenicity on the species of *Poaceae* members

The type strain of *P. stewartii* subsp. *indologenes* (LMG 2632^T) used as the positive control (for pearl millet and foxtail millet) caused brown necrotic lesions around the syringe-inoculated area in pearl millet and foxtail millet at 14 DPI (Supplementary Figure 2.1). The type strain of *P. stewartii* subsp. *stewartii* (LMG 2715^T) caused a necrotic lesion on corn but did not display any symptoms on pearl millet, foxtail millet and oat. All *P. stewartii* subsp. *indologenes* strains were able to cause necrotic symptoms on both pearl millet and foxtail millet at 14 DPI (Supplementary Figure 2.1; Table 2.4). The strains were moderate to highly aggressive except for NCPPB 2282 which was less aggressive on pearl millet (Table 2.4). Plants inoculated with PBS buffer showed no symptoms or lesions (<0.5 cm) at 14 DPI. Similarly, all *P. stewartii* subsp. *indologenes* strains except for NCPPB 2282 were pathogenic on oat. None of the *P. stewartii* subsp. *indologenes* caused symptoms on pearl millet, foxtail millet and oat. Positive control (*Pseudomonas coronafaciens* PCF 93-2) produced symptomatic lesions whereas PBS buffer caused no visible symptoms or lesions (<0.5 cm) on oat leaves at 21 DPI. *Pantoea stewartii* subsp. *indologenes* strains were re-isolated from infected leaves and identities confirmed using the subspecies-specific primer set. Bacterial colonies were not isolated from the PBS-inoculated

plants or from plants with <0.5 cm of lesion length. Hence, the plants that displayed <0.5 cm of lesion length were deemed non-pathogenic. These small lesions could result from the inoculation injury by the sterile needle.

(iii) Red onion scale necrosis assay

A red-scale necrotic strain of *P. ananatis* (PNA 97-1) used as a positive control was able to cause scale necrosis and red-pigment clearing at 7 DPI (Table 2.4). Four *P. stewartii* subsp. *indologenes* strains (PNA 03-3, PNA 14-12, PNA 14-11, and PNA 14-9) pathogenic on *Allium* seedlings caused scale pitting and clearing of red anthocyanin pigment on onion scales (Figure 2.2). The strain PNA 03-3 was highly aggressive among the four strains as it caused scale necrosis and red-pigment clearing with area >2 cm^2. The other three strains were less aggressive on red scale as they showed scale pitting with areas of red-pigment clearing <1 cm^2. None of the other strains caused scale pitting or red-pigment clearing at 7 DPI. Negative control seedlings did not cause any symptom on the red onion scales.

(iv) Whole bulb inoculation assay

All four *P. stewartii* subsp. *indologenes* strains pathogenic to *Allium* (Table 2.4) caused a rot in onion bulbs at 12 DPI as did the positive control (PNA 97-1). The strain PNA 03-3 showed 9.7% relative rotten tissue weight and was considered highly aggressive. The strain PNA 14-12 displayed moderate aggressiveness with a relative rotten tissue weight of 4.2% and the strains PNA 14-11 and PNA 14-9 were less aggressive as they caused bulb rot with a relative rotten tissue weight of <4%. All other strains of *P. stewartii* subsp. *indologenes* were non-pathogenic as they did not cause any symptoms on the bulb. Bulbs inoculated with PBS buffer developed no symptoms.

Screening of *P. stewartii* subsp. *indologenes* pv. *cepacicola* and *P. stewartii* subsp. *indologenes* pv. *setariae* strains for HiVir and *alt* gene clusters

HiVir gene cluster was amplified in the positive control (*P. ananatis* PNA 97-1) with primer set HiVir2_pF/HiVir2_pR. Among the four *P. stewartii* subsp. *indologenes* pv. *cepacicola* strains, only PNA 03-3 was amplified and none of the *P. stewartii* subsp. *indologenes* pv. *setariae* strains showed amplification with this primer-pair (Table 2.4). Similarly, *alt* gene cluster of positive control (*P. ananatis* PNA 97-1) was amplified with the primer pair AltF/AltR. Among all the *P. stewartii* subsp. *indologenes* pv. *cepacicola* strains, only PNA 03-3 showed amplification while no amplification was observed in any of the *P. stewartii* subsp. *indologenes* pv. *setariae* strains. Nuclease-free water used as a negative control did not show any amplification with any of the primer sets.

Screening for *hrcC* gene in *P. stewartii* subsp. *indologenes* pv. *cepacicola* and *P. stewartii* subsp. *indologenes* pv. *setariae* strains

The primer set PsiHrcCF/PsiHrcCR amplified a 553-nt sequence of the *hrcC* gene of the positive control (PNA 03-3). The *hrcC* gene was amplified in all the strains of *P. stewartii* subsp. *indologenes* pv. *cepacicola* (PNA 03-3, PNA 14-12, PNA 14-11, PNA 14-9) and *P. stewartii* subsp. *indologenes* pv. *setariae* (LMG 2632^{T}, PNA 15-2, PANS 07-4, PANS 07-6, PANS 07-10, PANS 07-12, PANS 07-14, PANS 99-15, NCPPB 1562, NCPPB 1877, NCPPB 2275, NCPPB 2281, NCPPB 2282) (Table 2.4). No amplification was observed in the negative control *P. ananatis* (PNA 97-1).

Fatty acid composition analysis in *P. stewartii* subsp. *indologenes* pv. *cepacicola* and *P. stewartii* subsp. *indologenes* pv. *setariae* strains

The FAME profiles of four *P. stewartii* subsp. *indologenes* pv. *cepacicola* strains were similar to that of 12 *P. stewartii* subsp. *indologenes* pv. *setariae*. The profile for the 16 *P. stewartii* subsp. *indologenes* strains (including both pathovars) were similar to the type strain of *P. stewartii* subsp. *indologenes* LMG 2632^T (Table 2.5; Supplementary Table 2.2). The fatty acid designated as "unknown 10.9525 (retention time)" was observed in only 50% of the *P. stewartii* subsp. *indologenes* pv. *setariae* strains (NCPPB 2275, NCPPB 1877, NCPPB 2282, NCPPB 1562, NCPPB 2281and PANS 99-15) but were absent in all *P. stewartii* subsp. *indologenes* pv. *cepacicola* as well as in the type strain (LMG 2632^T). The fatty acids; 14:0 2OH, 17:0 cyclo, and 18:2 ω6,9c were absent in *P. stewartii* subsp. *stewartii* LMG 2715^T, but were present in all *P. stewartii* subsp. *indologenes* strains from both pathovars including LMG 2632^T.

Substrate utilization by *P. stewartii* subsp. *indologenes* pv. *cepacicola* and *P. stewartii* subsp. *indologenes* pv. *setariae* strains

The results for the carbon substrate utilization and chemical sensitivity assay for *P. stewartii* subsp. *indologenes* pv. *cepacicola*, *P. stewartii* subsp. *indologenes* pv. *setariae* and *P. stewartii* subsp. *stewartii* (LMG 2715^T) are shown (Table 2.6 and Supplementary Table 2.3). Strains of *P. stewartii* subsp. *indologenes* pv. *setariae* utilized carbon substrates 3-methyl glucose (10/12), D-serine (5/12), L-arginine (5/12), L-pyroglutamic acid (2/12), D-lactic acid methyl ester (8/12), α-keto glutaric acid (3/12), γ-amino butyric acid (1/12), α-hydroxy butyric acid (6/12), Na butyrate (9/12), and grew in media containing 8% NaCl (5/12) and fusidic acid (8/12). In contrast, the *P. stewartii* subsp. *indologenes* pv. *cepacicola* strains were unable to utilize any of those substrates and were sensitive to 8% NaCl and fusidic acid. Some of the *P. stewartii* subsp. *indologenes* pv.

setariae strains utilized L-fucose (7/12) and acetoacetic acid (11/12) that was utilized by all four strains of *P. stewartii* subsp. *indologenes* pv. *cepacicola*. Similarly, some strains of *P. stewartii* subsp. *indologenes* pv. *setariae* were tolerant to pH 5 (9/12), 4% NaCl (11/12), guanidine HCL (11/12), and lithium chloride (2/12). On the other hand, all four strains of *P. stewartii* subsp. *indologenes* pv. *cepacicola* were tolerant to pH 5 and 4% NaCl and three out of four strains were tolerant to guanidine HCL and lithium chloride. All *P. stewartii* subsp. *indologenes* strains including LMG 2632^{T} were able to utilize 48 other carbon substrates and were tolerant to were tolerant to 12 different chemicals from the GENIII microplate (Supplementary Table 2.3).

Discussion

Pantoea stewartii subsp. *indologenes* causes a leafspot of foxtail millet and pearl millet, a rot of pineapple and one strain has also been isolated from cluster bean (*Cyamopsis tetragonolobus*) (Mergaert et al. 1993). Recently, *P. stewartii* subsp. *indologenes* strains were identified that caused symptoms similar to center rot of onion (Stumpf et al. 2018). These novel strains caused white streaking and tissue necrosis of onion foliage and also caused necrosis and rotting of onion bulbs, which is a characteristic of center rot of onion described by Gitaitis et al. (2002). These strains were also pathogenic on pearl millet. These host range distinctions warrant separate pathovar designations. The evidence in this manuscript supports the premise that the two novel pathovars of *P. stewartii* subsp. *indologenes* can be differentiated based on their ability to induce symptoms on different host species of different plant families (*Amaryllidaceae* and *Poaceae*). We also characterized these pathovars on the presence and absence of virulence genes (HiVir, *alt* and *hrcC*), fatty acid composition differences and substrate utilization patterns.

Prior to establishing pathovar designation, we evaluated the possibility of elevating subspecies '*indologenes*' to a 'species' level. Apart from Mergaert et al. (1993) and De Maayer et al. (2017), no detailed investigations were conducted regarding this proposition. We utilized *P. stewartii* subsp. *indologenes* strains from different culture collections (UGA-CPES and NCPPB) along with the type strain *P. stewartii* subsp. *indologenes* LMG 2632^T, *P. stewartii* subsp. *stewartii* LMG 2715^T, *P. ananatis* LMG 2665^T, *P. allii* LMG 24248^T and re-evaluated if subspecies "*indologenes*" could be elevated to a "species" level using dDDH, ANI and MLSA. Our results showed that the type strain of *P. stewartii* subsp. *indologenes* (LMG 2632^T) shared 92.7% dDDH value and 99.12% ANI value with *P. stewartii* subsp. *stewartii* (LMG 2715^T). The two type strains shared dDDH value greater than 91% and ANI values greater than 99% with all the test strains of *P. stewartii* subsp. *indologenes* (*n*=16). These observations indicate that the *P. stewartii* subsp. *indologenes* strains do belong to the species "*stewartii*" and a further argument on elevating "*indologenes*" to the species level is not valid according to the stated norm of species designation (70% for dDDH and 95~96 % for ANI) (Goris et al. 2007; Chun et al. 2018). Moreover, MLSA also supported this claim as all *P. stewartii* subsp. *indologenes* strains clustered with the type strains *P. stewartii* subsp. *indologenes* (LMG 2632^T) and *P. stewartii* subsp. *stewartii* (LMG 2715^T), and were distinct from the type strains of *P. ananatis* (LMG 2665^T), *P. allii* (LMG 24248^T), and *P. agglomerans* (LMG 1286^T). Collectively, the MLSA, dDDH and ANI indicated that the test strains were more genotypically related to *P. stewartii* subsp. *indologenes* and *P. stewartii* subsp. *stewartii* than to any other *Pantoea* sp. The test *P. stewartii* subsp. *indologenes* strains; however, could not be differentiated from *P. stewartii* subsp. *stewartii* based on the MLSA, dDDH and ANI assessments. Similar to our study, De Maayer et al. (2017) reported that the subspecies of *P. stewartii* (subsp. *stewartii* and subsp.

indologenes) could not be differentiated by dDDH and ANI values due to their close genomic relatedness but could be differentiated on their ability to utilize different substrates (Margaert et al., 1993; De Maayer et al. 2017).

According to ISPP, a pathovar is 'a strain or set of strains with the same or similar characteristics, differentiated at infra-subspecific level from other strains of the same species or subspecies on the basis of distinctive pathogenicity to one or more plant hosts' (Dye et al. 1980; Young et al. 2001). Our foliar pathogenicity assay on *Allium* species (onion, leek, chive, Japanese bunching onion) and on the members of *Poaceae* (oat, foxtail millet, pearl millet and corn) demonstrated a distinct host range among *P. stewartii* subsp. *indologenes* strains. Four strains of subspecies *indologenes* were pathogenic on the members of both *Allium* and *Poaceae*, whereas, other strains (*n*=13) were pathogenic on only the members of *Poaceae* (except corn). The *Allium*-pathogenic strains were designated as a newly proposed pathovar "*cepacicola*". In contrast, a sub-set of *P. stewartii* subsp. *indologenes* strains (*n*=13; including LMG 2632^T) that were pathogenic on oat, foxtail millet and pearl millet but not on *Allium* species (onion, leek chive, Japanese bunching onion) were designated as the newly proposed pathovar "*setariae*". Also, none of the strains of "pv. *cepacicola*" or "pv. *setariae*" were pathogenic on corn and hence, corn cannot be used as differential host to distinguish both pathovars. Interestingly, the type strain of *P. stewartii* subsp. *indologenes* (LMG 2632^T) also displayed host-range characteristics similar to the newly proposed pathovar "*setariae*" and hence, we propose that the type strain (LMG 2632^T) should be designated as a pathotype strain of *P. stewartii* subsp. *indologenes* pv. *setariae*. Likewise, "PNA 14-12" is proposed as the pathotype strain of *P. stewartii* subsp. *indologenes* pv. *cepacicola*. Except for corn, none of the Allium or Poacea hosts displayed any symptoms when inoculated with *P. stewartii* subsp. *stewartii* (LMG 2715^T).

Further detailed characterization of *P. stewartii* subsp. *indologenes* pv. *cepacicola* strains displayed differences in aggressiveness on foliar tissue of plant species of *Allium* and *Poaceae* (except corn). The strain PNA 03-3 was highly aggressive on leek, foxtail millet and pearl millet whereas moderately aggressive on onion, chive, Japanese bunching onion and oat. Other *P. stewartii* subsp. *indologenes* pv. *cepacicola* strains (PNA 14-9, PNA 14-11, PNA 14-12) were moderately-to-highly aggressive on onion, foxtail millet and pearl millet but were moderate-to-less aggressive on leek, chive, Japanese bunching onion and oat. The four *P. stewartii* subsp. *indologenes* pv. *cepacicola* strains (PNA 03-3, PNA 14-9, PNA 14-11, PNA 14-12) also caused onion bulb rot; however, the level of aggressive differed considerably among strains. PNA 03-3 was highly aggressive on onion bulbs whereas other *P. stewartii* subsp. *indologenes* pv. *cepacicola* strains were moderate-to-less aggressive. A similar trend of variability in aggressiveness among *P. stewartii* subsp. *indologenes* pv. *cepacicola* strains was observed in the RSN assay. The *P. stewartii* subsp. *indologenes* pv. *cepacicola* strain (PNA 03-3) was highly aggressive and produced a necrotic lesion area of ≥ 1 cm^2 whereas the other three strains (PNA 14-9, PNA 14-11, PNA 14-12) were less aggressive and produced a necrotic lesion area of <1 cm^2 on the red scale.

Detailed pathogenicity assay of *P. stewartii* subsp. *indologenes* pv. *setariae* strains revealed that the strains were able to cause symptoms neither on foliage of onion, leek, chive and Japanese bunching onion nor on the onion bulb and red scale. We observed that one of the *P. stewartii* subsp. *indologenes* pv. *setariae* strains (NCPPB 2282) despite being moderately aggressive on foxtail millet and less aggressive on pearl millet, was non-pathogenic on oat. We also observed that the *P. stewartii* subsp. *indologenes* pv. *setariae* strain (PNA 15-2), originally isolated from symptomatic onion foliage, was non-pathogenic on onion (foliage, bulb, scale) and

the foliage of leek, chive and Japanese bunching onion. It is possible that this strain was present as a secondary colonizer along with the primary causal agent in the same necrotic lesion. As a result, it was accidently isolated from onion tissue. Further detailed genomic studies may shed some light on the matter. Interestingly, this strain was highly aggressive on foxtail millet, and moderately aggressive on oat and pearl millet.

The presence of the onion pathogenicity (HiVir) and virulence (*alt*) factors of *P. ananatis* that is responsible for center rot symptoms were examined in the strains of both pathovars of *P. stewartii* subsp. *indologenes*. Except for one *P. stewartii* subsp. *indologenes* pv. *cepacicola* strain (PNA 03-3), none of the strains from either of the pathovars were positive for HiVir and *alt* in the PCR assays. These observations indicate that the strain *P. stewartii* subsp. *indologenes* pv. *cepacicola* (PNA 03-3) shares onion pathogenicity and virulence factors with *P. ananatis*. Other strains from *P. stewartii* subsp. *indologenes* pv. *cepacicola* that are pathogenic on onion lacked these factors and potentially use an alternative mechanism(s) to cause disease on onion. Future comparative genomic studies with *P. stewartii* subsp. *indologenes* pv. *cepacicola* and *P. stewartii* subsp. *indologenes* pv. *setariae* strains may shed light on the matter. The '*hrcC'* gene was amplified in all the strains of both pathovars of *P. stewartii* subsp. *indologenes*; *hrcC* is one of the known pathogenic factors in the *hrp*-mediated Type III secretion system in the *P. stewartii* subsp. *stewartii*-corn pathosystem. This observation may explain why strains from both pathovars were pathogenic on foxtail millet and pearl millet. However, mutational studies need to be conducted to further strengthen this claim.

Comparative evaluation of fatty acid (FAME) profiles of *P. stewartii* subsp. *indologenes* pv. *cepacicola* and *P. stewartii* subsp. *indologenes* pv. *setariae* strains was conducted. FAME profiles of *P. stewartii* subsp. *indologenes* pv. *cepacicola* were similar to that of *P. stewartii*

subsp. *indologenes* pv. *setariae* and all the test strains (*n*=16) had similar FAME composition as *P. stewartii* subsp. *indologenes* LMG 2632^T. However, the fatty acid (unknown 10.9525) was present in 50% of the *P. stewartii* subsp. *indologenes* pv. *setariae* strains but was absent in *P. stewartii* subsp. *indologenes* LMG 2632^T and in *P. stewartii* subsp. *indologenes* pv. *cepacicola* strains (100% of the strains). Further investigation with more strains from both pathovars is warranted to see if FAME (unknown 10.9525) is associated with *P. stewartii* subsp. *indologenes* pv. *setariae*.

Differences in substrate utilization patterns and chemical sensitivity were observed between the two pathovars. Strains of *P. stewartii* subsp. *indologenes* pv. *setariae* including LMG 2632^T were able to utilize 3-methyl glucose (10/12), Na butyrate (9/12) and fusidic acid (8/12) but none of the *P. stewartii* subsp. *indologenes* pv. *cepacicola* strains utilized these substrates. Future studies with a larger number of strains from both pathovars should be conducted and perhaps a semi-selctive medium using these substrates could be developed for isolating *Pantoea stewartii* subsp. *indologenes* pv. *setariae* strains.

Overall, we provided substantial evidence for creating two novel pathovars of *P. stewartii* subsp. *indologenes*; *Pantoea stewartii* subsp. *indologenes* pv. *cepacicola* (pathogenic on onion, leek, chive, Japanese bunching onion, oat, pearl millet and foxtail millet) and *P. stewartii* subsp. *indologenes* pv. *setariae* (pathogenic on oat, pearl millet and foxtail millet). The two pathovars can be differentiated on their ability to induce symptoms on hosts in the *Allium* species and members of *Poaceae*. We also propose that the type strain of *P. stewartii* subsp. *indologenes* (LMG 2632^T) should be designated as the pathotype strain of *P. stewartii* subsp. *indologenes* pv. *setariae*. Further, we demonstrated that these pathovars differ in the presence or absence of pathogenicity and virulence factors (HiVir, *alt* and *hrcC*), fatty acid composition and substrate

utilization profiles. The impact of these new pathovars in the center rot pathosystem of onion is yet to be determined.

Description of *P. stewartii* subsp. *indologenes* pv. *cepacicola* pv. nov.

Bacterial strains are gram negative, rod-shaped and produce yellow-pigmented colony on NA medium. All the strains can utilize L-fucose and acetoacetic acid and can grow at pH 5, 4% NaCl. Some strains can grow in guanidine HCl and lithium chloride but none of the strains can grow at 8% NaCl and fusidic acid. The fatty acid composition of the strains is listed in Table 2.5 and Supplementary Table 2.2. The four most abundant fatty acids are hexadecenoic acid ($C_{16:0}$), cis-7-octadecanoic acid ($C_{18:1\ cis\ 7}$), 3-hydroxy-tetradecanoic acid ($C_{14:0}$ 3OH) and cis-7-hexadecanoic acid ($C_{16:1\ cis\ 7}$). The strains lack the fatty acid with unknown identity (retention time: 10.9525) that is present in some of the *P. stewartii* subsp. *indologenes* pv. *setariae* strains. The strains of this pathovar can cause necrotic lesions on the foliage of *Allium* species: onion, leek, chive, and Japanese bunching onion and members of *Poaceae* family: oat, pearl millet and, foxtail millet. Moreover, the strains are pathogenic on onion bulbs and can clear red anthocyanin pigment on onion scale. The proposed pathotype strain is “PNA 14-12”.

Description of *P. stewartii* subsp. *indologenes* pv. *setariae* pv. nov.

Bacterial strains are gram negative, rod-shaped and can produce yellow-pigmented colony on NA medium. The pathovar is described based on the study of 13 strains used in the study. The strains of pv. *setariae* can utilize L-fucose and acetoacetic acid, 3-methyl glucose, D-serine, L-arginine, L-pyroglutamic acid, D-lactic acid methyl ester, α-keto-glutaric acid, γ-amino butyric acid, α-hydroxy-butyric acid and Na butyrate. Most of the strains can grow at pH 5, 4% NaCl, guanidine HCl and fusidic acid whereas some of the strains can grow in 8% NaCl and lithium chloride. The fatty acid composition of the strains is similar to that of pv. *cepacicola* (Table 2.5).

The four most abundant fatty acids are hexadecenoic acid ($C_{16:0}$), cis-7-octadecanoic acid ($C_{18:1\ cis\ 7}$), 3-hydroxy-tetradecanoic acid ($C_{14:0}$ 3OH) and cis-7-hexadecanoic acid ($C_{16:1\ cis\ 7}$). Some strains also constitute an unknown fatty acid (retention time: 10.9525) that is absent in pv. *cepacicola*. The strains can cause necrotic lesions on the members of *Poaceae* family: oat, pearl millet and foxtail millet but cannot cause any symptoms on the foliage of *Allium* species: onion, leek, chive, and Japanese bunching onion. Moreover, the strains are non-pathogenic on onion bulb and cannot clear red anthocyanin pigment on onion scale. The proposed pathotype strain is "LMG 2632".

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Table 2.1. List of primers used in this study.

Target	Primer name	Primer sequence (5' – 3')	Amplicon size (bp)	References
galE gene	3614galE	CGACCTGTTTGCCTCTCACC	267	Gehring et al. 2014
	3614galEc	CATCAGCTTGGAGGTGCCG		
*hrcC*gene	PsiHrcCF[a]	AAGATGGGCACGTTGAAACC	553	This study
	PsiHrcCR[a]	GTGATATTGCGGACCACACC		
HiVir cluster	HiVir2_pF	AATATCCATCAGTACCATT	857	Stice et al. in review
	HiVir2_pR	AATATCCATCAGTGCCATT		
Alt cluster	AltF	AGAATGCAGAACGGCTGGC	433	Stice et al. in review
	AltR	CCACCTGATTCATCATCAG		

[a] Primers were designed based on *hrcC* gene of the strain PNA 03-3 (GenBank accession number GCA_003201175.1).

Table 2.2. List of *Pantoea stewartii* subsp. *indologenes* strains used in phenotypic and genotypic characterization.

Strain name[a]	Host of origin	Place of isolation	Year of isolation
P. stewartii subsp. *indologenes*			
PNA 03-3	Onion	Georgia, USA	2003
PNA 14-9	Onion	Georgia, USA	2014
PNA 14-11	Onion	Georgia, USA	2014
PNA 14-12	Onion	Georgia, USA	2014
PNA 15-2	Onion	Georgia, USA	2015

PANS 07-4	Foxtail millet	Georgia, USA	2007	
PANS 07-6	Corn	Georgia, USA	2007	
PANS 07-10	Pearl millet	Georgia, USA	2007	
PANS 07-12	Pearl millet	Georgia, USA	2007	
PANS 07-14	Verbena	Georgia, USA	2007	
PANS 99-15	Crab grass	Georgia, USA	1999	
NCPPB[b] 2275	Pearl millet	India	1970	
NCPPB 1877	Guar pulse	Unknown	1966	
NCPPB 2282	Pearl millet	India	1956	
NCPPB 1562	Pearl millet	India	1963	
NCPPB 2281	Foxtail millet	India	1970	
LMG 2632[T]	Foxtail millet	India	1960	
P. stewartii subsp. *stewartii* LMG 2715[T]	Corn	USA		1970
P. ananatis LMG 2665[T]	Pineapple	Brazil		1965
P. allii LMG 24248[T]	Onion seed	South Africa	2008	

[a] PNA and PANS are strains isolated from onion and sources other than onion, respectively.
[b] NCPPB = The National Collection of Plant Pathogenic Bacteria, York, UK.
[T] Superscript "T" indicates type strain.

Table 2.3. Pairwise digital DNA-DNA hybridization (dDDH) and average nucleotide identity (ANI) for genomes of *Pantoea stewartii* subsp. *indologenes* and selected type-strains (^{T}) of *Pantoea* sp. expressed as percentages.

Strains	*P. stewartii* subsp. *indologenes* LMG 2632^T		*P. stewartii* subsp. *stewartii* LMG 2715^T		*P. ananatis* LMG 2665^T		*P. allii* LMG 24248^T	
	dDDH (%)	ANI (%)	dDDH (%)	ANI (%)	dDDH (%)	ANI (%)	dDDH (%)	ANI (%)
PNA 03-3	93.6	99.2	92.8	99.1	27.7	85.9	27.8	86.0
PNA 14-9	97.4	99.7	92.6	99.1	27.5	85.8	27.7	85.9
PNA 14-11	97.3	99.7	92.6	99.1	27.5	85.8	27.7	85.8
PNA 14-12	97.3	99.7	92.6	99.1	27.5	85.8	27.7	85.9
PNA 15-2	93.3	99.2	93.0	99.6	27.6	85.8	27.6	85.9
PANS 07-4	92.2	99.2	91.5	99.1	27.8	85.9	27.7	85.9
PANS 07-6	92.0	99.2	91.6	99.1	27.7	85.9	27.7	85.9
PANS 07-10	92.7	99.2	91.8	99.1	27.7	85.9	27.8	85.9
PANS 07-12	92.7	99.2	91.8	99.1	27.7	85.9	27.8	85.9
PANS 07-14	93.6	99.2	92.6	99.1	27.6	85.8	27.7	85.9
PANS 99-15	93.2	99.2	92.3	99.1	27.6	85.9	27.8	85.9
NCPPB 1562	93.5	99.2	92.5	99.1	27.6	85.8	27.7	85.9
NCPPB 1877	93.7	99.3	92.7	99.1	27.6	85.8	27.7	85.9
NCPPB 2275	93.7	99.3	92.7	99.1	27.6	85.8	27.7	85.9
NCPPB 2281	99.9	100.0	92.8	99.1	27.5	85.8	27.7	85.9
NCPPB 2282	93.5	99.2	92.5	99.1	27.6	85.8	27.7	85.9
P. stewartii subsp. *indologenes* LMG 2632^T	100.0	100.0	92.7	99.1	27.4	85.8	27.7	85.9
P. stewartii subsp. *stewartii* LMG 2715^T	92.7	99.1	100.0	100.0	27.8	85.9	28.0	85.9
P. ananatis LMG 2665^T	27.4	85.8	27.8	85.9	100.0	100.0	35.8	89.4
P. allii LMG 24248^T	27.7	85.9	28.0	85.9	35.8	89.4	100.0	100.0

Table 2.4. Host range and phenotypic characterization of *Pantoea stewartii* subsp. *indologenes* strains.

Strain	Host of origin	*hrcC*[a]	HiVir[b]	*alt*[c]	Whole bulb assay[d]	Red onion scale assay[e]	Strain aggressiveness based on foliar assay[f]							
							Allium species[g]				*Poaceae* species[h]			
							Onion	Leek	Chive	Japanese bunching onion	Oat	Foxtail millet	Pearl millet	Corn
PNA 03-3	Onion	+	+	+	+++	+++	**	***	**	**	**	***	***	-
PNA 14-9	Onion	+	-	-	+	+	**	*	**	*	*	***	***	-
PNA 14-11	Onion	+	-	-	+	+	**	*	*	**	*	***	***	-
PNA 14-12	Onion	+	-	-	++	+	***	**	*	*	*	**	**	-
PNA 15-2	Onion	+	-	-	-	-	-	-	-	-	**	***	**	-
PANS 07-4	Foxtail millet	+	-	-	-	-	-	-	-	-	**	***	**	-
PANS 07-6	Maize	+	-	-	-	-	-	-	-	-	*	**	**	-
PANS 07-10	Pearl millet	+	-	-	-	-	-	-	-	-	*	**	***	-
PANS 07-12	Pearl millet	+	-	-	-	-	-	-	-	-	**	**	***	-
PANS 07-14	Verbena	+	-	-	-	-	-	-	-	-	*	**	***	-
PANS 99-15	Crab grass	+	-	-	-	-	-	-	-	-	*	**	***	-
NCPPB 1562	Pearl millet	+	-	-	-	-	-	-	-	-	**	**	**	-
NCPPB 1877	Guar pulse	+	-	-	-	-	-	-	-	-	*	**	**	-
NCPPB 2275	Pearl millet	+	-	-	-	-	-	-	-	-	*	**	***	-
NCPPB 2281	Foxtail millet	+	-	-	-	-	-	-	-	-	**	***	***	-
NCPPB 2282	Pearl millet	+	-	-	-	-	-	-	-	-	-	**	*	-
LMG 2632^T	Foxtail millet	+	-	-	-	-	-	-	-	ND[i]	*	**	**	-
P. stewartii subsp. *stewartii* LMG 2715^T	Corn	ND	ND	ND	-	-	-	-	-	-	-	-	-	**

[a] Presence or absence of *hrcC* gene with primers designed based on *hrcC* gene of strain PNA 03-3 (Genebank accession number GCA_003201175.1).
[b] Hypothetical phosphonate gene cluster in *P. ananatis* associated with onion pathogenicity (Asselin et al. 2018).
[c] Gene cluster conferring allicin tolerance to *P. ananatis* (Stice et al., 2020).
[d] Ability of strain to cause scale maceration and water soaking on onion bulbs at 12 days post-inoculation (dpi). Non-pathogenic strain is represented by "-". Strains that caused relative rotten tissue weight of ≤4% were considered as less aggressive (+), 4-8% as moderately aggressive (++) and strains that resulted in ≥8% of relative rotten tissue weight were considered as highly aggressive (+++).
[e] Ability of strain to cause pitting and pigment clearing on red onion scales at 7 dpi. Strains unable to clear scale are represented by "-", strains causing <1 cm^2 scale clearing were considered less aggressive (+), strains clearing 1 cm^2 to 2 cm^2 of red scale were considered moderately aggressive (++), and those causing ≥2 cm^2 scale clearing were considered highly aggressive (+++).
[g] White streaking on leaves with water-soaked margins were observed at 7 dpi with bacterial suspension of 1×10^6 CFU/leaf.
[h] Streaking and necrotic lesions were observed on leaves (corn, pearl millet and foxtail millet: 14 dpi; oat: 21 dpi) inoculated with bacterial suspension of 1×10^6 CFU/leaf.
[f] Strains causing mean lesion length of <1.5 cm was considered as less aggressive (*), lesion length of 1.51-2.5 cm as moderately aggressive (**), and lesion length of >2.5 cm as highly aggressive (***) on onion, Japanese bunching onion, and chive. For leek, strains causing lesion length of <0.6 cm was considered as less aggressive (*), lesions length of 0.61-1.2 cm moderately aggressive (*), >1.2 cm highly aggressive (*). Strains causing a mean lesion length 0.51-1 cm was considered less aggressive (*), 1.1-1.5 cm as moderately aggressive (**), and lesions >1.5 cm as highly aggressive (***) on pearl millet, foxtail millet, oat and corn. Strains unable to develop lesions were considered as non-pathogenic (-).
[i] ND: Not determined.

Table 2.5. Cellular fatty acid compositions of *Pantoea stewartii* subsp. *indologenes* pv. *cepacicola*, *P. stewartii* subsp. *indologenes* pv. *setariae* and *Pantoea stewartii* subsp. *stewartii* LMG 2715^T, expressed as percentages of total fatty acid content using fatty acid methyl esterase (FAME) test.

Fatty acid	*Pantoea stewartii* subsp. *indologenes* pathovars						*Pantoea stewartii* subsp. *stewartii* LMG 2715^T
	pv. *cepacicola*				pv. *setariae* (12)[a]	pv. *setariae* LMG 2632^T	
	PNA 14-9	PNA 14-11	PNA 03-3	PNA 14-12			
unknown 10.9525[b]	-	-	-	-	0.80-2.24 (6)	-	-
12:0	4.80	5.21	4.72	4.91	4.37-6.25	5.06	5.15
14:0	3.70	3.94	3.56	4.09	3.14-4.73	3.82	5.67
14:0 2OH	2.15	2.17	2.10	1.96	1.42-3.16	2.23	-
14:0 3OH	12.38	12.74	11.97	12.66	10.93-15.61	12.92	11.81
16:0	33.99	33.44	34.03	34.10	29.49-34.44	34.78	38.93
16:0 N alcohol	-	-	-	-	0.44 (1)	-	-
16:1 ω7c	11.19	12.91	13.45	12.60	8.07-15.36	9.28	14.50
17:0	-	-	-	-	0.33(1)	-	-
17:0 cyclo	6.87	5.43	4.59	4.65	3.02-7.37	7.57	-
18:0	1.58	1.41	1.60	1.46	1.45-2.57	1.63	3.69
18:1 ω7c	22.11	22.15	23.23	22.84	20.75-23.77	20.96	20.26
18:2 ω6,9c	0.69	0.59	0.46	0.73	0.40-0.78	0.73	-
19:0	-	-	-	-	0.3-0.39 (2)	0.31	-
19:0 cyclo ω8c	0.53		0.29		0.47-0.70 (3)	0.72	-

[a] Number in parenthesis represents number of strains containing particular fatty acid. Details of fatty acid component of each strain in listed in the Table 2.2.

[b] Numerical value represents the retention data as equivalent chain length value.

Table 2.6. Differential substrate utilization and chemical sensitivity profiles of *Pantoea stewartii* subsp. *indologenes* pv. *cepacicola* and *P. stewartii* subsp. *indologenes* pv. *setariae* strains based on BIOLOG GENIII microplate assay.

	Pantoea stewartii subsp. *indologenes* pathovars			*P. stewartii* subsp. *stewartii* LMG 2715^T
	pv. *cepacicola*[a]	pv. *setariae*[b]	pv. *setariae* LMG 2632^T	
Carbon source	Number of strains positive/total strains (*n*=4)	Number of strains positive/total strains (*n*=12)		
3-Methyl Glucose	0/4[c]	10/12	+	-
L-Fucose	4/4	7/12	-	-
D-Serine	0/4	5/12	+	-
L-Arginine	0/4	5/12	+	-
L-Pyroglutamic Acid	0/4	2/12	-	-
D-Lactic Acid Methyl Ester	0/4	8/12	(+)[d]	-
α-Keto-Glutaric Acid	0/4	3/12	-	-
γ-Amino Butyric Acid	0/4	1/12	-	-
α-Hydroxy-Butyric Acid	0/4	6/12	-	-
Acetoacetic Acid	4/4	11/12	+	-
Na butyrate	0/4	9/12	+	-
Chemical sensitivity[e]				
pH 5	4/4	9/12	T[f]	S[h]
4% NaCl	4/4	11/12	(T)[g]	T
8% NaCl	0/4	5/12	S	S
Fusidic Acid	0/4	8/12	(T)	T
Guanidine HCl	3/4	11/12	T	(T)
Lithium Chloride	3/4	2/12	S	T

[a] Strains used: PNA 03-3, PNA 14-12, PNA 14-11, PNA 14-9
[b] Strains used: PNA 15-2, PANS 07-4, PANS 07-6, PANS 07-10, PANS 07-12, PANS 07-14, PANS 99-15, NCPPB 1562 , NCPPB 1877, NCPPB 2275, NCPPB 2281, NCPPB 2282
[c] Number of strains positive to the total number of strains tested.
[d] Strain weakly able to utilize the carbon source is designated as (+).
[e] Chemical sensitivity assay tests the sensitivity of a strain to the respective chemical.
[f] Letter "T" is designated for a strain able to tolerate the chemical.
[g] (T) represents the ability of a strain to weakly tolerate the chemical.
[h] Letter "S" is designated for strains sensitive to the chemical.

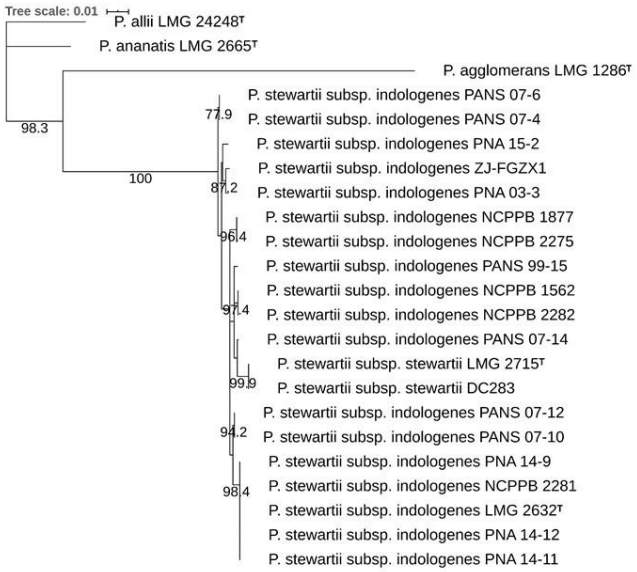


Figure 2.1. Phylogenetic relationships among the *Pantoea stewartii* subsp. *indologenes* inferred from the multi-locus sequence analysis using 2,256 concatenated partial nucleotide sequences from amplification of four housekeeping genes (*atpD*, *gyrB*, *infB*, and *rpoB*). The phylogenetic tree was built with the maximum likelihood method using the PHYLIP package and with a custom model selected from the best substitution model. *Pantoea stewartii* subsp. *indologenes* strains (PANS 07-14, PANS 99-15, PNA 15-2, PANS 07-6, PANS 07-4, NCPPB 2275, NCPPB 1877, NCPPB 1562, NCPPB 2282, PNA 03-3, ZJ-FGZX1, PANS 07-12, PANS 07-10, PNA 14-9, NCPPB 2281, LMG 2632^{T}, PNA 14-12 and PNA 14-11) were used. For comparison, strains of *P. stewartii* subsp. *stewartii* (LMG 2715^{T}, and DC283), *P. allii* (LMG 24248^{T}), *P. ananatis* (LMG 2665^{T}) and *P. agglomerans* (LMG 1286^{T}). The superscript 'T' indicates type strains. Bootstrap values above 70% (from 1,000 replicates) are shown.

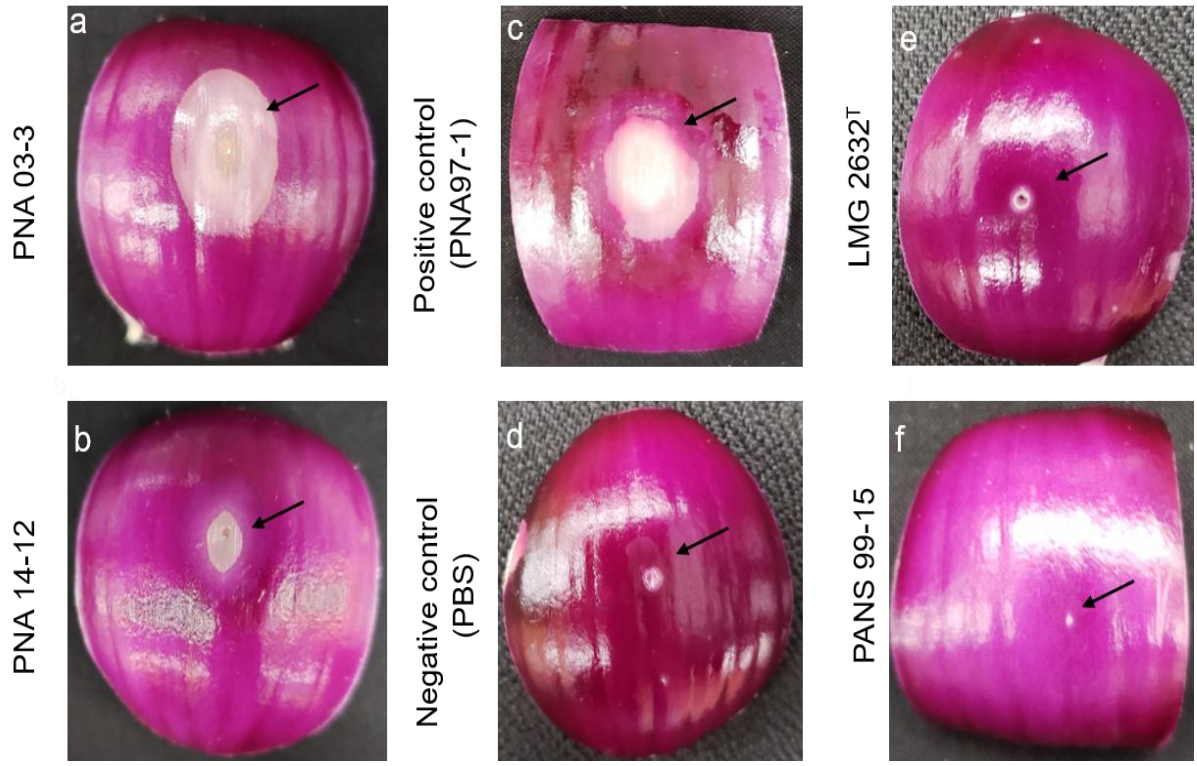


Figure 2.2: Red-scale necrosis assay with representative strains of *P. stewartii* subsp. *indologenes* pv. *cepacicola* and *P. stewartii* subsp. *indologenes* pv. *setariae*. The scale of red onion (cv. Red Barrett) was wounded at the center with a sterile needle and inoculated with a bacterial suspension of 1×10^6 CFU/scale using a sterile pipette tip. At 7 days post inoculation (DPI), the area of red pigment clearing with scale pitting was measured. Strains unable to cause red pigment clearing were considered as non-pathogenic. Two representative strains of *P. stewartii* subsp. *indologenes* pv. *cepacicola,* a) PNA 03-3 and b) PNA 14-12 were pathogenic as they cleared red pigment and caused scale pitting at 7 DPI. Two representative *P. stewartii* subsp. *indologenes* pv. *setariae* strains e) LMG 2632^T and f) PANS 99-15 were unable to cause scale clearing at 7 DPI and were considered non-pathogenic. Scales inoculated with the positive control *P. ananatis* PNA 97-1 (c) caused scale pitting and red pigment clearing symptoms but

scales inoculated with negative control (d) was unable to cause any symptoms. Three replications were performed for each strain and total two independent experiments were performed. Arrow in each panel shows the point of inoculation.

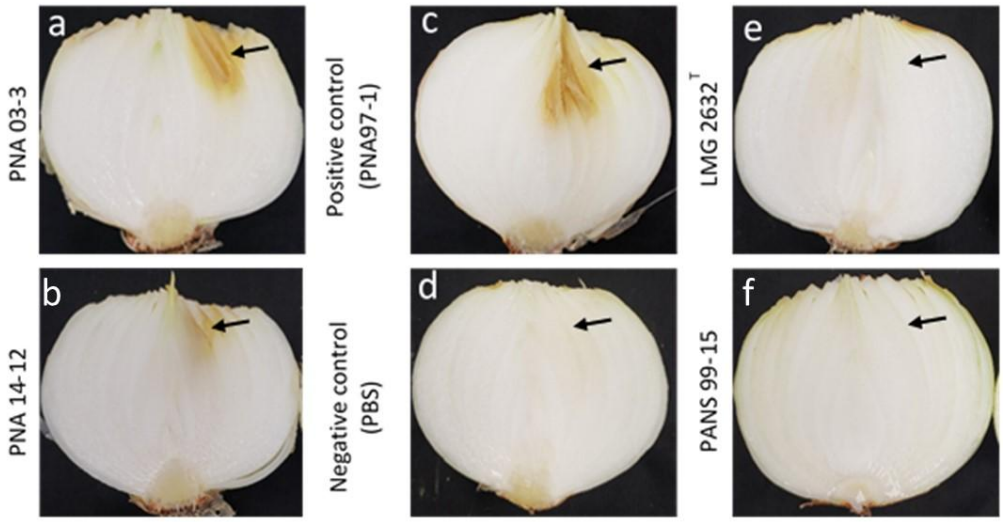


Figure 2.3. Whole-onion bulb assay for internal rot with artificial inoculation of representative strains of *Pantoea. stewartii* subsp. *indologenes* pv. *cepacicola* and *P. stewartii* subsp. *indologenes* pv. *setariae* strains. Onion bulbs (cv. Century) were inoculated longitudinally at the shoulder with a syringe and a sterile needle containing a volume of 400 µl (1×10^8 CFU/ml). After 12 days post inoculation (DPI), pathogenicity of each was determined based on the necrotic lesion and appearance of rotten internal tissue. Aggressiveness of each strain was also determined using relative weight of the rotten tissue to the entire weight of the bulb. Strains

unable to cause symptoms on the scales were considered as non-pathogenic. Two representative strains of *P. stewartii* subsp. *indologenes* pv. *cepacicola*; a) PNA 03-3 and b) PNA 14-12 were pathogenic on the bulb as they caused internal bulb rot at 12 DPI. Two representative *P. stewartii* subsp. *indologenes* pv. *setariae* strains; e) LMG 2632^{T} and f) PANS 99-15 were non-pathogenic on bulbs at 12 DPI. Bulbs inoculated with the positive control (c) *P. ananatis* PNA 97-1 displayed interanl bulb rot whereas bulb inoculated with negative control (d) PBS did not show any interanl rot symptoms. Three replications were performed for each strain and total two independent experiments were performed. Arrow in each panel shows the depth at which inoculation was done.

Supplementary Table 2.1. List of strains used in the preliminary screen with *galE* gene based *Pantoea stewartii* subsp. *indologenes*-specific primers.

Strains	**Place of isolation**	**Isolation source**	**Band**
PNA 92-2	unknown	onion	-
PNA 92-7	unknown	onion	-
PNA 97-1	Toombs Co. GA	onion	-
PNA 97-2	unknown	onion	-
PNA 97-3	unknown	onion	-
PNA 97-4	unknown	onion	-
PNA 97-5	unknown	onion	-
PNA 97-6	unknown	onion	-
PNA 97-7	unknown	onion	-
PNA 97-8	Toombs Co. GA	onion	-
PNA 97-9	Georgia	onion	-
PNA 97-10	Toombs Co. GA	onion	-
PNA 97-11	Toombs Co. GA	onion	+
PNA 98-1	Tattnall Co. GA	onion	-
PNA 98-2	Tift Co. GA	onion	-
PNA 98-3	Dougherty	onion	-
PNA 98-4	Tattnall Co. GA	onion	-
PNA 98-5	Vidalia Region GA	onion	-
PNA 98-6	Tift Co. GA	onion	-
PNA 98-7	Tift Co. GA	onion	-
PNA 98-8	Vidalia Region GA	onion	-
PNA 98-9	Vidalia Region GA	onion	-
PNA 98-10	Vidalia Region GA	onion	-
PNA 98-11	Evans Co. GA	onion	-
PNA 98-12	Toombs Co. GA	onion	-
PNA 99-1	MT Vernon, GA	onion	-
PNA 99-2	Tattnall Co. GA	onion	-
PNA 99-3	Tift Co. GA	onion	-
PNA 99-4	Tattnall Co. GA	onion	-
PNA 99-5	Tattnall Co. GA	onion	-
PNA 99-6	Toombs Co. GA	onion	-
PNA 99-7	Tattnall Co. GA	onion	-
PNA 99-8	Wheeler Co. GA	onion	-
PNA 99-9	Tattnall Co. GA	onion	-
PNA 99-10	Toombs Co. GA	onion	-
PNA 99-11	unknown	onion	-
PNA 99-12	unknown	onion	-
PNA 99-13	unknown	onion	-

PNA 99-15	Toombs Co. GA	onion	-
PNA 99-16	Toombs Co. GA	onion	-
PNA 99-17	Toombs Co. GA	onion	-
PNA 200-1	Toombs Co. GA	onion	-
PNA 200-3	Tift Co. GA	onion	-
PNA 200-4	Tift Co. GA	onion	-
PNA 200-5	Tift Co. GA	onion	-
PNA 200-7	Tift Co. GA	onion	-
PNA 200-8	Tift Co. GA	onion	-
PNA 200-9	Tift Co. GA	onion	-
PNA 200-10	Tift Co. GA	onion	-
PNA 200-11	Tift Co. GA	onion	-
PNA 200-12	Tift Co. GA	onion	-
PNA 200-13	Tift Co. GA	onion	-
PNA 02-12	Tift Co. GA	onion	-
PNA 02-13	Tift Co. GA	onion	-
PNA 03-1	Tift Co. GA	onion	-
PNA 03-2	Tift Co. GA	onion	-
PNA 03-3	Georgia	onion	+
PNA 05-1	Vidalia Region GA	onion	-
PNA 06-1	Wayne Co. GA	onion	-
PNA 06-3	Wayne Co. GA	onion	-
PNA 06-4	Wayne Co. GA	onion	-
PNA 06-5	CPES Bact. Lab - parent strain Tat 1/15/b	onion	-
PNA 06-6	CPES Bact. Lab - parent strain Tat 2/5/a	onion	-
PNA 06-7	CPES Bact. Lab - parent strain Pans 99-4	onion	-
PNA 06-8	CPES Bact. Lab - parent strain Pans 99-25	onion	-
PNA 06-11	Vidalia Region GA/Nischwitz	thrips	-
PNA 06-12	Vidalia Region GA/Nischwitz	Leaf Rinse from thrips	-
PNA 06-13	Vidalia Region GA/Nischwitz	Leaf Rinse from thrips	-
PNA 06-14	Vidalia Region GA/Nischwitz	Leaf Rinse from thrips	-
PNA 06-15	Vidalia Region GA/Nischwitz	Leaf Rinse from thrips	-
PNA 06-16	Vidalia Region GA/Nischwitz	Leaf Rinse from thrips	-

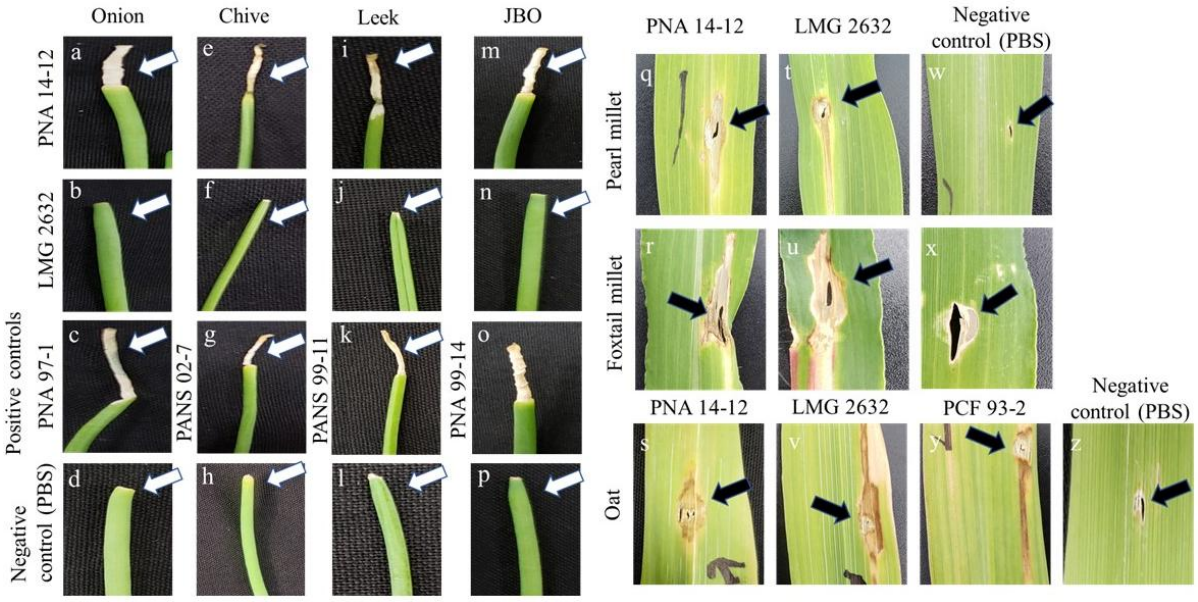
PNA 06-18	Vidalia Region GA/Nischwitz	Leaf Rinse from thrips	-
PNA 06-19	Vidalia Region GA/Nischwitz	Leaf Rinse from thrips	-
PNA 06-18	Vidalia Region GA/Nischwitz	Leaf Rinse from thrips	-
PNA 06-19	Vidalia Region GA/Nischwitz	Leaf Rinse from thrips	-
PNA 07-6	Wayne Co. GA	onion	-
UK-1	Wayne Co. GA	onion	-
UK-2	Wayne Co. GA	onion	-
PNA 07-7	Toombs Co. GA	onion	-
PNA 07-8	Toombs Co. GA	onion	-
PNA 07-9	Toombs Co. GA	onion	-
PNA 07-10	Toombs Co. GA	onion	-
PNA 07-11	Toombs Co. GA	onion	-
PNA 07-12	Toombs Co. GA	onion	-
PNA 07-15	Toombs Co. GA	onion	-
PNA 07-16	CPES	pepper	-
PNA 07-17	CPES	pepper	-
PNA 07-18	CPES	pepper	-
PNA 07-19	CPES	pepper	-
PNA 07-20	Tift Co. GA	onion	-
PNA 07-21	Tift Co. GA	onion	-
PNA 07-22	Tift Co. GA	onion	-
PNA 08-1	Tattnall Co. GA	onion	-
PNA 08-2	Tattnall Co. GA	onion	-
PNA 09-1	National Onion Labs, Collins GA	onion	-
PNA 09-2	National Onion Labs, Collins GA	onion	-
PNA 11-1	Vidalia Region GA	onion	-
PNA 11-2	unknown	onion	-
PNA 11-3	unknown	onion	-
PNA 13-1	Lyons, GA	onion	-
PNA 14-1	unknown	onion	-
PNA 14-2	unknown	onion	-
PNA 14-3	unknown	onion	-
PNA 14-4	unknown	onion	-
PNA 14-5	unknown	onion	-
PNA 14-6	unknown	onion	-
PNA 14-7	unknown	onion	-
PNA 14-8	unknown	onion	-
PNA 14-9	unknown	onion	+

PNA 14-10	unknown	onion	-
PNA 14-11	unknown	onion	+
PNA 14-12	unknown	onion	+
PNA 18-5	Vidalia Region GA	onion	-
PNA 18-10	Vidalia Region GA	onion	-
PNA 18-1	Black Shank Farms, Tifton, GA	onion	-
PNA 18-2	Black Shank Farms, Tifton, GA	onion	-
PNA 18-1S	Vidalia Region GA	onion	-
PNA 18-2S	Vidalia Region GA	onion	-
PNA 18-4S	Vidalia Region GA	onion	-
PNA 18-3S	Vidalia Region GA	onion	-
PNA 18-5S	Vidalia Region GA	onion	-
PNA 18-6S	Vidalia Region GA	onion	-
PNA 18-7S	Vidalia Region GA	onion	-
PNA 18-8S	Vidalia Region GA	onion	-
PNA 18-9S	Vidalia Region GA	onion	-
PNA 18-10s	Vidalia Region GA	onion	-
PANS 14-13	Tifton, GA	F. pusley	-
PANS 14-14	Tifton, GA	F. pusley	-
PNA 14-17	Tifton, GA	F. pusley	-
PNA 14-18	Tifton, GA	F. pusley	-
PNA 14-19	Tifton, GA	F. pusley	-
PNA 14-20	Tifton, GA	F. pusley	-
PNA 14-21	Tifton, GA	F. pusley	-
PNA 15-1	Tattnall Co. GA	onion	-
PNA 15-2	Tattnall Co. GA	onion	+
PNA 15-3	tattnall Co. GA	onion	-
PANS 99-1	Tift Co. GA	Florida pusley	-
PANS 99-2	Tift Co. GA	Florida pusley	-
PANS 99-3	Tift Co. GA	Florida pusley	-
PANS 99-4	Tift Co. GA	Florida pusley	-
PANS 99-5	Tift Co. GA	prairie verbena	-
PANS 99-6	Tift Co. GA	prairie verbena	-
PANS 99-7	Tift Co. GA	prairie verbena	-
PANS 99-8	Tift Co. GA	Florida pusley	-
PANS 99-9	Tift Co. GA	prairie verbena	-
PANS 99-10	Tift Co. GA	prairie verbena	-
PANS 99-11	Tift Co. GA	crab grass	-
PANS 99-12	Tift Co. GA	crab grass	-
PANS 99-14	Tift Co. GA	crab grass	-
PANS 99-15	Tift Co. GA	crab grass	+

PANS 99-16	Tift Co. GA	crab grass	-
PANS 99-18	Tift Co. GA	Florida pusley	-
PANS 99-19	Tift Co. GA	Florida pusley	-
PANS 99-20	Tift Co. GA	Florida pusley	-
PANS 99-21	Tift Co. GA	Florida pusley	-
PANS 99-22	Tift Co. GA	crab grass	-
PANS 99-23	Vidalia Region GA	yellow nut sedge	-
PANS 99-24	Vidalia Region GA	cowpea	-
PANS 99-25	Vidalia Region GA	bristly starbur	-
PANS 99-26	Vidalia Region GA	hyssop spurge	-
PANS 99-27	Vidalia Region GA	Florida beggarweed	-
PANS 99-28	Tift Co. GA	Florida pusley	-
PANS 99-29	Tift Co. GA	crab grass	-
PANS 99-30	Tift Co. GA	crab grass	-
PANS 99-31	Tattnal Co. GA	Texas millet	-
PANS 99-32	Vidalia Region GA	Florida pusley	-
PANS 99-33	Coffee Co. GA	Florida pusley	-
PANS 99-34	Coffee Co. GA	Florida pusley	-
PANS 99-35	Terrell Co. GA	Florida pusley	-
PANS 99-36	Terrell Co. GA	Florida pusley	-
PANS 200-1	Reidsville, GA	slender amaranth	-
PANS 200-2	Reidsville, GA	pink purslane	-
PANS 01-01	Tift Co. GA	10 thrips from onion leaf	-
PANS 01-02	Tift Co. GA	10 thrips from onion leaf	-
PANS 01-03	Tift Co. GA	10 thrips from onion leaf	-
PANS 01-04	Tift Co. GA	10 thrips from onion leaf	-
PANS 01-05	Tift Co. GA	1 adult tobacco thrip	-
PANS 01-06	Tift Co. GA	1 adult tobacco thrip	-
PANS 01-07	Tift Co. GA	tobacco thrip	-
PANS 01-08	Tift Co. GA	tobacco thrip	-
PANS 01-09	Tift Co. GA	thrip feces from peanut leaf	-
PANS 01-10	Tift Co. GA	thrip feces from peanut leaf	-
PANS 02-1	Tift Co. GA	1 adult tobacco thrip from peanut	-
PANS 02-2	Tift Co. GA	1 adult tobacco thrip from peanut	-
PANS 02-3	Tift Co. GA	1 adult tobacco thrip from peanut	-
PANS 02-4	Tift Co. GA	1 adult tobacco thrip from peanut	-
PANS 02-5	Tift Co. GA	thrips from peanut blossoms	-
PANS 02-6	Tift Co. GA	thrips from peanut blossoms	-
PANS 02-7	Tift Co. GA	thrips from peanut blossoms	-
PANS 02-8	Tift Co. GA	thrips infected peanut leaf	-

PANS 02-10	Tift Co. GA	thrips infected peanut leaf	-
PANS 02-11	Tift Co. GA	thrips infected peanut leaf	-
PANS 02-12	Tift Co. GA	thrips from peanut blossoms	-
PANS 02-13	Tift Co. GA	thrips from peanut leaves	-
PANS 02-14	Tift Co. GA	thrips peanut leaf	-
PANS 04-1	Tift Co. GA	1 adult tobacco thrip from peanut	-
PANS 04-2	Tift Co. GA	1 adult tobacco thrip from peanut	-
PNA 2-5p	unknown	onion	-
PNA 1-12p	unknown	onion	-
1-12-b-c	unknown	unknown	-
11-15b-c	unknown	unknown	-
PNA 07-14	Toombs Co. GA	onion	-
PANS 07-1	Georgia, USA	unknown	-
PANS 07-2	Georgia, USA	unknown	-
PANS 07-3	Georgia, USA	unknown	-
PANS 07-4	Georgia, USA	Foxtail millet	+
PANS 07-5	Georgia, USA	unknown	-
PANS 07-6	Georgia, USA	Corn	+
PANS 07-7	Georgia, USA	unknown	-
PANS 07-8	Georgia, USA	unknown	-
PANS 07-9	Georgia, USA	unknown	-
PANS 07-10	Georgia, USA	Pearl millet	+
PANS 07-11	Georgia, USA	unknown	-
PANS 07-12	Georgia, USA	Pearl millet	+
PANS 07-15	Georgia, USA	unknown	-
PANS 07-13	Toombs Co. GA	verbena	-
PANS 07-14	Toombs Co. GA	verbena	+

Note: Supplementary Table 2.2 and Supplementary Table 2.3 are uploaded as additional file for chapter 2.



Supplementary Figure 2.1. Foliar inoculation assay on *Allium* sp. and *Poaceae* sp. using representative strains of *Pantoea. stewartii* subsp. *indologenes* pv. *cepacicola* (PNA 14-12) and *P. stewartii* subsp. *indologenes* pv. *setariae* (LMG 2632^{T}). The tip of the foliage of *Allium* sp. (onion, chive, leek and Japanese bunching onion (JBO)) was cut with sterile scissors and inoculated with the bacterial suspension of 1×10^{6} CFU/leaf with a micropipette. After 7 days post inoculation (DPI), pathogenicity and aggressiveness of the strains was determined based on the length lesion on the leaves. The foliage of oat, pearl millet and foxtail millet were inoculated with a syringe containing bacterial suspension (1×10^{6} CFU/leaf). Leaf streaking and necrotic lesions were observed on the inoculated leaves and the lesion length was measured to determine the aggressiveness of strains (pearl millet and foxtail millet: 14 dpi; oat: 21 dpi). Phenotype on each host-bacterial strain combination can be observed in panels (a-d for onion), (e-h for chive), (i-l for leek), (m-p for JBO), (q, t and w for pearl millet), (r, u and x for foxtail millet) and (s, v, y and z for oats). Arrow in each panel indicates necrotic lesion or inoculation site on foliage.

CHAPTER 3

EVALUATING OPTIONS TO INCREASE THE EFFICACY OF BIOCONTROL AGENTS FOR THE MANAGEMENT OF *PANTOEA* SPP. UNDER FIELD CONDITIONS

Koirala, S., Myers, B., Shin, G. Y., Gitaitis, R., Kvitko, B. H., & Dutta, B. 2023. Evaluating options to increase the efficacy of biocontrol agents for the management of *Pantoea* spp. under field conditions. Plant Disease, 107(9), 2701-2708.

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Abstract

Center rot of onion is caused by plant pathogenic *Pantoea* species which can lead to significant yield losses in the field and during storage. Conventional growers use foliar protectants such as a mixture of copper bactericides and an ethylene-bis-dithiocarbamate (EBDC) fungicide to manage the disease; however, organic growers have limited management options besides copper-protectants. Biocontrol agents (BCAs) provide an alternative; however, their efficacy could be compromised due in part to their inability to colonize the foliage. We hypothesized that pre-treatment with peroxide (OxiDate 2.0: a.i. hydrogen peroxide and peroxyacetic acid) enhances the colonizing ability of the subsequently applied BCAs leading to effective center rot management. Field trials were conducted in 2020 and 2021 to assess the efficacy of peroxide, BCAs (Serenade ASO: *Bacillus subtilis* and BlightBan: *Pseudomonas fluorescens*) and an insecticide program (tank mix of spinosad and neem oil) to manage center rot. We observed no significant difference in foliar AUDPC between the peroxide pre-treated *P. fluorescens* plots and only-*P. fluorescens* treated plots in 2020 and 2021. Peroxide pre-treatment before *B. subtilis* significantly reduced the foliar AUDPC as compared to stand-alone *B. subtilis* treatment in 2020; however, no such difference was observed in 2021. Similarly, peroxide pre-treatment before either of the BCAs did not seem to reduce the incidence of bulb rot as compared to the stand-alone BCAs treatment in any of the trials. Additionally, our foliar microbiome study showed comparatively higher *P. fluorescens* retention on peroxide pre-treated onion foliage; however, at the end of the growing season, *P. fluorescens* was drastically reduced and was virtually non-existent (<0.002% of the total reads). Overall, the pre-treatment with peroxide had a limited effect in improving the foliar colonizing ability of BCAs and consequently a limited effect in managing center rot.

Introduction

Center rot disease in onion is of significant economic importance as outbreaks in the field can lead to considerable yield losses (Walcott et al. 2002; Gitaitis et al. 2003). The onset of the disease generally starts at the central leaf displaying white streaks along with some water-soaking symptoms (Gitaitis et al. 2003). Eventually, the lesions may turn necrotic and cause leaf bleaching and wilting (Dutta et al. 2014). The pathogen can further progress into the bulb leading to the discoloration and rotting of neck tissue and liquefaction of the internal scales (Gitaitis et al. 2002; Schwartz and Otto 2000; Carr et al. 2013). The disease can be caused by the members of the genus *Pantoea*: *P. ananatis*, *P. allii, P. agglomerans,* and *P. stewartii* subsp. *indologenes*. Within the four bacterial species, *P. ananatis* is more broadly associated with center rot, particularly in the south-eastern United States (Stice et al. 2020). This bacterial pathogen is difficult to control in the field due to its broad host range and different mode of survival and transmission.

Common sources of inocula for *P. ananatis* include infested seeds, weeds and thrips. The bacterium can survive epiphytically on different monocot and dicot weed species and crop plants found in and around onion fields (Gitaitis et al. 2002, 2003; Dutta and Gitaitis 2020). The bacterium can be harboured in the guts of onion thrips (*Thrips tabaci*) and tobacco thrips (*Frankliniella fusca*) and be transmitted through feces to healthy onion seedlings in a non-propagative and non-circulative manner (Gitaitis et al. 2003; Dutta et al. 2016). Thrips feeding also facilitates pathogen invasion of the neck and disease development in onion leaves (Riley et al. 2014; Grode et al. 2017; Stumpf et al. 2021).

Conventional onion growers use foliar protectants such as a mixture of copper-containing bactericides and an EBDC fungicide (e.g. Mancozeb; Corteva Agrisciences, IN) to manage

bacterial diseases in onion (Gent and Schwartz 2005; Harrison et al. 2008; Pfeufer and Gugino 2018). However, organic onion growers have limited chemical control options which makes the management of this disease more challenging. Biocontrol agents (BCAs) can provide an alternative to chemicals for disease control in organic onion. The QST 713 strain of *Bacillus subtilis* is commercially available as an aqueous suspension (Serenade ASO®; Bayer CropScience, MO) and is used to control fungal and bacterial diseases in a broad range of crops including onion (Kinsella et al. 2009, Lahlali et al. 2013; Abbasi and Weselowski 2015; Reiss and Jorgensen 2017; Matzen et al. 2019). *B. subtilis* is known to produce lipopeptides and surfactant antibiotics that are involved in antibiosis (Bonmatin et al. 2003; Ongena et al. 2007; Kinsella et al. 2009) and can also activate induced resistance against invading pathogens in the host (Ongena et al. 2007; Fischer et al. 2013, Lahlali et al. 2013). On the other hand, *P. fluorescens* A506 strain, commercially available as BlightBan A506 (NuFarm Americas, Burr Ridge, IL), can suppress the growth of pathogens by potentially creating a shortage of nutrients and niche required for their colonization and optimal growth (Wilson and Lindow 1993), a process called “pre-emptive exclusion or competitive exclusion”.

Effective management of disease by BCAs requires successful occupation of the foliar niche and early establishment of BCAs on onion foliage. This could potentially be achieved by reducing the native microbial populations on the foliar surfaces before introducing BCAs. Reactive oxygen species (ROS) such as hydrogen peroxide are known to have direct antimicrobial activity against several fungal and bacterial plant pathogens (Peng and Kuc 1992; Kotchoni et al. 2007; El-Mougy et al. 2008) and can be used as an effective disinfectant. OxiDate 2.0 (Biosafe Systems, East Hartford, CT) with a.i. hydrogen peroxide and peroxyacetic acid is an Organic Materials Review Institute-listed product available for use in organic

production. Our study aimed to evaluate the effect of pre-treatment with peroxide to improve the efficacy of subsequently applied BCAs (*B. subtilis* or *P. fluorescens*) in managing center rot disease in onion leaves and bulbs. Similarly, we assessed the dynamics of the microbial community on *P. fluorescens*-treated onion foliage over time by analyzing their taxonomic distribution. The foliar microbiome composition of *P. fluorescens* plots pre-treated with peroxide was compared with that of plots treated with *P. fluorescens* only. Additionally, we also assessed the efficacy of an organically approved thrips control program (neem oil and spinosad) alongside BCAs and peroxide in managing center rot.

Materials and methods

Field experiments

Field trials were conducted during the 2019-2020 and 2020-2021 onion-growing seasons on a certified organic farm at the University of Georgia Coastal Plain Experimental Station (UGA-CPES). The soil series at the UGA-CPES experimental site was fine-loamy, kaolinitic, thermic plinthic kandiudults. Onion cultivars (cv.) 'Sweet Jasper' was transplanted on December 4 of 2019 for the 2020 trial and cv. 'Granex Yellow PPR' was transplanted on December 3 of 2020 for 2021 trial in a 6 m long and 1.2 m wide treatment plot with a 3-m bare-ground buffer between adjacent plots. Four rows of onions with approximately 30 plants per row were transplanted in each plot with a spacing of 0.28 m between rows and 0.18 m between plants. Onion transplants were obtained from a local organic onion grower in Tattnall County, Georgia (GA), USA and were 45-days old. Treatments were arranged in a randomized complete block design (RCBD) with four replications per treatment. Each block was separated by a 1.2 m wide alley planted with cover crops (Sunn hemp). Cover crops were mowed at intervals to restrict

their growth beyond 12 cm. Plants were irrigated using overhead sprinkler irrigation and mechanical and hand weeding were performed as needed.

Treatments

Foliar applications of peroxide and/or biocontrol agents (*B. subtilis* and *P. fluorescens*) were started in February depending upon the environmental condition and plant growth in the particular year. In 2020 trial, peroxide (OxiDate 2.0) was applied at 0.94 l/ha then 2 h later either *B. subtilis* (Serenade; 9.35 L/ha) or *P. fluorescens* (BlightBan; 374.7 g/ha) was applied at 64 DAT. The 2 h time frame was deemed safe for BCA use after peroxide based on a pilot study under greenhouse conditions to determine the impact of peroxide on population of both BCAs in onion foliage (*unpublished data*). In 2021, peroxide and either of the BCAs were applied at the same rate at 85 DAT. Peroxide was applied only once during the season before the application of either of the BCAs, while BCAs were applied for a total of three times during the onion growth period. The second and third application of BCA was done at 88 DAT and 107 DAT in 2020 and 106 DAT and 126 DAT in 2021, respectively. Challenge inoculation was performed in all the treatment plots one week after the first BCA application by spray-inoculating the onion foliage with a suspension of a pathogenic strain of *P. ananatis* (PNA 97-1) containing 10^5 CFU/ml. The non-treated control plots were also inoculated with the same pathogen population and no baseline non-inoculated control plots were used during the study. Insecticide program (IP), a tank mix of spinosad (a.i. 30 g/ha) and neem oil (a.i. 2 L/ha) were applied at approximately two to three-week intervals after the thrips incidence reached the threshold of two thrips per plant. In 2020, IP was applied first at 103 DAT and the second and third applications were done at 116 DAT and 130 DAT. In 2021 study, IP was applied at 116, 128 and 140 DAT. All the treatments were applied with a CO_2 backpack sprayer and a broadcast boom equipped with three XR8003

flat-fan nozzles (TeeJet Technologies, Wheaton, IL) calibrated at 345 kPa and delivering 467.5 l/ha.

Data collection and analysis

Disease data collection

After symptoms of center rot first appeared on onion foliage (~110-120 DAT and approximately four weeks after challenge inoculation), plots were rated for disease severity. The severity of center rot in onion plots was assessed four times at an interval of 7 to 10 days using a scale from 0% to 100%, where 0% = no plant with visible disease symptom in the plot and 100% = all plants in the plot showing disease symptom. The disease severity on the plots was assessed for each treatment by calculating the area under the disease progress curve (AUDPC). Additionally, the center rot incidence in onion bulbs was evaluated post-harvest. Mature bulbs randomly selected from the middle two rows of the plot (40 bulbs/plot) were undercut and field cured for 3 h. Bulb tops and roots were clipped and hand-harvested. Onion bulbs were then stored for 30 days in a cold storage maintained at 4°C and 70% RH. The bulbs were cut into half from the bulb neck and the total number of bulbs showing symptoms of center rot per plot was recorded. The percent incidence of center rot was calculated as: $Incidence\ of\ center\ rot\ in\ bulbs\ (\%) = \frac{number\ of\ bulbs\ infected\ with\ center\ rot}{total\ number\ of\ bulbs\ assessed} \times 100$. Putative center rot symptoms were confirmed by isolating the pathogen on nutrient agar (NA) medium from a subset of symptomatic bulbs, followed by colony-PCR using *P. ananatis*-specific primers (Asselin et al. 2016).

Statistical analysis

Analysis of Variance (ANOVA) was conducted on the AUDPC value and the percent incidence of center rot in bulbs for 2020 and 2021 field trials. Blocks were considered random effects, whereas peroxide, BCAs (*P. fluorescens*, *B. subtilis* or no-BCAs) and IP treatment were

considered fixed effects. Similarly, mean separations among the treatments were carried out using LSMeans differences Student's t-test at $\alpha = 0.05$. Both ANOVA and Student's t-test were carried out using JMP pro 16.0.0 (SAS, Cary, NC) and graphs were constructed using Sigma plot 14.0 (Systat Software Inc., San Jose, CA).

Foliar microbiome assessment

Foliar sampling, DNA isolation and sequencing

During the 2021 field trial, a metagenomics survey was conducted on the onion foliage in peroxide pre-treated *P. fluorescens* plots, *P. fluorescens*-only-treated plots and non-treated control plots to monitor the population dynamics of *P. fluorescens* A506 on onion leaves. For bulk foliar sampling, six leaves from the plot/replicate were randomly selected and pooled together by treatment. Leaves were cut from plants with sharp sterile blades at approximately 14 cm from the top and collected in a resealable plastic bag. New blades and gloves were used to collect each sample in order to reduce the contamination between the treatments. Leaves were not trimmed further to reduce the onion host DNA contamination in the samples. Foliar samples were collected at 85 DAT, 1 h post application of *P. fluorescens* (t1) and at 135 DAT, which was nine days after the third *P. fluorescens* application (t2) (Supplementary Table 3.1). The short interval between *P. fluorescens* application and sample collection time of t1 was chosen with the goal of observing the persistence of the *P. fluorescens* in peroxide pre-treated plants compared to those treated with *P. fluorescens*. Similarly, timepoint t2 was chosen to evaluate the bacterial composition and diversity of onion leaf tissues later in the growing season. Metagenomics results from t1 and t2 samplings were compared to observe any temporal changes in microbial population.

Leaves pooled from each treatment were suspended in 350 ml of sterile 1X phosphate-buffer saline (PBS) solution and subjected to sonication in a Bransonic Ultrasonic bath (Emerson, MO, US) for 18 min. Leaf washate was transferred to 50 ml falcon tubes after carefully avoiding the onion leaf tissues and centrifuged for 24 min at 4,000 rpm using an Eppendorf 5810R centrifuge (Eppendorf Ag, Hamburg, Germany). The resulting pellet at the bottom of the tubes was transferred to a 1.7 ml centrifuge tube and DNA was extracted using Omega E.Z.N.A bacterial DNA extraction kit (Omega Biotek, Norcorss, GA), as per the manufacturer's instructions. DNA was quantified using NanoDrop Lite spectrophotometer (Thermo Fisher Scientific, WI, US) and ~40 µl of ≥30 ng/µl genomic DNA per sample was sent to Novogene (Sacramento, CA) for shotgun metagenomic sequencing. Briefly, for metagenomics library preparation, the DNA was fragmented and end repairing and phosphorylation were performed, which was followed by A-tailing and adaptor ligation. The DNA libraries were pooled and 150 bp paired-end sequencing was performed using NovaSeq PE150.

Metagenomic data trimming, quality filtering and estimation of coverage

The raw sequence reads were uploaded to NCBI Sequence Read Archive under the BioProject accession number PRJNA904248. The quality of raw FASTQ data obtained from shotgun sequencing was assessed using FastQC (Andrews 2010). Low-quality bases as well as adapter sequences were trimmed using fastp v0.23.2 (Chen et al. 2018). Additionally, the in-built Trimmomatic (Bolger and Giorgi 2014) tool in KneadData (https://github.com/biobakery/kneaddata) trimmed low-quality reads shorter than 50 bp and the reads with phred quality score cut-off of 33. The onion host "contaminant" reads were filtered using default parameters of KneadData after aligning the metagenomic data with the complete genome of *Allium cepa* (GCA_905187595.1) obtained from the NCBI database. The remaining

high-quality reads obtained after data filtering were used for further analysis. Moreover, the abundance-weighted average coverage of each metagenomic sample was estimated using a *k*-mer-based database-independent tool, Nonpareil v3.3.4 (Rodriguez-R et al. 2018).

Taxonomic profiling of metagenomic samples

Taxonomic labels were assigned to the high-quality reads using Kraken2 v2.1.1 (Wood et al. 2019) that classified reads to a specific taxon based on the exact k-mer alignments. Kraken2 identifies the genus with 99.9% precision and has a genus sensitivity of 89.5% and provides a qualitative profile of the microbial community in a sample. Default parameters of Kraken2 were used for this study which had an updated version of the NCBI-nucleotide database. The resulting taxonomic distribution of the metagenomic samples was visualized using Sankey flow diagrams in Pavian (Breitwieser and Salzberg. 2020).

Results

AUDPC for foliar disease assessment

Peroxide and BCA treatment had significant effect on the foliar AUDPC both in the 2020 and 2021 trials (Table 3.1) compared to the non-treated controls. A significant peroxide by BCA interaction was observed in 2020 ($P<0.001$) and 2021 ($P<0.01$). On the other hand, the IP for thrips control had no significant effect on the foliar AUDPC and no two-way interaction was observed between IP and peroxide or BCAs in either year. Similarly, there were no three-way interactions among peroxide, BCAs, and the IP in any of the field trials (Table 3.1).

Mean separation of the peroxide by BCAs interaction in 2020 showed that the non-treated control had the highest mean AUDPC value (2,000.5) as compared to any other combinations of BCAs or peroxide (Figure 3.2). Interestingly, peroxide pre-treatment before *P. fluorescens* did not reduce foliar severity as compared to *P. fluorescens*-only treatment. *B.*

subtilis treated after peroxide significantly reduced foliar severity (mean AUDPC 935.3) as compared to only *B. subtilis* treatment (mean AUDPC 1,165.6) (Figure 3.2). However, the mean AUDPC of the peroxide-pre-treated *B. subtilis* plots was not significantly different from peroxide-only-treated plots nor from the mean of any of the *P. fluorescens* treated plots.

The severity of foliar symptoms of center rot was found to be generally lower in 2021 as compared to 2020. Post-hoc analysis of BCA by peroxide interaction in 2021 also showed that the mean AUDPC of peroxide pre-treated *P. fluorescens* plots was similar to *P. fluorescens*-only treated plots. The highest mean foliar AUDPC value (990.94) was again observed in the non-treated plots (Figure 3.2). In contrast to the 2020 trial, peroxide pre-treatment before *B. subtilis* had no significant effect on mean AUDPC as compared to *B. subtilis*-only treatment.

Center rot incidence in the bulb

BCAs significantly affected the incidence of center rot in onion bulbs in 2020 (*P=0.014*) as well as in 2021 (*P=0.021*) (Table 3.1). However, peroxide treatment had no effect on the bulb rot incidence in both years. Significant BCA by peroxide interaction was observed in 2020 (*P=0.041*), although, no such interaction occurred in 2021. IP had no significant effect on center rot bulb symptom incidence in any trial. Furthermore, IP did not interact with peroxide and/or BCAs in any of the field trials.

Post-hoc analysis of the BCA by peroxide interaction in 2020 showed that the non-treated control plots had the highest incidence of center rot (18.75%) as compared to other treatment combinations (Table 3.2). Peroxide pre-treatment before either *P. fluorescens* or *B.subtilis* had no significant effect on the center rot incidence in the bulb as compared to the standalone application of BCAs.

Metagenomic data trimming, quality filtering and estimation of coverage

Shotgun metagenomic sequencing yielded 12.4 to 17.7 G of raw data per sample that was used for downstream analysis. The lowest number of raw reads was 41.2 (million) in the *P. fluorescens*_t1 sample, whereas the non-treated_t2 sample had the highest number of raw reads 59.1 (million) (Supplementary Table 3.1). Low-quality reads in the samples ranged from 3.6% to 6.7% of the raw reads, which were trimmed by fastp and the in-built Trimmomatic tool of KneadData. KneadData detected and filtered out onion (host) reads ranging from 3.8% to 9.2% of the raw reads from the samples. The high-quality reads per sample used for further analysis ranged from 86.7% to 92.0% of the raw data (Figure 3.3A). The nonpareil rarefaction curves suggested that the portion of the total microbial community of foliage covered by the sequencing effort was >95% per sample (Figure 3.3B). Similarly, we evaluated the Nonpareil index of sequence diversity (Nd), a coverage-independent measure of the diversity of the sampled community, which correlates with the alpha diversity estimated from the 16S rRNA gene profiles (Rodriguez-R et al. 2018). The Nd value of the samples used in our study ranged from 15.2 to 17.8.

Taxonomic profiling of metagenomic samples

Kraken2 was used for profiling the microbial community on onion foliage and classified bacteria at the genus as well as species level in all the metagenomic samples. Most of the reads in the samples were classified into specific taxon; however, a few reads from each sample remained unclassified. The proportion of unclassified reads ranged from 2.2% to 29.8%. Interestingly, bacterial reads were identified even at the strain level that allowed for the direct comparison of the proportion of *P. fluorescens* A506 reads between *P. fluorescens* plots pre-treated with peroxide and only *P. fluorescens*-treated plots. At t1, 10.2% of the total reads were classified as

P. fluorescens A506 in peroxide pre-treated *P. fluorescens* plots as compared to only 0.9% in *P. fluorescens*-only treated plots (Figure 3.4A and 3.4C; Supplementary Table 3.2). Similarly, 36% of the total reads were classified under the genus *Pseudomonas* in peroxide pre-treated *P. fluorescens* plots; however, *P. fluorescens*-only plot had only 3.2% of the reads classified as *Pseudomonas* (Supplementary Table 3.2). Surprisingly, the proportion of reads that mapped to *P. fluorescens* A506 strain drastically reduced at t2 and were lower than 0.002% of the total reads in both *P. fluorescens* plots treated with or without peroxide (Figure 3.4B and 3.4D; Supplementary Table 3.2). Moreover, at t1, the highest percentage of the reads, approximately 40.7% in peroxide pre-treated *P. fluorescens* plots and 78% in peroxide non-treated *P. fluorescens* plots, were identified as *P. agglomerans* suggesting that it was the most predominant species occupying onion foliage at that time of the onion growing season. On the other hand, at t2, most of the reads were classified as *Pantoea* spp. in all the samples, irrespective of the treatments (Figure 3.4B, 3.4D and 3.4F).

In the case of non-treated control, an insignificant proportion of reads (<0.0002%) were classified as *P. fluorescens* A506 at t1 and t2 (Supplementary Table 3.2). The presence of *P. fluorescens* A506 in non-treated check could be partly attributed to wind and rainfall dispersal. *P. agglomerans* was the dominant species in the non-treated check plots both at t1 and t2.

Discussion

In this study, we assessed if the prior application of peroxide on onion foliage can improve the efficacy of subsequently applied BCAs, *B. subtilis* or *P. fluorescens*, in reducing the center rot severity in onion foliage and bulbs. Our approach of using peroxide on onion foliage prior to the BCA application was based on the hypothesis that peroxide would lower the microbial populations on the onion phyllosphere, which would promote the colonizing ability of the

subsequently applied BCA. Onion foliage well-colonized by BCAs could potentially preclude the invasion of bacterial foliar pathogens including *Pantoea spp.* thus reducing center rot in onion fields. Early establishment of microbes is known to either reduce the amount of available resources for late-arriving species or manipulate the foliar micro-environment and alter the microbial community of the foliage (Fukami 2015; Egel et al. 2019). Several reports have illustrated this sort of 'priority effects' where the early arriving species influence the microbiome composition of the plant phyllosphere (Braun-Kiewnick et al. 2000; Chase 2003; Carlstrom et al. 2019; Halliday et al. 2020; Seybold et al. 2020).

The 2020 and 2021 field data were evaluated separately due to a high magnitude of variation in the foliar severity as well as bulb rot incidence between the two trials. In both the 2020 and 2021 trials, peroxide and BCAs had significant effects on AUDPC values (Table 3.1). Moreover, peroxide by BCAs interaction was significant in both trials ($P<0.05$). Since our study was aimed at elucidating the effect of peroxide on the efficacy of BCAs, we focused our analysis on their interactions rather than their individual effects. In both trials, the non-treated control had the highest mean AUDPC values, which were significantly higher than any other treatment combinations for that respective year (Figure 3.2). Therefore, any combination of peroxide and BCAs seemed to reduce center rot severity on onion foliage better than the non-treated check. Interestingly, in both trials, stand-alone peroxide treatment seemed to achieve a similar level of foliar disease control as BCAs applied with or without peroxide. The anti-microbial activity of hydrogen peroxide has been noted to reduce the total indigenous bacterial populations by half in fairway turf (Mercier 2005). We speculate that the similar anti-microbial activity of peroxide on the pathogenic bacterial populations could contribute to the reduction in foliar disease severity.

We found that the mean AUDPC for peroxide pre-treated *P. fluorescens* plots were not significantly different from the *P. fluorescens*-only-treated plots in both the 2020 and 2021 field trials. Similarly, although the mean AUDPC value of the peroxide pre-treated *B. subtilis* plots was significantly lower than *B. subtilis*-only treated plots in 2020; this difference was not observed in 2021. In the 2020 trial, the mean AUDPC value of the peroxide pre-treated *B. subtilis* plot was similar to that of the peroxide-only plot and hence we could not observe a clear effect of peroxide pre-treatment on the efficacy of *B. subtilis*. Therefore, our field experiments could not establish a consistent effect of peroxide pre-treatment in improving the efficacy of *B. subtilis* to manage foliar disease. Altogether, our findings from the study of foliar severity suggested that peroxide pre-treatment before BCA may not play any role in improving the efficacy of BCA to control center rot as compared to the stand-alone BCA application.

Evaluation of center rot incidence in onion bulbs showed that the BCAs had a significant effect on center rot incidence in the bulbs in both trials (Table 3.1). BCA by peroxide interaction was significant in 2020; however, no such interactions were observed in 2021. Percent incidence of center rot in bulbs was the highest (18.75%) in non-treated plots as compared to other treatments for that year (Table 3.2). The bulb rot incidence in the plot receiving *B. subtilis* only did not vary significantly from that of peroxide pre-treated *B. subtilis* plots and no considerable differences were observed in the percent incidence of bulb rot in *P. fluorescens* plots treated with or without peroxide in 2020. In 2021 trial, no differences in bulb rot incidence were observed among any of the treatments. Our data on bulb rot incidence also suggested that peroxide pre-treatment does not necessarily improve the efficacy of BCAs (Table 3.2). Additionally, we could not establish a consistent role of peroxide in managing the center rot in the bulb.

Spinosad and neem oil product has been generally reported to reduce onion thrips population in organic production system (Iglesias et al. 2021a, 2021b). However, these studies have primarily focused on the direct effect of thrips population in bulb yield and the implication of thrips incidence on onion disease has not been clearly studied. Our study did not find any significant effect of IP (tank mix of spinosad and neem oil) on foliar AUDPC or center rot incidence in the bulb. Similarly, no three-way interactions were observed between peroxide, BCAs, and the IP.

Generally higher disease severity in onion leaves and bulbs was observed in 2019-2020. This might be explained by the slightly higher average maximum (20.7°C) and average minimum temperature (9.8°C) observed in the 2019-2020 onion growing season as compared to 20.2°C and 8.8°C, respectively in 2020-2021 (Figure 3.1). Center rot is generally more prevalent in warmer months and is considered to be a warm-weather disease (Gitaitis et al. 2002; Harrison et al. 2008); therefore, even though a lower amount of rainfall (4.02 mm) occurred during 2019-2020 onion season as compared to 4.8 mm in 2020-2021 season, higher temperature could have had more influence on center rot intensity than rainfall.

Our sequencing effort captured on average >95% of the total microbial community of the foliage in each sample and their Nd values (15.2 to 17.8) were comparable to the levels of microbial complexity associated with other crop hosts such as tomatoes (Newberry et al. 2019) and even humans and other animals (Rodriguez-R et al. 2018). Assessment of the foliar microbiome composition in peroxide pre-treated *P. fluorescens* plots at t1 DAT in 2021 (1 h after the first *P. fluorescens* application) showed that a higher proportion of *P. fluorescens* A506 retained in the onion foliage as compared to *P. fluorescens*-only-treated plot (Figure 3.4A and 3.4C). Although this was a noteworthy observation, we could not conclude just from this

observation that pre-treatment of peroxide improved the foliar colonizing ability of *P. fluorescens* since this time point was too early for the bacterium to establish and colonize the onion phyllosphere. More microbiome data at several different time points needs to be evaluated to further compare the *P. fluorescens* populations between peroxide pre-treated or peroxide non-treated *P. fluorescens* plots. Surprisingly, later in the growing season (t2), the proportion of reads classified as *P. fluorescens* A506 drastically reduced to <0.002% of the total reads in both peroxide pre-treated as well as peroxide non-treated *P. fluorescens* plots (Figure 3.4B and 3.4D). Our study used higher concentration of *P. fluorescens* A506 (~10^7 CFU/ml) as compared to the pathogen (~10^5 CFU/ml) but irrespective of the treatments, *Pantoea* species was found to be predominant on onion foliage from the beginning to the late onion-growing season. *Pantoea* spp. has been widely reported to be associated with onion foliage and bulb and is also considered as one of the most predominant bacterial pathogens of onion in Georgia (Stice et al. 2020, Dutta and Gitaitis 2020). Although our finding was based on the year showing less disease pressure, the widespread distribution of *Pantoea* spp. points towards its similar prevalence in onion fields in other cropping seasons. This could imply that even if peroxide pre-treatment improves the foliar colonizing ability of *P. fluorescens*, *Pantoea* species could outcompete *P. fluorescens* and re-establish themselves in the foliar niche over time. Mercier (2005) suggested that the indigenous species of fairway turf were able to stabilize their population even after *P. fluorescens* A506 was provided an edge for its establishment by pre-treatment with peroxide. The reduced *P. fluorescens* populations on onion foliage in *P. fluorescens* treated plots with the progression of growing season irrespective of peroxide pre-application could potentially explain the insignificant difference between peroxide pre-treated *P. fluorescens* plot and only *P. fluorescens* treated plots in managing center rot. Since we did not study the dynamics of

microbial community in *B. subtilis* treated plots, we could only speculate similar population dynamics of *B. subtilis* on the onion foliage of peroxide pre-treated and peroxide non-treated *B. subtilis* plots. A similar study on peroxide pre-treatment before the foliar application of Sustane (aerobically composted turkey litter: Sustane Natural Fertilizers, Inc., Cannon Falls, MN) found an inconsistent effect of peroxide (OxiDate) in enhancing its efficacy in reducing the early blight and Septoria leaf spot diseases in tomato (Egel et al. 2019).

As observed in this research, stand-alone peroxide treatment might have a similar ability to reduce center rot as BCAs with or without peroxide pre-treatment. Multiple applications of peroxide alternated with BCAs could be an alternative approach to reduce disease severity; however, it may not be applicable as it could also disrupt the growth and development of beneficial microbes along with the plant-pathogenic organisms. Nevertheless, this experiment along with the testimonials of onion growers about the efficacy of peroxide merits further inquiry on the effect of this product in reducing center rot severity. Similarly, our experiments used only three total BCA applications each at 2-3 week intervals; however, more frequent applications of BCAs after peroxide could favour higher niche occupation by beneficial microbes and prolong their survival on the foliage. Overall, our multi-year field trials indicate that while peroxide on its own might have some level of disease control in the field, it may not play any significant role in improving the efficacy of subsequently applied BCAs. Additionally, *Pantoea* spp. could predominate the foliar surface of onion throughout the onion growing season, therefore, better center rot management in organic onion would require further investigation into novel beneficial micro-organisms that could establish well on onion foliage and surpass the growth of *Pantoea* spp.. Overall, the current research could potentially provide some guidance for the modification

of the center rot management program utilizing peroxide and BCAs in organically managed onion crops.

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Table 3.1. Analysis of variance results (*P*-values) for peroxide (OxiDate 2.0; Biosafe Systems, East Hartford, CT), biocontrol agents *Pseudomonas fluorescens* (Blightban; NuFarm Americas, Burr Ridge, IL) or *Bacillus subtilis* (Serenade ASO; Bayer CropScience, MO) and insecticide program (tank mix of spinosad and neem oil) and their two-way and three-way interactions to affect area under disease progress curve (AUDPC) and percent incidence of center rot in 2020 and 2021 trial.

	AUDPC[d]		**Center rot incidence in onion bulb (%)**	
Factor	**2020**	**2021**	**2020**	**2021**
Peroxide[a]	<0.001**	0.026*	0.503	0.179
BCA[b]	<0.001**	<0.001**	0.014*	0.021*
IP[c]	0.088	0.135	0.295	0.333
Peroxide × BCA	<0.001**	0.007**	0.041*	0.144
Peroxide × IP	0.319	0.397	0.390	0.849
BCA × IP	0.706	0.021*	0.413	0.105
Peroxide × BCA × IP	0.282	0.342	0.266	0.882

[a] Peroxide vs. No peroxide.
[b] *P. fluorescens* vs. *B. subtilis* vs No BCA.
[c] Insecticide program (neem oil + spinosad) vs. No IP.
[d]Analysis of Variance was conducted on AUDPC values and percent incidence of center rot in onion bulbs to determine the treatment effect. Mean separations were done using LSMeans differences Student's t-test ($P<0.05$). *P*-values significant at 5% and 1% levels of significance are denoted by * and ** respectively.

Table 3.2. Percent incidence of center rot in onion bulbs from plots treated with biocontrol agents *Pseudomonas fluorescens* (Blightban; NuFarm Americas, Burr Ridge, IL) or *Bacillus subtilis* (Serenade ASO; Bayer CropScience, MO) either with or without pre-treatment with peroxide (OxiDate 2.0; Biosafe Systems, East Hartford, CT).

	Center rot incidence in onion bulb (%)[a]	
	2020	2021
Peroxide		
P. fluorescens	6.87 b[b]	4.45 A
B. subtilis	10.00 b	1.21 A
None	9.37 b	1.79 A
No Peroxide		
P. fluorescens	5.62 b	2.05 A
B. subtilis	6.25 b	1.39 A
None	18.75 a	1.67 A
P-value	0.041*	0.144

[a] Center rot incidence in bulb was calculated as (number of onion bulbs infected with center rot/total number of bulbs assessed) × 100.
[b]Within each year, the mean percent incidence of center rot followed by the same letter do not differ significantly at $P = 0.05$ (LSMeans difference Student's t-test). *P* value significant at a 5% level of significance is denoted by *.

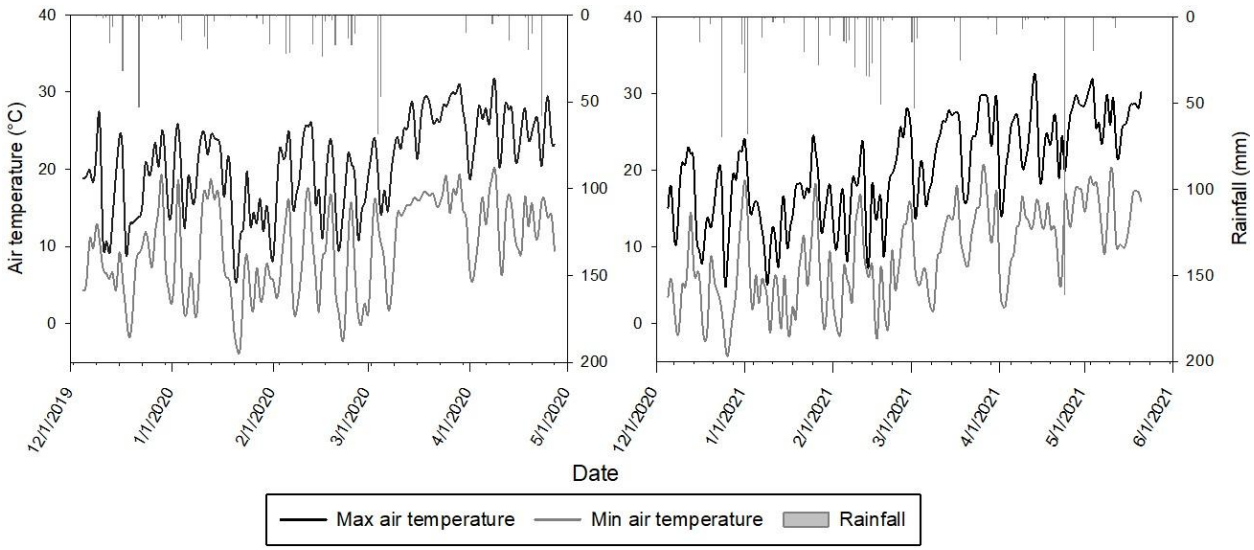

Figure 3.1. Maximum and minimum air temperature, and amount of rainfall during onion growing seasons in 2020 and 2021. Data retrieved from the Georgia Automated Environmental Monitoring Network at University of Georgia, Tifton, Georgia.

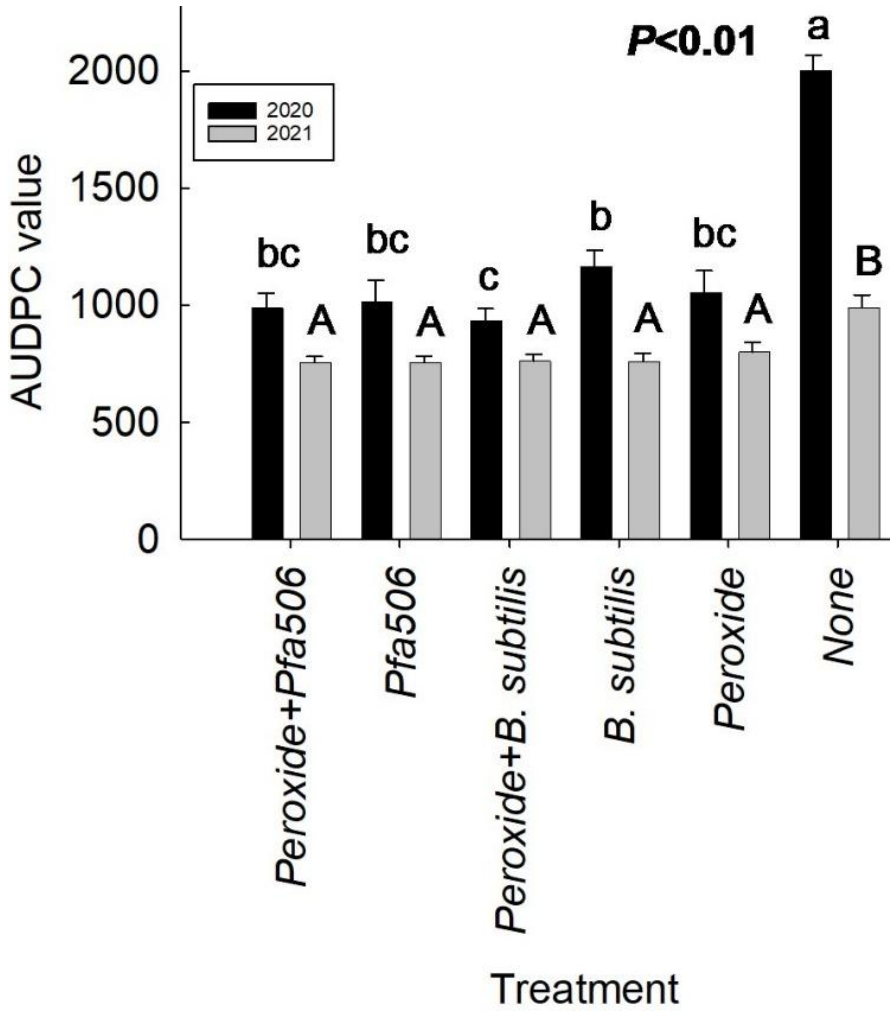

Figure 3.2. Area under disease progress curve (AUDPC) of center rot severity in onion foliage treated with *Pseudomonas fluorescens* (BlightBan; NuFarm Americas, Burr Ridge, IL) or *Bacillus subtilis* (Serenade ASO; Bayer CropScience, MO) with or without peroxide (OxiDate 2.0; BioSafe systems LLC, East Hartford, CT) in 2020 and 2021 field trials. For both trials, the foliar severity on each plot was recorded four times at an interval of 7-10 days. The AUDPC was calculated using MS Excel as follows: $\Sigma_{i=1}$ $[(Y_{i+n1} + Y_i)]/2$ $[X_{i+1}-X_i]$ where Yi = foliar severity caused by center rot at the ith observation, Xi = time in days at the ith observation and n = total number of observations. Error bars represent mean AUDPC ± standard error. For each year,

mean AUDPC values with the same letter are not significantly different according to LSMeans differences Student's t-test (P<0.05).

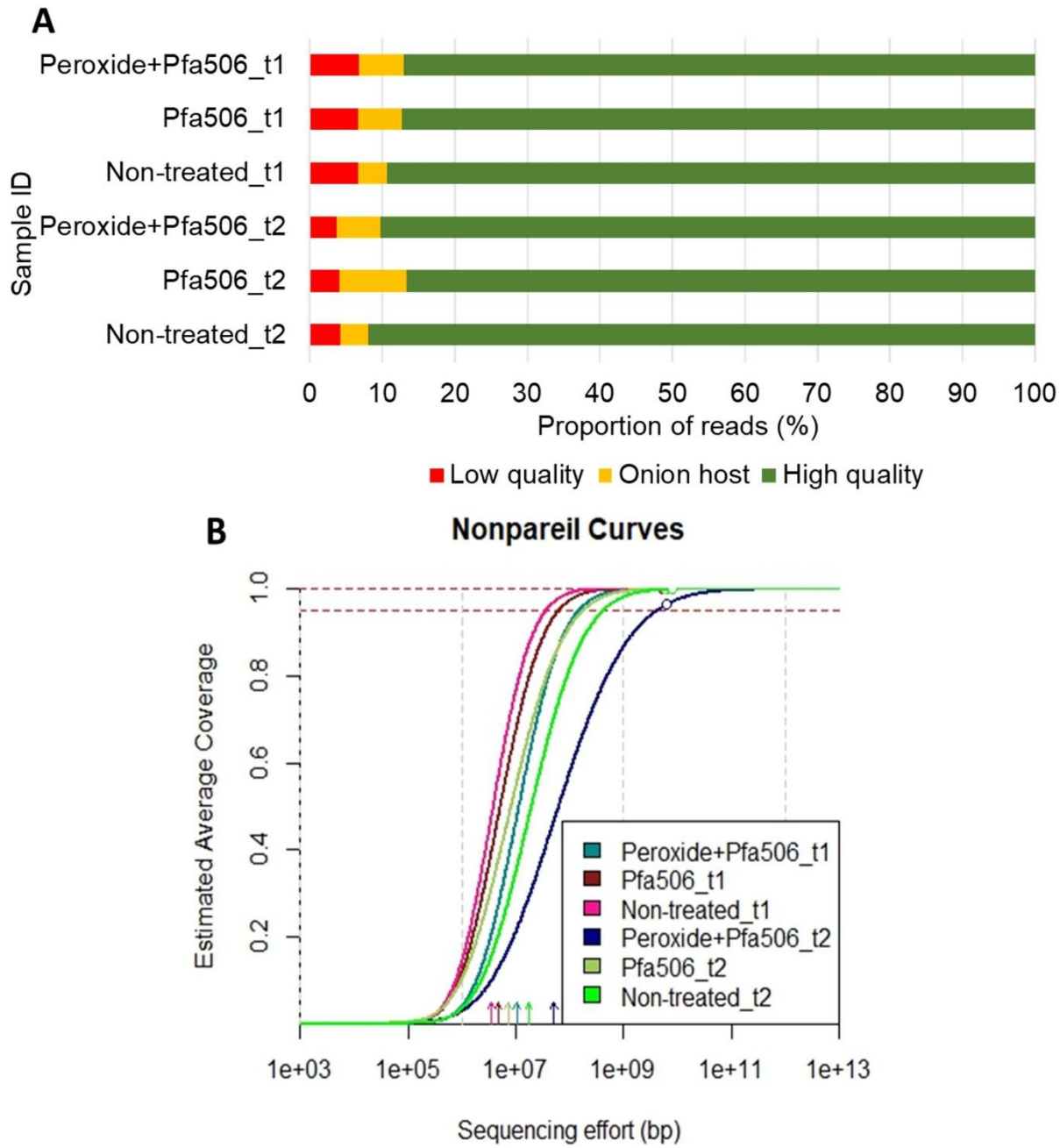

Figure 3.3. Bar graph showing the proportion of low-quality, onion host and high-quality reads per sample, and a Nonpareil rarefied curve showing estimated average coverage. A) Stacked bar graph showing the percentage of low-quality (red), onion host (yellow) and high-quality (green) reads for each sample sequenced for this study. B) Nonpareil curves; empty circle in each curve indicates the degree of average coverage of a microbial community of foliage per sequencing effort. The line after the circle is a Nonpareil projection curve. The lower dashed horizontal line indicates 95% coverage and the upper line indicates 99% coverage. The upward pointing arrows on X-axis represent sequence diversity (N_d) that correlates with alpha diversity (Rodriguez-R et al. 2018). In each sample ID, t1 and t2 indicate the DNA extraction time points 85 and 135 days after transplanting, respectively. Pfa506 represents samples treated with *Pseudomonas fluorescens* A506 strain (BlightBan; NuFarm Americas, Burr Ridge, IL) and peroxide represents samples treated with OxiDate 2.0 (Biosafe Systems, East Hartford, CT).

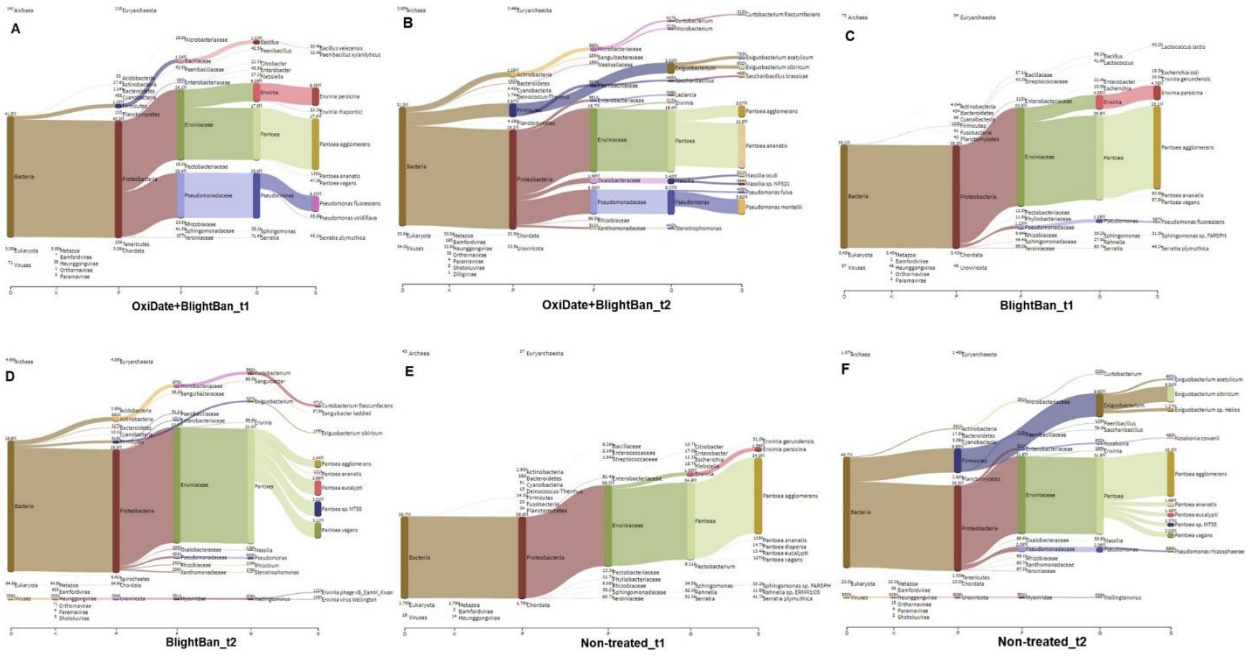

Figure 3.4. Sankey flow diagrams showing the taxonomic distribution of microbiome associated with onion leaves treated with peroxide+*Pseudomonas fluorescens* A506, *P. fluorescens* A506 (BlightBan; NuFarm Americas, Burr Ridge, IL), and non-treated check at two different time points. Bacterial DNA extracted from the washate of onion foliage was sequenced and Kraken2 was used to assign taxa to the reads. The taxonomically classified reads were visualized using Pavian. The Division, Kingdom, Phylum, Family, Genus and Species are designated at the bottom of each figure as D, K, P, F, G and S respectively. Similarly, the number assigned on each taxa represent the number of total reads classified as that taxon. Peroxide (OxiDate 2.0; Biosafe Systems, East Hartford, CT) was applied at 85 days after onion transplanting (DAT) as a one-time application followed by the first *P. fluorescens* application 2h after peroxide. A) Peroxide+Pfa506_t1 represents the microbiome associated with onion foliage in peroxide pre-treated *P. fluorescens* plot, 1 h after the first *P. fluorescens* application (85 DAT). B) Peroxide+Pfa506_t2 represents the microbiome composition of onion foliage after 9 days of the last *P. fluorescens* application (135 DAT). C) Pfa506_t1 and D) Pfa_t2 shows the distribution of

different taxa on the onion foliage in *P. fluorescens*-only plots at 85 DAT and 135 DAT, respectively. E) Non-treated_t1 and F) Non-treated_t2 show the microbiome present on onion foliage of control plots at 85 DAT and 135 DAT, respectively. Pfa506 represents samples treated with *P. fluorescens* A506 strain (BlightBan; NuFarm Americas, Burr Ridge, IL) and peroxide represents samples treated with OxiDate 2.0 (Biosafe Systems, East Hartford, CT).

Supplementary Table 3.1. Sample ID, sampling time, raw data output, number of raw reads and number of high-quality reads obtained after quality filtering.

Sample name	Sampling time (DAT)[a]	Raw sequences (Gbp)	Raw reads (m)	Reads after quality filtering (m)
Peroxide[b]+Pfa506[c]_t1	85	14.7	49	42.7
Pfa506_t1	85	12.4	41.6	36
Non-treated_t1	85	12.6	42	37.5
Peroxide+ Pfa506 _t2	135	13.7	45.8	41.4
Pfa506_t2	135	13.3	44.4	38.5
Non-treated_t2	135	17.7	59.1	54.4

[a] Days after transplanting
[b] OxiDate® 2.0 (Biosafe Systems, East Hartford, CT)
[c] *P. fluorescens* (BlightBan®; NuFarm Americas, Burr Ridge, IL)

Note: Supplementary Table 3.2 is provided as an additional file for Chapter 3.

CHAPTER 4

INTEGRATING BIOCONTROL AGENTS WITH COPPER FOR CENTER ROT MANAGEMENT IN ONION

Koirala, S., Shin, G. Y., Kvitko, B., & Dutta, B. 2024. Integrating biocontrol agents with copper for center rot management in onion. Crop Protection, 106785.

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Abstract

Center rot of onion caused by *Pantoea* species can lead to substantial yield losses in the field and during storage. Growers rely on copper-based products or their combination with ethylenebisdithiocarbamate fungicides to manage center rot. Biocontrol agents (BCAs) are occasionally used alone or in combination with copper. The optimum intervals for alternate application of copper and BCAs have not been established and the efficacy of tank-mixing copper with BCAs over their solo applications remains largely understudied. We evaluated the ability of *Bacillus subtilis* QST 713 (Serenade ASO) or *Bacillus mycoides* isolate J (LifeGard WG) applied alternately or as tank mixes with copper (Nordox 75 WG) to manage center rot in onion leaves and bulbs. Alternating the application of BCAs with copper at longer intervals (10- or 15-d) could achieve a similar level of center rot control as their alternate treatment at shorter intervals (5-d). Under low disease pressure, two to three applications could be eliminated from the spray program without compromising disease suppression. We also found that *B. subtilis* QST 713 and *B. mycoides* isolate J strains from respective products were copper-sensitive at concentrations >250 ppm. Our concurrent study on the tank mix of BCAs with copper showed that standalone copper treatment had a similar level of disease control as their tank mix application. Frequent applications of copper may control disease relatively better than treatments lacking copper products but the integration of BCA with copper in spray programs may largely depend upon their compatibility and modes of action of the BCA products.

Introduction

Center rot is a yield-limiting disease of onion that affects onion leaves and bulbs (Gitaitis and Gay, 1997). Phytopathogenic *Pantoea* spp., namely *P. ananatis*, *P. agglomerans*, *P. allii* and *P. stewartii* subsp. *indologenes* are responsible for center rot symptoms that typically start from the

central leaves of onion and can often progress to bulbs (Gitaitis and Gay, 1997; Edens et al., 2006; Nischwitz et al., 2007; Brady et al., 2011; Stumpf et al., 2018). White streaking in leaves is commonly observed upon the onset of infection, which is accompanied by water-soaked margins (Gitaitis et al., 2002), characteristic of bacterial disease symptoms. Severe infection leads to bleaching of leaves (Nischwitz et al., 2007) and results in the potential collapse of the whole plant (Schwartz and Otto, 2002; Gitaitis and Gay, 1997). The pathogen can progress into the bulb via the bulb apex and cause yellow-brown discoloration of scales leaving the tissues intact (Carr et al., 2013). Occasional bulb colonization by secondary microbes potentially leads to tissue maceration and bulb rot, resulting in foul order in pre- or post-harvest onions (Gitaitis et al., 2002, 2003; Nischwitz et al., 2007; Carr et al., 2013).

Since the first report of its epidemic in 1997 in Vidalia sweet onions, center rot continues to pose as one of the significant bacterial threats to onion production in the Vidalia region (Gitaitis and Gay, 1997; Gitaitis et al., 2002; Dutta et al., 2014). As with other bacterial disease management in onion, growers rely heavily on copper-based compounds to manage center rot (Gent and Schwartz, 2005; Harrison et al., 2008; Pfeufer and Gugino, 2018; Belo et al., 2023), which are typically mixed with ethylenebisdithiocarbamate (EBDC) fungicides and applied at 7-10-d intervals (Agarwal et al., 2015; Dutta and Gitaitis, 2020). Yet, several independent studies have noted the limited efficacy of copper bactericides in managing this disease (Gitaitis et al., 2003; Nischwitz et al., 2007; Dutta et al., 2014; Grode et al., 2019; Stumpf et al., 2021). The reason for the limited effect has not been fully understood but copper-tolerant strains and thrips populations in onion fields have been attributed to play an important role. *In vitro* screening of predominant center rot-causing pathogens, *P. ananatis* and *P. agglomerans* has indicated the prevalence of copper-tolerant isolates in onion fields (Nischwitz et al., 2007; Tho et al., 2019).

Although these strains were tolerant to copper products at a concentration of ~200 µg/ml, which is below the recommended rates of field treatment, they may exacerbate disease severity in the field. Similarly, tobacco thrips (*Frankliniella fusca*) and onion thrips (*Thrips tabaci*) have been implicated as a source of inocula for this pathogen (Wells et al., 2002; Gitaitis et al., 2003). *Pantoea* spp. in the contaminated feces of thrips may gain access to the internal tissue of onion through the wounds created by thrips while feeding (Dutta et al., 2014, 2016). Thrips often colonize the tightly folded leaves of onion neck, allowing the pathogens to evade surface-protectants (Gitaitis et al., 2003; Dutta et al., 2014; Stumpf et al., 2021) and reducing the efficacy of bactericidal sprays in disease management.

Several plant activators and biocontrol agents (BCAs) have been tested for their efficacy in bacterial disease management with or without their integration with copper. Plant defense inducer, acibenzolar-*S*-methyl, combined with cupric hydroxide significantly reduced center rot in bulbs and foliage as compared to non-treated control under greenhouse conditions (Stumpf et al., 2021). However, this treatment effect was observed only in a thrips-restricted environment; treatment did not reduce disease severity in the presence of thrips (Stumpf et al., 2021). *Bacillus subtilis* QST 713 (Serenade ASO; Bayer CropScience, St. Louis, MO), a biocontrol agent known to have antimicrobial properties and an inducer of plant natural defense (Bonmatin et al., 2003; Nagorska et al., 2007; Ongena et al., 2007; Onega and Jacques 2008; Lahlali et al., 2013), was shown to slightly reduce center rot symptoms in onion foliage as compared to the non-treated control (Koirala et al., 2023). Moreover, their study showed that the treatment of peroxide before *B. subtilis* on onion foliage may not improve the efficacy of *B. subtilis* in center rot management. Another biocontrol agent, *B. mycoides* isolate J, commercially available as LifeGard (Certis Biologicals, Columbia, MD), is known to trigger systemic resistance (Bargabus et al., 2002). It

has been shown to reduce Cercospora leaf spot of sugar beet caused by *Cercospora beticola* and anthracnose of cucumber caused by *Glomerella cingulata* var. *orbiculare* (Bargabus et al., 2002; Neher et al., 2009). However, it has varying levels of efficacy against bacterial diseases in onion including center rot (Dutta et al., 2021; Belo et al., 2023).

The study of potential alternatives to conventional chemicals is important as their excessive use poses a risk to environmental safety and increases the cost associated with production. Optimizing BCA application timing and incorporating it effectively into spray programs may equip growers with improved center rot management techniques. Our study used a multi-pronged approach to understand the efficacy of *B. subtilis* QST 713 (BsQST) and *B. mycoides* isolate J (BmJ) in managing center rot in foliage and bulbs when combined with copper. This study investigated whether alternating BCAs with copper at shorter intervals have a more pronounced effect against center rot as compared to their alternate application at longer intervals. We also assessed the copper sensitivity of *Bacillus* spp. at varying copper concentrations and evaluated the effectiveness of tank mixes of BCAs and copper in managing center rot.

Materials and Methods

Experimental design

Two independent trials were conducted in 2021-2022 and 2022-2023 at the Black Shank Farm, Tifton, GA. Experiments on i) alternate application of BCAs and copper and ii) tank mix treatment of BCAs and copper were undertaken in two consecutive seasons. Onion seedlings of cv. Pleothera (2021-2022) and cv. Sapelo (2022-23) were transplanted into each plot that had four rows of onions. The row-to-row spacing within a plot was 0.28 m and the plant-to-plant spacing was 0.18 m. The experiment on the alternate application of treatments was arranged in a

completely randomized design (CRD), while the experiment on tank mix treatment utilized a randomized complete block design (RCBD). Each experiment had four replications. The plots were 3-m-long and 1.2-m-wide for the first experiment and the latter experiment utilized 6 m × 1.2 m plots. A 3-m non-planted break separated the adjacent plots.

Approximately 70 days old onions were transplanted in the first week of December in 2021-22 while in 2022-23, due to frost injury, onions had to be replanted in mid-January and were 115 days old. Treatments were initiated ~ 60 days after transplanting (DAT) in both seasons when the plants were at four to seven true leaf stage. Onion transplanting and the first treatment application were synchronized for both experiments. Treatments were applied with a CO_2-pressurized backpack sprayer and a broadcast boom equipped with three XR8003 flat-fan nozzles (TeeJet Technologies, Wheaton, IL) delivering 375 L/ha at 60 psi. Center rot disease in onions across all plots, including non-treated control plots, resulted from artificial inoculation with a bacterial suspension (*P. ananatis* PNA 97-1) containing 10^5 colony-forming units (CFU)/ml. For inoculum preparation, a pure colony from a 48-h old nutrient agar was dipped into a 3 ml nutrient broth and shaken overnight in a rotary shaker (MaxQ 4450, Thermo Fisher Scientific; Waltham, MA) at 200 rpm and 28°C. One ml of the culture was centrifuged at 8,000 rpm for 2 min (Centrifuge 5430, Eppendorf, Boston, MA) and the supernatant was discarded. The bacterial pellet was resuspended in 0.01 M of phosphate buffer saline (PBS) and adjusted to an optical density of 0.3 at OD600nm (~1×10^8 CFU/ml) using a spectrophotometer (Bio Photometer, Eppendorf, Boston, MA). CO_2-pressured backpack sprayer used for treatment applications was utilized for spray inoculation.

To minimize thrips impact on treatment effect, Tolfenpyrad (Torac; Nichino America, Wilmington, DE) was treated in all plots including non-treated control plots at 2.3 L/ha.

Treatment was initiated after the first appearance of thrips in the field and was applied twice per season. For weed suppression, herbicides oxyfluorfen (Goal 2XL) and pendimethalin (Prowl 3.3 EC) were applied each at 2.3 L/ha within two DAT. Plots were irrigated using sprinkler irrigation and fertilization was performed as per the UGA Cooperative extension guidelines.

Biocontrol agents and copper products

This study evaluated Organic Materials Review Institute (OMRI)-listed copper-based bactericide and BCAs in managing foliar disease severity and bulb rot incidence caused by *Pantoea* spp. (Table 4.1 and 4.2). Treatments included individual applications of cuprous oxide (Nordox 75 WG; 7.5 g/L; 75% metallic Cu equivalent) and BCAs; BsQST (Serenade ASO; 9.5 L/ha) and Bmj (LifeGard WG; 0.33 g/L), or combinations thereof. For the study on alternate treatments, BCA was alternated with copper at 5-, 10- or 15-d intervals, while for the tank mix treatment study, BCA mixed with copper was applied at every 7-d interval. Applications were limited to eight or fewer per season in both experiments except for the conventional check in the alternate treatment study (Tables 4.1 and 4.2).

Field treatments

Alternate treatment of BCAs and copper

Cuprous oxide was treated alternately with either BsQST or BmJ at every 5-, 10- or 15-d intervals. Cuprous oxide or BCAs were also treated individually at every 7-d interval to compare their efficacy against their alternate applications (Table 4.1). In 2021-2022, depending upon the interval of application, a total of six to eight applications per treatment were made starting from February 3, 2022. Cuprous oxide was alternately applied with BCA at every 5- or 10-d interval for a total of eight times as compared to six alternate applications made for 15-d intervals. Similarly, eight applications of BCAs or copper alone were made at every 7-d interval.

Treatments also had a conventional check, where the mixture of chlorothalonil at 1250 g a.i./ha (Bravo Weather Stik; Syngenta, Greensboro, NC) and cuprous oxide (Nordox 75 WG at 7.5 g/L) was alternated with the mixture of penthiopyrad at 350 g a.i./ha (Fontelis; Corteva Agriscience, Wilmington, DE) and Nordox 75 WG (7.5 g/L) at 7-d intervals. Conventional check comprised of chemicals applied by growers for disease management in Vidalia, GA. Challenge-inoculation was performed in all plots on February 28, 2022, including non-treated control plots, after briefly allowing BCAs to colonize the foliage.

In 2022-2023, the treatment initiation date and the overall number of applications differed from the previous season (Table 4.1). Alternate applications of BCAs and cuprous oxide were made a total of three to eight times depending upon the interval of application. Applications were initiated on March 19, 2023, for all treatments. Cuprous oxide was alternated with BCA at every 5- or 10-d interval for a total of eight and four times respectively, while only three alternate applications of BCAs and copper were made at every 15-d interval. Since onions had to be replanted due to frost injury, plant establishment took longer than in 2021-2022 leaving a shorter window for applications, which limited the overall number of applications (Table 4.1). Only six stand-alone applications of either of the BCAs or cuprous oxide were made at 7-d intervals from March 19, 2023, to April 28, 2023. The number of conventional treatments was also limited to six in 2022-2023. Challenge inoculation was done on April 18, 2023. Treatment in both seasons also included an inoculated non-treated control in which no treatment applications were made.

Tank mix treatment of BCAs and copper

Tank mixes of either BsQST or BmJ with copper were evaluated to compare their efficacy against their stand-alone treatment or their alternate applications. Treatments included tank mix application of BCAs and cuprous oxide at 7-d intervals, stand-alone applications of BCAs or cuprous oxide, or the alternate application of BCAs and cuprous oxide at every 7-d interval (Table 4.2). In 2021-2022, foliar application of treatments was initiated on February 3, 2022, in sync with another study in the same season. A total of eight applications were made for each treatment and the challenge inoculation was performed on February 28, 2022. In 2022-2023, all applications were limited to six due to a shorter window for application as explained above (Table 4.2). Treatment application was initiated on March 19, 2023, and all the plots were spray inoculated with pathogens on April 18, 2023. Treatment in both seasons included an inoculated non-treated control but did not include a conventional check.

Disease data collection

Disease severity assessments in onion leaves were initiated ~105 DAT for both experiments in 2021-2022. Between March 22 and April 13, 2022, three assessments were made on 32-40 plants per plot for the experiment on alternate treatment of BCAs and copper and 60-70 plants per plot for the study on tank mix treatment. In 2022-2023, foliar disease assessments for both experiments were done between April 18 and May 2, 2023. A minimum rating was assigned to all the plots in the first rating in 2022-2023 as foliar disease was considerably low during that period (Supplementary Table 4.2 and Supplementary Table 4.4). Severity ratings were performed using the Horsfall-Barratt (H-B) scale as described by Bock et al., 2009, wherein each plant with a specific percent range of infection was assigned a category from 0 to 11 (where 0 to 11 represents 0, 0 to 3, 3 to 6, 6 to 12, 12 to 25, 25 to 50, 50 to 75, 75 to 87, 87 to 94, 94 to 97,

97 to 100, and 100% area diseased, respectively). The H-B scale divides the percent scale into 12 logarithmic-based severity intervals ranging from 0 to 100%. The intervals are symmetrically distributed around 50% based on the assumption that the eye perceives infected tissue below 50% disease and healthy tissue above 50% disease (Horsfall Barratt., 1945; Bock et al., 2010). Each H-B category was converted back to the respective mid-point percentage value for all plants and the mean percent infection per plot was calculated. The mean percent infection obtained from three individual ratings was used to calculate the area under disease progress curve (AUDPC) using the function described by Shaner and Finey, 1976 (Jegera and Viljanen-Rollinson, 2001).

Center rot incidence in onion bulbs was also evaluated in all trials. Bulbs were harvested from the middle two rows (26-42 bulbs/plot) for all trials except the tank mix experiment in 2021-2022 that used 50-70 bulbs/plot. Onions were undercut and field-cured for two days before harvesting. In mid-May of both seasons, bulb tops and roots were clipped and onions were manually harvested. Onion bulbs were then stored for 30 days in cold storage at 2 °C and 70% relative humidity. After storage, the bulbs were cut in half longitudinally from the bulb apex and the total number of bulbs showing symptoms of center rot was recorded. The percent incidence of center rot was calculated as:

$$\text{Bulb rot incidence (\%)} = \frac{\text{number of bulbs infected with center rot}}{\text{total number of bulbs assessed}} \times 100$$

Bacterial pathogens from a subset of symptomatic bulbs were re-isolated on nutrient agar (NA) for detection. The 16S rRNA gene was amplified with primers 27F and 1492R (Frank et al., 2008) and sequenced using Sanger sequencing by Eurofins genomics (Louisville, KY). The isolated pathogen was confirmed as *Pantoea* spp. by subjecting the sequences to a BLAST search against the National Center for Biotechnology Information (NCBI) database.

Data analysis

The foliar disease severity (AUDPC) and percent center rot incidence in the bulb for both experiments were analyzed independently for each season. For tank-mix treatment study, blocks were considered as random effect and treatments as fixed effects, while the alternate treatment experiment that utilized CRD did not account for any random effect. Treatment effects on foliar disease for alternate application experiment were evaluated using a linear model (lm); whereas a linear mixed-effects model (lmer) was fitted for the study on tank-mix treatments. ANOVA was performed using Type III sum of squares on the foliar severity data. When the F value ($P < 0.05$) was significant, pairwise mean comparisons were performed using the estimated marginal means (emmeans) package in R (V. 4.2.0). As for the evaluation of treatment effect on bulb rot incidence, a non-parametric test (Kruskal-Wallis) followed by a Dunn's test was used whenever there was a significant difference among treatments ($P < 0.05$).

Copper sensitivity assay with biocontrol agents

Bacterial strains of products Serenade ASO (*B. subtilis* QST 713) and LifeGard WG (*B. mycoides* isolate J) were tested for their sensitivity to copper at different concentrations. The products were serially diluted in sterile 1X PBS and streaked onto NA medium to obtain a pure culture. A bacterial colony was selected from a 36-h-old culture incubated at 28 °C and transferred to a tube with 5 ml nutrient broth (Difco). The broth was shaken overnight at 200 rpm on a rotary shaker (MaxQ 4450, Thermo Scientific, Waltham, MA) at 28°C. One ml bacterial suspension was transferred to a 1.7 ml tube and centrifuged (Centrifuge 5430, Eppendorf, Boston, MA) at 10,000 rpm for 1 min. Subsequently, the supernatant was discarded, and the pellets were resuspended in sterile 1X PBS. The bacterial concentration was adjusted to an optical density (OD) value of 0.3 at 600 nm (~10^8 CFU/ml), using a Biophotometer (Eppendorf,

Boston, MA). Casitone yeast extract glycerol medium (CYE-G) (Zevenhuizen et al., 1979; Tho et al., 2019) modified by excluding glycerol, was amended with filter-sterilized copper sulfate pentahydrate ($CuSO_4*5H_2O$) stock to achieve concentrations of 50, 100, 250, 500 and 1000 ppm. CYE medium without copper sulfate was used as a positive control. The pH for CYE medium at different concentrations was adjusted to 7-7.5 using filter-sterilized 1 N NaOH. An aliquot of one µl bacterial suspension was dropped onto the CYE media. Each CYE plate had twelve technical replicates per strain and a total of two independent experiments were conducted. Plates were incubated for 30 h at 28 °C before observing the bacterial growth.

Results

Alternate treatment of BCAs and copper

The disease pressure in onion was low in all plots including the non-treated checks in both seasons as depicted by the low mean percentage of foliar infection during each severity rating (Supplementary Table 4.1 and Supplementary Table 4.2). In 2021-2022, the highest mean disease severity was 21.25 % for the plot treated alternately with BsQST and cuprous oxide at 5 d intervals, while in 2022-2023 it was 15.88% for the plot alternately treated with Bmj and cuprous oxide at every 15-d interval. In 2021-2022, there was no significant difference between any of the treatments ($P = 0.6908$) i.e., solo applications of BCAs or cuprous oxide or their alternate applications at different intervals (Figure 4.1). The BmJ treated plot had the lowest AUDPC value (144.6), while BmJ alternated with cuprous oxide at 15-d intervals had the highest AUDPC value (196.5). The center rot incidence in bulbs was not significantly different among any of the treatments ($P = 0.1291$). The percent center rot incidence in bulbs was generally low for all plots and ranged from 0.7% in the Bmj-treated plot to 6.8% in the non-treated control (Table 4.3).

In 2022-2023, treatments had statistical differences ($P = 0.04746$) among each other. Plots treated with cuprous oxide (75.6) or conventional check (62) had significantly lower AUDPC values compared to the non-treated control plots (109.5). However, all other treatments showed a similar level of foliar disease reduction to the non-treated check (Figure 4.1). Alternating BCAs with copper at shorter intervals (5- or 10-d) did not significantly differ from their application at longer intervals (15-d) in managing center rot in foliage. Moreover, the percentage of center rot incidence in bulbs was considerably low (≤ 1.62 %) in this trial as compared to the first season and treatments had a statistically similar effect in reducing bulb rot ($P = 0.4601$).

Tank mix treatment of BCAs and copper

Similar to the experiment on alternate treatment of BCAs and copper, this experiment had considerably low disease pressure in both seasons. In 2021-2022, the mean percent infection was highest for plots treated with Bmj (15.05%), whereas, in 2022-2023, the highest severity was in non-treated plots (18.43 %). Treatments were significantly different in 2021-2022 ($P = 0.002572$). Tank mix of BsQST with cuprous oxide (139.1) or BmJ with cuprous oxide (145.8) showed significantly lower AUDPC as compared to the non-treated control plots (199.1). BmJ mixed with cuprous oxide had a similar level of foliar disease reduction as the plots treated with cuprous oxide (146.5); however, both treatments had significantly lower AUDPC value as compared to the stand-alone BmJ (183.6) treated plots. Tank mix of BsQST and cuprous oxide did not significantly reduce foliar severity as compared to their independent applications (Figure 4.2). In general, the foliar disease severity of plots treated with copper alone was relatively lower as compared to BCAs alone or BCAs alternated with copper at 7-d intervals. Furthermore, treatments were not significantly different from each other in reducing the bulb rot incidence (*P*

= 0.444). The highest center rot incidence in bulbs was observed in non-treated plots (4.6%) and lowest in plots treated with the tank mix of BsQST and copper (0.57%).

Significant differences among treatments were observed in 2022-2023 ($P < 0.001$). Tank mix of cuprous oxide and BsQST (97.6) or the mix of cuprous oxide and Bmj (85.9) had similar AUDPC values as the individual applications of BsQST (102.7), Bmj (103.2) or cuprous oxide (83.8). However, all these treatments significantly reduced foliar severity compared to the non-treated control (139.9) (Figure 4.2). The cuprous oxide mixture with Bmj or its mix with BsQST had a statistically similar effect on AUDPC as the stand-alone application of either of the BCAs or copper. Although not significant, plots treated with the tank mix of cuprous oxide and BCAs (Bmj or BsQST) had relatively lower AUDPC values than cuprous oxide alternated with Bmj (116.6) or cuprous oxide alternated with BsQST (119.1). The bulb rot incidence was not significantly different among any of the treatments in this trial ($P = 0.8371$). Moreover, there was no bulb rot in plots treated with the mix of BmJ and cuprous oxide, cuprous oxide alone and cuprous oxide alternated with BsQST. The highest incidence was only 1.25% in plots alternately treated with cuprous oxide and BmJ (Table 4.4).

Copper sensitivity assay with biocontrol agents

The growth of BsQST and BmJ was visually assessed in CYE media amended with $CuSO_4*5H_2O$ at different copper concentrations. Both *Bacillus* spp. strains were able to grow in CYE media without copper sulfate i.e., non-treated control. BmJ exhibited reduced growth with increasing copper sulfate concentrations; it grew well at 50 ppm, showed weak colony growth at 250 ppm but was unable to grow at 500 ppm or higher. On the other hand, BsQST displayed very weak colony growth at 50 ppm and did not form colonies at concentrations beyond 100 ppm (Supplementary Figure 4.1).

Discussion

Application of copper-based products mixed with the group M ethylenebisdithiocarbamates (EBDCs) fungicides is generally recommended for bacterial disease management in Vidalia onions (Dutta and Gitaitis, 2020; Harrison et al., 2008). Growers' testimonials, alongside several reports, suggest a growing interest in incorporating plant defense inducers and BCAs into spray programs (Pfeufer & Gugino, 2018; Stumpf et al., 2021). Our study focused on the prospect of combining BCAs with copper and underscored the importance of understanding the tank mix compatibility between BCAs and copper used for center rot management.

Throughout the experiment, we observed that the area of leaf infected and the bulb rot incidence for all plots was low, which may underrepresent the epidemic levels of disease severity registered in the Vidalia region. Center rot dynamics in the field is modulated by an interplay of several factors including insect vectors, environmental factors, host characteristics and weeds surrounding the onion fields. Onion thrips (*Thrips tabaci*) and tobacco thrips (*Frankliniella fusca*) are known to transmit *Pantoea* spp. in onions (Dutta et al., 2014). Thrips feeding may also promote disease severity in leaves (Grode et al., 2017) and inadvertently support pathogen transmission from foliage to the bulb. Similarly, several weed species commonly prevalent in onion fields can harbor this pathogen (Gitaitis et al., 2002) potentially serving as an inoculum source. Our application of insecticide to minimize the role of thrips in treatment effect could have had indirect impact in reducing center rot severity in plots. This, combined with the use of herbicide, may have contributed to the overall low center rot prevalence in the field.

Our alternate treatment experiment evaluated the efficacy of copper alternated with Bmj or BsQST at different intervals for center rot management in onion foliage and bulbs. In the first trial, treatments had no significant effect on foliar severity in any of the plots including the non-

treated check. In the repetition trial, the level of foliar disease reduction achieved by cuprous oxide alternated with BCAs (Bmj or BsQST) at 5- or 10-d intervals was comparable to that of their alternate application at 15-d intervals. Frequent applications of cuprous oxide alone (at 7-d intervals) or its alternation with BCAs at shorter intervals (5- or 10-d) had none to only slight reduction in foliar severity as compared to longer alternation intervals of BCAs and copper (15-d). Similarly, in both seasons, the center rot incidence in bulbs was not significantly different among the treatments and several treatment plots even had no bulb rot incidence. Under the condition of low disease development, our results indicated that the interval of alternate treatment of BCAs and copper may potentially be stretched apart, and the number of applications of BCAs and copper may be restricted to four to six per season.

Gent and Schwartz (2005) also observed that an increased number of copper hydroxide applications may not necessarily translate to reduced *Xanthomonas* leaf blight in onions. Protective application in a timely manner targeting the most susceptible plant growth stage (i.e., bulb initiation) was shown to have an equivalent effect to its frequent applications throughout the season (Gent and Schwartz, 2005). Stand-alone applications of BsQST or BmJ were unable to reduce foliar disease as compared to the non-treated control in both trials. BsQST treatment was shown to vary across trials in managing bacterial spot severity in tomatoes (Roberts et al., 2008). In only two of their four trials, BsQST reduced disease severity better than the non-treated check. Similarly, reports also suggest inconsistent effects of BmJ on bacterial disease management including center rot (Belo et al., 2023). Strayer-Scherer et al., 2024 reported that BmJ alone was unable to reduce bacterial spot as compared to the non-treated control in greenhouse studies, although their field study showed variable results over different trials.

Observations from our tank mix treatment study implied that copper amended with BCAs may have better efficacy over BCAs alone in managing center rot, but the effectiveness may not surpass the application of copper alone. We found that the mixture of BsQST and copper had statistically similar foliar disease suppression as their independent applications in both trials. Roberts et al., 2008, showed that copper mixed with BsQST provides some advantage over BsQST formulations alone in managing tomato bacterial spot caused by *Xanthomonas perforans* and *X. euvesicatoria*. This added effect of tank mixing, however, did not outweigh the conventional copper-mancozeb treatment. On the other hand, BmJ mixed with copper did not show consistency in reducing foliar severity. In 2021-2022, Bmj copper mixture showed a significant reduction of foliar disease as compared to BmJ-treated plots; however, the disease reduction was comparable to that of stand-alone copper-treated plots. In the second trial, independent application of BmJ or copper had a similar level of disease control as their application as a mixture. Similar to our findings, Strayer-Scherer et al., 2024, also suggested that treatment with BmJ or Bmj mixed with copper hydroxide in greenhouse-grown tomatoes may not significantly reduce bacterial spot as compared to copper hydroxide alone.

Our *in vitro* copper sensitivity assay also showed that BsQST and BmJ were sensitive to copper sulfate at concentrations above 100 ppm and 250 ppm, respectively. The recommended rate of copper application in the field including cuprous oxide (Nordox 75 WG) is usually higher than 1000 ppm. Results from the lab-based artificial testing conditions should be interpreted with caution as they may underrepresent the status of BCAs during tank mixing. Nevertheless, considering the sensitivity of these BCAs products to copper at these rates, we hypothesize that the enhanced effect of BCAs mixed with copper over BCAs alone may be attributed solely to the activity of copper. Additionally, tank mix treatment of BCAs with copper may not have higher

efficacy as compared to standalone copper treatment. A few extension reports suggest that the activity of the Serenade product (a.i. BsQST) is modulated mostly by the lipopeptides produced in the fermentation broth rather than the BsQST population in foliage (Schilder, A., 2012; Davis, 2022). Therefore, even if the strain is sensitive to copper, the activity of the Serenade ASO, which already contains lipopeptides, should be least affected. However, contrary to our speculation, Abbasi and Weselowski., 2014, found that copper hydroxide mixed with aqueous BsQST may not significantly reduce bacterial spot severity in tomatoes as compared to its mixture with wettable powder form of BsQST (Abbasi and Weselowski, 2014). Moreover, stand-alone BsQST treatment in neither of the forms reduced disease severity better than their respective tankmixes with copper hydroxide. In the case of BmJ product LifeGard, wettable granules are applied rather than the formulation broth. We also speculate that due to copper sensitivity of Bmj, its mixture with copper had similar activity against *Pantoea* spp. as compared to copper alone.

Biocontrol agents pose as a potential alternative to synthetic chemicals for disease management in crops and surging farmers' interest in their use warrants further inquiry into their efficacy under different conditions. Incorporating BCAs with copper into spray programs directly relates to added cost and time allotted for disease management. Farmers need to be apprised of the associated costs and more importantly the effectiveness of integrating these components into their spray schedule for disease management. Our multi-year trial showed that BCAs alone may not provide an alternative solution to copper-based products for center rot management in onion. Treatment with copper or its tank mix with BCAs at optimum intervals may still have some effect as compared to their sporadic applications or lack thereof. These treatments may even yield better results if onion growth stages most susceptible to bulb infection

are targeted i.e., first leaf senescence or bulb initiation and swelling (Stumpf et al., 2017). This study also highlights the importance of testing different types and forms of BCAs and their compatibility with copper at different concentrations for center rot management. Alternative plant defense activators such as acibenzolar-*S*-methyl that may not be affected by copper or other non-synthetic compounds could be evaluated in the future for their synergistic effect in center rot management.

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Table 4.1. Description of the sequence and interval of *Bacillus* spp. and copper treatment for the experiment on alternate treatment of BCAs and copper.

Trt No.	Product and rate of application	Application sequence[e]	Alternate application interval (days)[f]
1	*B. subtilis*[a] /	1, 3, 5, 7	5
	Cuprous oxide	2, 4, (6), 8	5
2	*B. mycoides*[b] /	1, 3, 5, 7	5
	Cuprous oxide	2, 4, (6), 8	5
3	*B. subtilis* /	1, 3, 5, 7	10
	Cuprous oxide[c]	2, (4), 6, 8	10
4	*B. mycoides* /	1, 3, 5, 7	10
	Cuprous oxide	2, (4), 6, 8	10
5	*B. subtilis* /	1, (3), 5,	15

	Cuprous oxide	2, 4, 6	15
6	*B. mycoides* /	1, (3), 5,	15
	Cuprous oxide	2, 4, 6	15
7	*B. subtilis*	1–8 (6)	Solo/7
8	*B. mycoides*	1–8 (6)	Solo/7
9	Cuprous oxide	1–8 (6)	Solo/7
10	Conventional chemicals[d]	1–10 (6)	7
11	Non-treated check	-	-

[a] Serenade ASO (Bayer Crop Science, MO) applied at 9.5 L/ha
[b] LifeGard WG (Certis biologicals, MD) applied at 0.33 g/L
[c] Nordox 75 WG (Nordox AS, Oslo, Norway) applied at 7.5 g/L
[d] Treatments included tank mix application of chlorothalonil at 1250 g a.i./ha (Bravo Weather Stik; Syngenta, Greensboro, NC) + Nordox 75 WG (7.5 g/L) alternated with penthiopyrad at 350 g a.i./ha (Fontelis; Corteva Agriscience, Wilmington, DE) + Nordox 75 WG (7.5 g/L)
[e] Numbers outside parenthesis represent applications made in 2021-2022; numbers enclosed by parenthesis denote application limits in the 2022-2023 trial.
[f] Treatments with alternate applications are denoted by "/".

Table 4.2. Description of the sequence and interval of *Bacillus* spp. and copper treatment for the experiment on tank-mix of BCAs and copper.

Trt No.	Product and rate of application	Application sequence	Type of application[e]	Application interval (days)
1	*B. subtilis*[a] + Cuprous oxide	1 – 8 (6)[d]	Tank-mix	7
2	*B. mycoides*[b] + Cuprous oxide	1 – 8 (6)	Tank-mix	7
3	*B. subtilis* / Cuprous oxide[c]	1, 3, 5, 7 2, 4, (6), 8	Alternate	7
4	*B. mycoides* / Cuprous oxide	1, 3, 5, 7 2, 4, (6), 8	Alternate	7
5	*B. subtilis*	1 – 8 (6)	Solo	7
6	*B. mycoides*	1 – 8 (6)	Solo	7
7	Cuprous oxide	1 – 8 (6)	Solo	7
8	Non-treated check	-	-	-

[a] Serenade ASO (Bayer Crop Science, MO) at 9.5 L/ha
[b] LifeGard WG (Certis biologicals, MD) applied at 0.33 g/L
[c] Nordox 75 WG (Nordox AS, Oslo, Norway) applied at 7.5 g/L
[d] Numbers outside parenthesis represent applications made in 2021-2022; numbers enclosed by parenthesis denote application limits in the 2022-2023 trial.
[e] Treatments with alternate applications are denoted by "/" and tank-mix are represented as "+".

Table 4.3. Center rot incidence in onion bulbs for plots treated with *Bacillus* spp. or copper alone or their alternate application at different intervals in field trials conducted during 2021-2022 and 2022-2023.

Treatment[a]	**Center rot incidence in bulbs (%) Mean ± SE**[f]	
	2021/22	**2022/23**
B. subtilis[b] / Cu_15	5.00 ± 1.05	0.00 ± 0.00
B. mycoides[c] / Cu_15	6.63 ± 2.71	0.00 ± 0.00
B. subtilis / Cu[d]_10	2.67 ± 1.91	1.62 ± 0.97
B. mycoides / Cu_10	6.69 ± 2.93	0.78 ± 0.78
B. subtilis / Cu_5	4.91 ± 0.95	0.00 ± 0.00
B. mycoides / Cu_5	4.39 ± 2.71	0.81 ± 0.81
B. subtilis _7	0.76 ± 0.76	0.96 ± 0.96
*B. mycoides*_7	0.69 ± 0.69	0.00 ± 0.00
Cu_7	4.30 ± 1.45	0.00 ± 0.00
Convn[e]_7	0.83 ± 0.83	0.00 ± 0.00
Non-treated	6.79 ± 2.51	0.66 ± 0.66

[a] Treatments with alternate applications are denoted by "/" and numbers after "_" represent the interval in days between alternate applications.
[b] Serenade ASO (Bayer Crop Science, MO) applied at 9.5 L/ha
[c] LifeGard WG (Certis biologicals, MD) applied at 0.33 g/L
[d] Nordox 75 WG (Nordox AS, Oslo, Norway) applied at 7.5 g/L
[e] Conventional treatments included alternate application of chlorothalonil at 1250 g a.i./ha (Bravo Weather Stik; Syngenta, Greensboro, NC) + Nordox 75 WG (7.5 g/L) and penthiopyrad at 350 g a.i./ha (Fontelis; Corteva Agriscience, Wilmington, DE) + Nordox 75 WG (7.5 g/L)
[f] For the respective year, mean percent center rot incidence values were not significantly different among any of the treatments according to the estimated marginal means (emmeans) test in R software at $P<0.05$ significance level.

Table 4.4. Center rot incidence in onion bulb for plots treated with *Bacillus* spp. or copper alone or their tank mix application for field trials conducted during 2021-2022 and 2022-2023.

Treatment[a]	**Center rot incidence in bulbs (%)** **Mean ± SE**[f]	
	2021/22	**2022/23**
B. subtilis[b] + Cu[c]_7	0.57 ± 0.57	0.71 ± 0.71
B. mycoides[d] + Cu_7	1.85 ± 0.118	0.00 ± 0.00
B. subtilis / Cu_7	2.71 ± 1.58	0.00 ± 0.00
B. mycoides / Cu_7	1.95 ± 1.16	1.25 ± 1.25
*B. subtilis*_7	3.76 ± 1.26	0.63 ± 0.63
*B. mycoides*_7	3.78 ± 0.867	0.63 ± 0.63
Cu_7	1.98 ± 0.943	0.00 ± 0.00
Non-treated	4.61 ± 1.88	0.61 ± 0.61

[a] Treatments with alternate applications are denoted by "/" and tank-mix are represented as "+". Numbers after "_" represent the interval in days between treatment applications.
[b] Serenade ASO (Bayer Crop Science, MO) applied at 9.5 L/ha
[c] Nordox 75 WG (Nordox AS, Oslo, Norway) applied at 7.5 g/L
[d] LifeGard WG (Certis biologicals, MD) applied at 0.33 g/L
[f] For the respective year, mean percent center rot incidence values were not significantly different among any of the treatments according to the estimated marginal means (emmeans) test in R software at $P<0.05$ significance level.

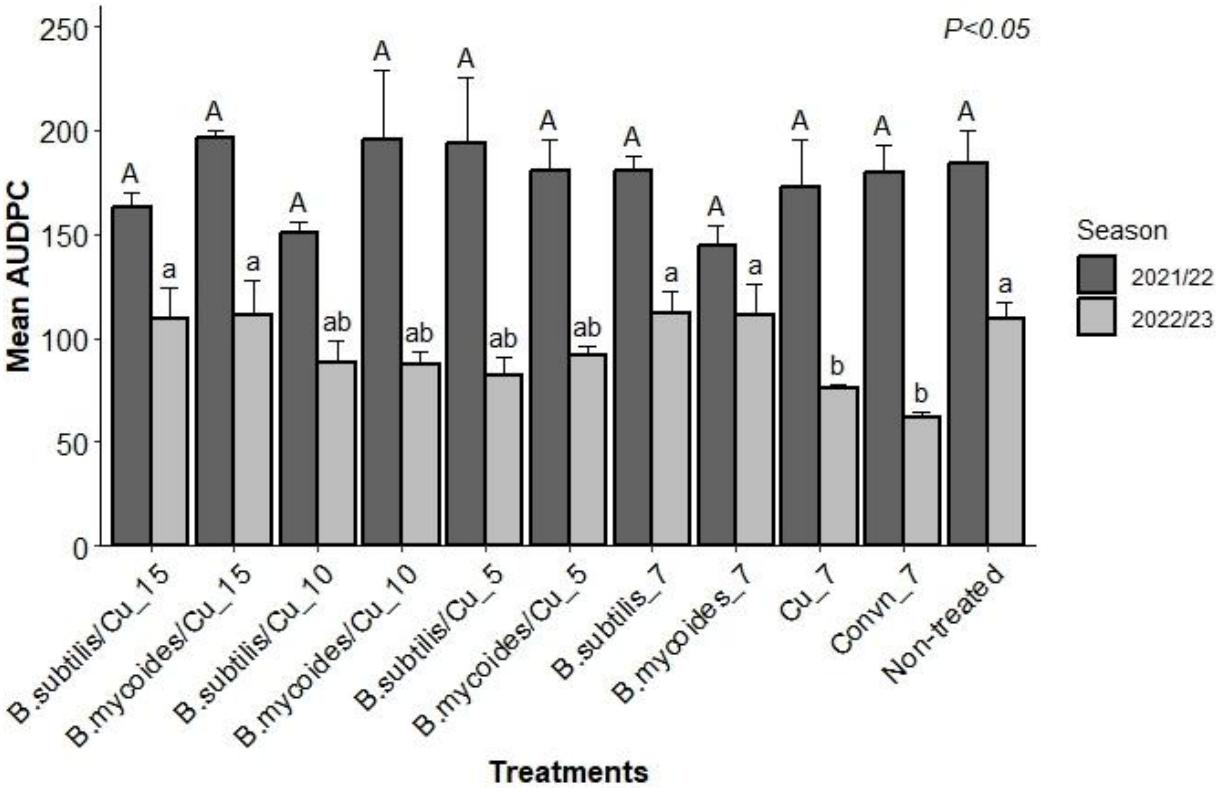


Figure 4.1. Area under disease progress curve of *B. subtilis* QST 713 (Serenade ASO; Bayer Crop Science)*, B. mycoides* isolate J (LifeGard WG; Certis biologicals) and copper (Nordox 75 WG; Nordox AS) treated plots or their alternate application ("/") in seasons 2021/22 and 2022/23. Foliar severity per treatment was recorded three times at an interval of 10 days using the Horsfall-Barratt scale. The AUDPC was calculated using MS Excel as follows: $\Sigma_{i=1}$ [(Y_{i+n1} +Y_i)]/2 [X_{i+1}-X_i] where Y*i* = foliar severity caused by center rot at the *i*th observation, X*i* = time in days at the *i*th observation and n = total number of observations. Bars represent mean AUDPC ± standard error. For each year, mean AUDPC values with the same letter are not significantly different according to the estimated marginal means (emmeans) test in R software at *P*<0.05 significance level.

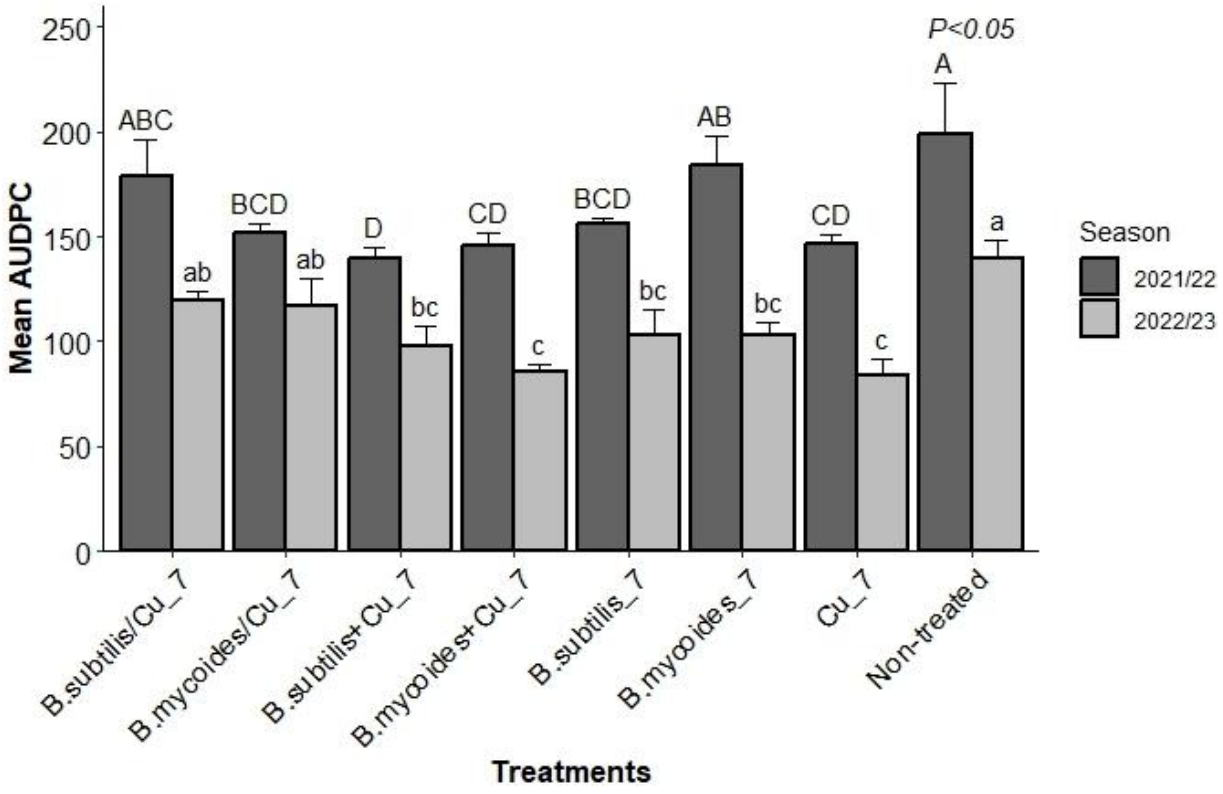


Figure 4.2. Area under disease progress curve of *B. subtilis* QST 713 (Serenade ASO; Bayer Crop Science)*, B. mycoides* isolate J (LifeGard WG; Certis biologicals) and copper (Nordox 75 WG; Nordox AS) treated plots or their tank mix ("+") and alternate application ("/") in seasons 2021/22 and 2022/23. Foliar severity per treatment was recorded three times at an interval of 10 days using the Horsfall-Barratt scale. The AUDPC was calculated using MS Excel as follows: $\Sigma_{i=1}$ $[(Y_{i+n1} + Y_i)]/2$ $[X_{i+1}-X_i]$ where Y*i* = foliar severity caused by center rot at the *i*th observation, X*i* = time in days at the *i*th observation and n = total number of observations. Bars represent mean AUDPC ± standard error. For each year, mean AUDPC values with the same letter are not significantly different according to the estimated marginal means (emmeans) test in R software at $P<0.05$ significance level.

Supplementary Table 4.1. Mean percentage of foliar infection during 2021-2022 trial for alternate treatment of BCAs and copper.

Treatments	Average % of infection		
	22-Mar, 2022	3-Apr, 2022	13-Apr, 2022
Convn	4.5	10.09210526	6.945945946
Convn.1	5.565789474	8.447368421	18.3875
Convn.2	5.513513514	9.364864865	9.041666667
Convn.3	5.484375	7.328125	6.677419355
LG	4.916666667	8.305555556	10.74324324
LG.1	4.776315789	5.407894737	7.297297297
LG.2	5.318181818	5.863636364	7.453125
LG.3	5.272727273	6.272727273	9.03030303
LG-Nor_10	6.7	14.66176471	19.52380952
LG-Nor_10.1	5.863636364	8.181818182	10.63235294
LG-Nor_10.2	5.911764706	8.418918919	12.51219512
LG-Nor_10.3	5.115384615	4.730769231	6.352941176
LG-Nor_15	5.1	11.18571429	8.65625
LG-Nor_15.1	5.921052632	12.26315789	7.573529412
LG-Nor_15.2	5.863636364	10.21212121	8.757575758
LG-Nor_15.3	5.272727273	9.954545455	9.720588235
LG-Nor_5	4.147058824	9.3125	13.57317073
LG-Nor_5.1	4.887096774	9.64516129	8.785714286
LG-Nor_5.2	4.808823529	9.642857143	15.82051282

LG-Nor_5.3	4.897058824	6.264705882	7.308823529
Non-treated	7.272727273	7.272727273	10.33823529
Non-treated.1	7.257575758	12.60606061	10.68333333
Non-treated.2	4.985294118	7.720588235	8.794117647
Non-treated.3	6.016129032	6.741935484	11.64516129
Nor	5	4.875	8.166666667
Nor.1	5.294117647	10.57142857	18.5
Nor.2	6.409090909	9.757575758	8.765625
Nor.3	5.756756757	6.689189189	5.625
Ser	5.885714286	9.428571429	12.95588235
Ser.1	5.914285714	9.042857143	8.324324324
Ser.2	5.882352941	7.676470588	11.78378378
Ser.3	5.25	7.625	9.357142857
Ser-Nor_10	5.9625	7.2	8.602564103
Ser-Nor_10.1	6	7.662162162	7.902777778
Ser-Nor_10.2	5.528571429	6.685714286	9.078947368
Ser-Nor_10.3	4.757142857	5.871428571	7.985294118
Ser-Nor_15	5.647058824	8.514705882	11.17647059
Ser-Nor_15.1	5.166666667	7.625	8.541666667
Ser-Nor_15.2	5.411764706	5.411764706	10.34848485
Ser-Nor_15.3	5.5	8.46875	7.939393939
Ser-Nor_5	4.125	14.375	21.25
Ser-Nor_5.1	5.785714286	5.785714286	8.5

Ser-Nor_5.2	5.818181818	12.33333333	8.075757576
Ser-Nor_5.3	5.628571429	5.2	8.681818182

Supplementary Table 4.2. Mean percentage of foliar infection during 2022-2023 trial for alternate treatment of BCAs and copper.

Treatments	Average % of infection		
	18-Apr, 2023	25-Apr, 2023	2-May, 2023
Convn	1.5	5.357142857	7.2
Convn	1.5	5.027027027	5.416666667
Convn	1.5	4.666666667	7.819444444
Convn	1.5	4.378378378	5.594594595
LG	1.5	7.153846154	8.328947368
LG	1.5	6.875	7.697368421
LG	1.5	13.63513514	12.85135135
LG	1.5	12.69444444	11.5
LG-Nor_10	1.5	6.039473684	8.171052632
LG-Nor_10	1.5	6.486842105	9.945945946
LG-Nor_10	1.5	6.6	7.855263158
LG-Nor_10	1.5	9.131578947	11.43589744
LG-Nor_15	1.5	7.385714286	9.585714286
LG-Nor_15	1.5	8.692307692	9.894736842
LG-Nor_15	1.5	6.882352941	9.283783784

LG-Nor_15	1.5	15.39473684	15.88157895
LG-Nor_5	1.5	6.7625	8.08974359
LG-Nor_5	1.5	8.783783784	11.06756757
LG-Nor_5	1.5	7.5	9.894736842
LG-Nor_5	1.5	6.851351351	9.608108108
Non-treated	1.5	7.756410256	10.24358974
Non-treated	1.5	8.342105263	12.1125
Non-treated	1.5	7.783783784	11.33783784
Non-treated	1.5	12.02631579	13.63157895
Nor	1.5	6.25	7.581081081
Nor	1.5	6.128571429	8.527777778
Nor	1.5	5.605263158	6.868421053
Nor	1.5	6.897058824	7.676470588
Ser	1.5	7.653846154	9.921052632
Ser	1.5	7.675675676	8.815789474
Ser	1.5	10.05405405	13.51351351
Ser	1.5	12.63157895	13.77631579
Ser-Nor_10	1.5	5.067567568	6.986842105
Ser-Nor_10	1.5	6.560606061	8.297297297
Ser-Nor_10	1.5	6.882352941	9.986111111
Ser-Nor_10	1.5	10.31944444	12.31944444
Ser-Nor_15	1.5	6.041666667	7.657894737
Ser-Nor_15	1.5	6.932432432	10.32432432

Ser-Nor_15	1.5	10	13.36111111
Ser-Nor_15	1.5	13.25	15.33333333
Ser-Nor_5	1.5	7.675	10.07894737
Ser-Nor_5	1.5	5.314285714	6.971428571
Ser-Nor_5	1.5	4.542857143	8.351351351
Ser-Nor_5	1.5	9.621621622	8.474358974

Supplementary Table 4.3. Mean percentage of foliar infection during 2021-2022 trial for tank-mix treatment of BCAs and copper.

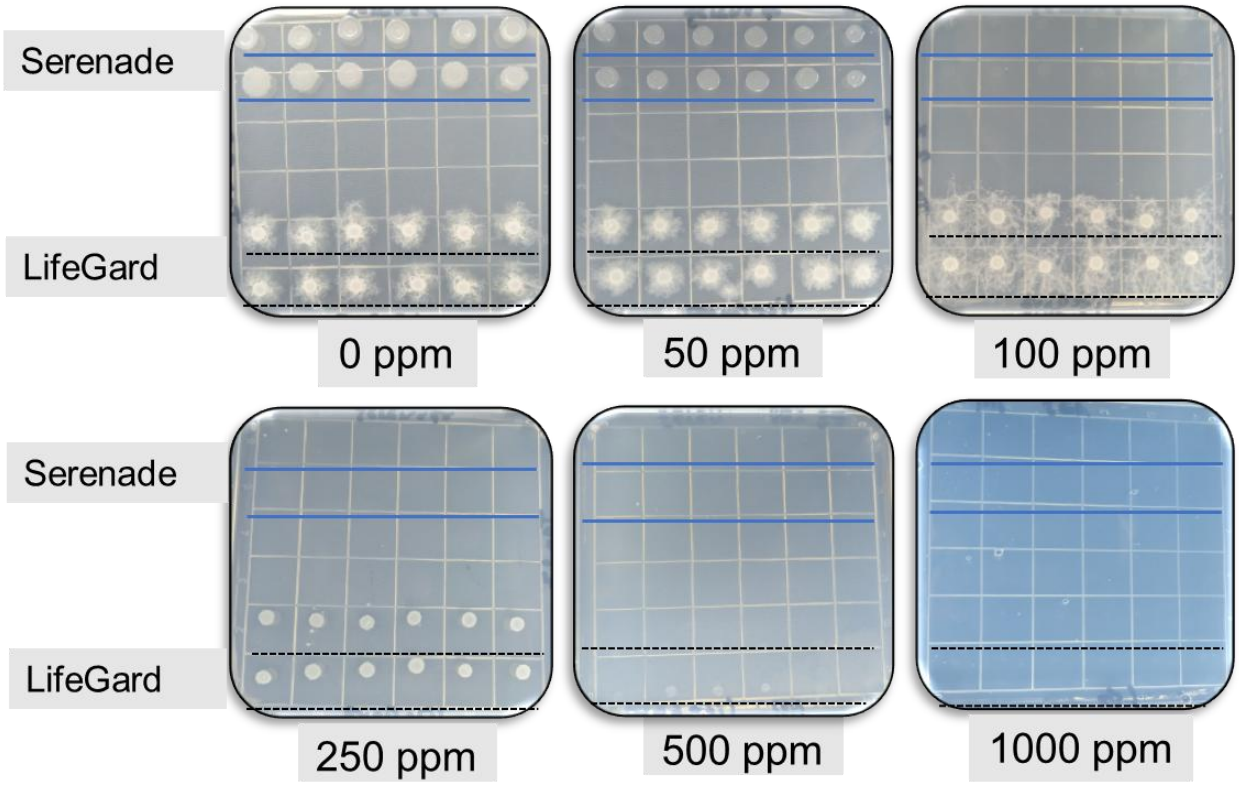
Treatments	Block	Average % of infection		
		22-Mar, 2022	3-Apr, 2022	13-Apr, 2022
LG	108	3.916667	9.606667	10.11039
LG	203	6.112676	10.01449	15.05556
LG	308	4.424658	6.465278	11
LG	408	5.614286	8.385714	10.81618
LG+Nor	101	5.25	6.8	8.033784
LG+Nor	204	5.014286	7.208333	10.28814
LG+Nor	307	4.150685	5.506849	9.886667
LG+Nor	404	4.58	6.304054	8.884615
LG-Nor	106	4.183099	5.956522	10.25
LG-Nor	206	5.442857	6.431507	10.20423
LG-Nor	306	4.3125	7.890411	10.10959

LG-Nor	407	4.507463	6.448529	9.892857
Non-treated	102	5.819444	12.96528	11.15333
Non-treated	202	6.5	14.02778	11.125
Non-treated	305	5.1375	6.3	11.0443
Non-treated	405	5.798611	6.145833	11.25625
Nor	107	4.065789	8.067568	9.240506
Nor	207	5.21831	5.166667	10.15541
Nor	304	4.069444	6.417808	9.340278
Nor	402	4.86	6.268421	9.625
Ser	104	3.986842	7.763889	10.08228
Ser	205	5.506667	5.980263	10.43151
Ser	301	5.333333	6.683099	10.95775
Ser	401	5.387324	6.39726	10.0625
Ser+Nor	208	4.5	6.759494	8.881579
Ser+Nor	303	4.992857	4.979167	8.134328
Ser+Nor	403	4.506757	6.849315	8.756757
Ser-Nor	105	4.461039	6.121622	9.782353
Ser-Nor	201	4.880282	10.5	9.263889
Ser-Nor	302	4.952055	7.208333	10.15079
Ser-Nor	406	4.434783	13.00725	10.31884

Supplementary Table 4.4. Mean percentage of foliar infection during 2022-2023 trial for tank-mix treatment of BCAs and copper.

Treatments	Block	Average % of infection		
		18-Apr, 2023	25-Apr, 2023	2-May, 2023
LG	108	1.5	10.075	12.22951
LG	203	1.5	8.769841	10.98387
LG	308	1.5	6.065217	8.58209
LG	408	1.5	9.313433	11.72794
LG+Nor	101	1.5	7.292308	9.25
LG+Nor	204	1.5	6.90625	9.59375
LG+Nor	307	1.5	6	8.080645
LG+Nor	404	1.5	7.569231	9.730769
LG-Nor	106	1.5	12.95385	14.81343
LG-Nor	206	1.5	8.054688	8.923077
LG-Nor	306	1.5	9.515152	11.0597
LG-Nor	407	1.5	9.523077	12.44615
Non-treated	102	1.5	13.07937	14.63846
Non-treated	202	1.5	15.24219	18.42969
Non-treated	305	1.5	10.88406	11.74242
Non-treated	405	1.5	9.692308	11.32576
Nor	107	1.5	7.569231	8.746032
Nor	207	1.5	5.485075	6.333333
Nor	304	1.5	8.095588	10.375

Nor	402	1.5	6.391304	9.320896
Ser	104	1.5	13.70149	13.09375
Ser	205	1.5	6.46875	9.65873
Ser	301	1.5	7.714286	9.784615
Ser	401	1.5	7.463768	8.169118
Ser+Nor	103	1.5	9.943548	11.36508
Ser+Nor	208	1.5	7.820313	9.650794
Ser+Nor	303	1.5	7.413043	9.402985
Ser+Nor	403	1.5	7.76087	9.297101
Ser-Nor	105	1.5	10.36567	12.6
Ser-Nor	201	1.5	13.03788	13.9697
Ser-Nor	302	1.5	8.407692	11.23016
Ser-Nor	406	1.5	8.960938	10.7377



Supplementary Figure 4.1: Copper sensitivity test for *Bacillus subtilis* QST 713 strain (Serenade ASO) and *Bacillus mycoides* isolate J (LifeGard WG) strains at different concentrations of copper sulfate pentahydrate (CuSO4*5H20). Bacterial isolates were grown in casitone yeast extract media (excluding glycerol) as described by Zevenhuizen et al. (1979) and Tho et al. (2019). Observations were recorded after 30 h of growth at 28 °C.

CHAPTER 5

SURVIVABILITY OF *PANTOEA STEWARTII* SUBSPECIES *INDOLOGENES* IN CROP RESIDUE AND ITS TRANSMISSION RISK TO ONIONS IN POACEAE-ALLIUM CROPPING SYSTEMS

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Abstract

Pantoea stewartii subspecies *indologenes* (*Psi*) isolates can cause disease in several Poaceae hosts, including millets and rice and were recently known to cause foliar and bulb symptoms characteristic of center rot in onions. Cover crops such as millet and cash crops like corn are commonly grown in the summer after onion harvest in Vidalia, Georgia, USA. However, the risk of pathogen transmission to onions in the cropping systems where summer crops precede onion planting is mostly unknown. We evaluated the survivability of *Psi* in corn and pearl millet residues and assessed its ability to colonize onions transplanted into infested soil. Our microplot study showed that millet and corn residues support the transient survival of *Psi*. The presence of the pathogen in the soil also overlapped with the presence of onion transplants. However, despite planting onion seedlings in *Psi*-infested soil, no bacterial colonization was observed in their rhizosphere and foliar surfaces. Moreover, no visible symptoms of center rot were observed in onion foliage and bulbs, indicating a lesser risk of vertical transmission in the Poaceae-*Allium* cropping system. We further investigated genetic determinants for bacterial survival in millet residue and bare soil by creating deletion mutants of the genes responsible for exopolysaccharides, flagellar motility, quorum sensing and pathogenicity in a *Psi* pathovar *cepacicola* strain PNA 14-12. All mutant strains persisted for at least 24 days in millet residue at high population levels and colonies of all the strains remained detectable in bare soil until 44 days. Exopolysaccharide seemed to play a minor role in pathogen survival, but none of the other targeted genes contributed to the bacterial survival in millet residue and bare soil. Overall, our findings suggest that summer crop residues play an important role in the survival of *Psi* in fields under an onion-millet/corn cropping scheme; however, the risk of *Psi* transmission from millet or corn residue to onions appears

minimal. Despite this observation, crop residues should be incorporated into the soil to facilitate decomposition before onion transplanting.

Introduction

Bacterial pathogens are among the major threats to onion (*Allium cepa*) production worldwide (Belo et al., 2023). Center rot, caused by several phytopathogenic *Pantoea* species, is a widely prevalent onion disease in the Southeastern United States. Since its first outbreak reported on Vidalia sweet onions in Georgia, in 1997, the pathogen has been widely reported to cause significant yield losses in onions across the United States including Colorado, New York and Michigan (Gitaitis and Gay, 1997; Carr et al., 2010; Schwartz and Otto 2000; Tho et al., 2015). Disease onset in the field starts from the central foliage, which typically displays symptoms of white streaking and water-soaked lesions (Gitaitis et al., 2003), followed by leaf bleaching and wilting (Schwartz and Otto, 2000; Dutta et al., 2014). Further pathogen progression into bulbs can lead to brownish discoloration of the internal scales and bulb rotting (Gitaitis et al., 2002; Schwartz and Otto, 2000; Carr et al., 2013).

Pantoea ananatis, *P. agglomerans* and *P. allii* are widely known as the causal agents of center rot, although their distribution in onion fields may vary by geographical location. *P. stewartii* subsp. *indologenes* (*Psi*) strains isolated from onions collected from the Toombs County, GA, in 2014, were recently shown to cause center rot following Koch's postulate (Stumpf et al., 2018). Prior to their findings, Psi had not been associated with onion disease; rather, it has long been known as the causative agent of bacterial blight in pearl millet (*Pennisetum glaucum*) and foxtail millet (*Setaria italica*) (Mergaert et al., 1993; De Maayer et al., 2017). Various reports have also associated *Psi* with leafspot and blights of *Oryza sativa*, *Dracaena sanderiana* and *Bougainvillea spectabilis* (Azizi et al., 2019; Zhang et al., 2020; Hu et

al., 2022). In 2021, we characterized seventeen *Psi* strains and proposed two distinct pathovars within *Psi* based on their pathogenicity on different *Allium* and Poaceae species under greenhouse conditions (Koirala et al., 2021). *Psi* strains able to infect *Allium* species, including those causing center rot in onion, were designated as *Psi* pv. *cepacicola*, while its counterpart pathovar, *Psi* pv. *setariae*, included strains unable to cause disease on any of the *Allium* species tested [onion, leek (*Allium porrum*), chive (*Allium schoenoprasum*) and Japanese bunching onion (*Allium fistulosum*)]. All seventeen strains were pathogenic to several members of Poaceae [pearl millet, foxtail millet and oats (*Avena sativa*)] tested in the study (Koirala et al., 2021). Zhao et al. (2023) later confirmed the presence of phosphonate biosynthetic gene clusters in *Psi* pv. *cepacicola* strains, which produce phosphonate toxins responsible for the characteristic lesion development on onion leaves and bulbs (Asselin et al., 2018; Polidore et al., 2021; Zhao et al., 2023). Similarly, the same study implicated the Type-III secretion system (T3SS) in *Psi* as the primary virulence factor for pathogenicity on the foliage of pearl millet.

Onions are typically transplanted in November-December in Georgia, USA, and harvested in the late spring from April–May (Boyhan and Torrance, 2002; Harrison et al., 2008). Onion growers in the Vidalia region of GA commonly practice double cropping, a system referring to the production of two different crops in the same year (Watson 2016). After onion harvest, some growers plant cover crops such as Sunn hemp (*Crotalaria juncea*), cowpea (*Vigna unguiculate*), sorghum (*Sorghum bicolor*) and pearl millet, and cash crops such as corn (*Zea mays*), peanut (*Arachis hypogaea*), cotton (*Gossypium* spp.) and soybean (*Glycine max*) (Pollock-Moore 2014; Cover crops, n.d.). Cover crops offer several benefits such as soil organic matter enrichment, reduced evapotranspiration, weed inhibition and improvement of soil quality and fertility (Clarke 2008; Schwartz 2013). Typically, summer cover crops are planted in late

April-August (Little 2013) and incorporated into the soil by mowing, undercutting or rolling (Rodale institute 2011) at full maturity through deep tillage, while cash crops are harvested and the remaining stubble is later plowed before land preparation for onion transplanting (Boyhan and Torrance, 2002). The crops planted in the summer between onion crops will be henceforth referred to as summer crops.

The susceptibility or tolerance of the crops to pathogens is not the selection criterion for the choice of summer crops by onion growers in Georgia (Watson 2016). Further, knowledge on the role of high-residue systems in disease occurrence is limited for most crop producers in Georgia (Reberg-Horton et al., 2012). Some plant-pathogenic bacteria can survive in infected plant parts, crop residues and soil in amounts sufficient to initiate field outbreaks (Schuster and Coyne, 1974; Silva Junior et al., 2020). Lettuce debris infected with *Xanthomonas campestris* pv. *vitians*, which cause leafspot, survived for a few months in the field, resulting in disease outbreaks in the subsequent lettuce crops (Barak et al., 2001). *Clavibacter michiganensis,* causing bacterial canker in tomato, can survive for up to two years in infested plant debris and serve as a source of primary inoculum on subsequent tomato crops (Gleason et al., 1991; Fatmi and Schaad, 2002). *Erwinia psidii*, closely related to the genus *Pantoea*, survived in buried *Eucalyptus* leaf debris at very low populations for two months (Lanna-Filho et al., 2021) and *P. ananatis* (causal agent of maize spot disease) was reported to survive in the corn residue for at least 60 days (Sauer et al., 2015). However, no clear relationship between *Pantoea* species survival in crop residue and its transmission risk to subsequent crops has not been established, particularly in the double-cropping system in GA, USA. Moreover, little is known about the ability of *Pantoea* to survive in the rhizosphere of onion seedlings that are transplanted in the winter in the Vidalia region, GA. Knowledge of plant residue as a survival niche for pathogens is

pivotal to developing effective disease management strategies. This information can be more crucial for pathogens such as *Psi*, which can infect various crop species planted in the same season (such as millets and onions).

Moreover, understanding the genetic factors that facilitate pathogen persistence in crop residues could be crucial to devise targeted management strategies. Several plant pathogenic bacteria can persist under nutrient-limiting conditions by entering a hypobiotic state, characterized by reduced metabolic activity (Roszak and Colwell 1987; Leben 1981; Martins et al., 2018). Evidence of such a dormant-like state has not been documented in *P. stewartii* and the genetic determinants of pathogen persistence in the residue and bare soil remain poorly understood. While genes associated with stress tolerance or epiphytic survival may play important roles in pathogen persistence in the environment, they have not been characterized in *Psi*. Due to the limited availability of functional genomic data for *Psi*, we prioritized genes whose roles have been experimentally validated in the sister-subspecies *P. stewartii subsp. stewartii* (*Pss*), focusing on those functionally linked to key survival traits in both host and non-host environments. In *Pss*, the stewartan exopolysachharide (EPS), is essential for Stewart's wilt symptom development in corn (Dolph et al., 1998; Beck von and Farrand, 1995; Herrera et al., 2008). EPS also helps in the formation of multicellular community structure that aids bacterial survival in nature (Herrera 2008). While *Psi* does not infect corn (Koirala et al., 2021), EPS could support *Psi* persistence in in the residue and soil environments for extended periods. Flagellar motility is another trait linked to host colonization and biofilm formation (Herrera et al., 2008; Ramsey and Whiteley, 2004; Roper 2011), both of which are relevant for bacterial persistence under environment stress. We also examined T3SS, a known pathogenicity factor in *Psi* on millet, for its role in bacterial survival as a previous study demonstrated that a T3SS-

defective mutant (Δ*hrcC*) reached significantly lower population levels than the wild-type strain of *Psi* (Zhao et al., 2023). Additionally, quorum sensing (QS), a cell-density-dependent regulatory system in bacteria, was examined also for its role in *Psi* survivability under adverse conditions. QS can regulate genes responsible for EPS biosynthesis, motility, biofilm development, surface attachment and host colonization (Koutsoudis et al., 2006; Herrera et al., 2008; Doblas-Ibanez et al., 2019; von Bodman et al., 1998).

This study aimed to elucidate the potential risk of *Psi* transmission from crop residue to the winter-grown onions. We evaluated the survivability of *Psi* in foliage and residue of corn and pearl millet as well as their colonization ability on onion roots and shoots transplanted in the infested soil. We hypothesized that *Psi* can survive in the summer crop residue for an extended period in the soil and the cultural practices such as tilling and weeding and other abiotic factors may facilitate bacterial spread to the rhizosphere and shoots of onions, thus helping the pathogen to colonize onion plants. We also explored the potential role of exopolysaccharide production (*epsG*), pathogenicity (*hrcC*), flagellar motility (*motB*) and quorum sensing (*esaIR*) genes in pathogen fitness by analyzing the survival and growth of the gene-deletion mutant strains of *Psi* strain (PNA 14-12) in pearl millet residue and bare soil.

Materials and Methods

Bacterial strains and growth

Psi pv. *cepacicola* (PNA 14-12 strain) used in this study was originally isolated from an infected onion sample collected in Toombs County, Georgia, USA, in 2014. The bacterial strain was preserved at - 80°C in a 15% glycerol solution and maintained at the University of Georgia (UGA), Tifton. In 2021, PNA 14-12 was proposed and designated as the pathotype-strain of *Allium*-infecting pathovar of *Psi*; pv. *cepacicola* (Koirala et al., 2021). A rifampicin-resistant

derivative generated via spontaneous mutation, designated as PNA 14-12^{Rif}, was used in this study (Zhao et al., 2023). The rif-resistant strain exhibited growth on nutrient agar (NA) comparable to that of the wild-type (WT) isolate and was pathogenic on onion.

For inoculation, bacterial cultures were grown overnight in NA medium amended with rifampicin (30 µg/ml) and cycloheximide (50 µg/ml) at 28°C. A single colony from a 48-h-old NA plate was transferred into a 3-ml nutrient broth supplemented with rifampicin and incubated overnight at 28°C in a rotary shaker (MaxQ 4450, Thermo Fisher Scientific; Waltham, MA) at 200 rpm. One ml of the culture was centrifuged at 10,000 rpm for 2 min (Centrifuge 5430, Eppendorf, Boston, MA) and the supernatant was discarded. The bacterial pellet was resuspended in 0.1 M phosphate buffer saline (PBS) and adjusted to an optical density of 0.3±0.05 at OD600nm (~1 × 10^8 CFU/ml) using a spectrophotometer (Bio Photometer, Eppendorf, Boston, MA). The suspension was diluted with sterile PBS to obtain a final concentration of approximately 1 × 10^5 CFU/ml before inoculation.

Summer crops and growth conditions

Corn (*Zea mays*) and pearl millet (*Pennisetum glaucum*) were planted in plastic containers (80 cm × 44 cm × 33 cm; length × width × depth) filled with 1:1 mixture of field soil from the Blackshank farm at UGA, Tifton and a Sta-green commercial potting mix (Spectrum Brands, Middleton, WI). The soil at BSF is a Tifton loamy sand, a fine-loamy, kaolinitic, thermic Plinthic Kandiudults of the Tifton soil series (Dutta et al., 2017). The selected cultivars, dent corn (cv. DKC 68-69) and pearl millet (cv. TifGrain 102), have no known resistance against *Pantoea stewartii*. Ten-day-old seedlings were transplanted into microplots, each consisting of three rows with four plants per row. The plant-to-plant and row-to-row spacing in each microplot consisted of 18 cm and 14 cm, respectively. Treatments were replicated four times and arranged

in a randomized complete block design (RCBD). In the 2021 trial, crops were transplanted on March 24 and in the 2022-23 and 2023-2024 trials, transplanting was done on July 5, 2022, and June 1, 2023, respectively.

Microplots were established in an open field at the HortHill farm, UGA, Tifton. Periodic fertilization with 15-9-12 NPK (Osmocote; The Scotts Company, Marysville, OH) and overhead irrigation were provided to support optimal plant growth, and the microplots were kept weed-free throughout the study.

Plant inoculations and soil incorporation of summer crops

At full maturity, plants were inoculated by spraying the foliage with 300 ml suspension containing ~10^5 CFU/ml of onion-pathogenic *Psi* strain (PNA 14-12^{Rif}). The pathogen suspension was prepared as described above. The shoots were thoroughly drenched from overhead using a fine mist of inoculum generated by a 32 oz all-purpose plastic spray bottle. The pathogen was allowed to be established on the foliage of the cover crops before crop incorporation into the soil. Approximately 12 days post-inoculation (DPI), foliage and stems were finely chopped with a disinfected pair of scissors and incorporated into the soil at a depth of 10-12 cm. In the 2021 trial, crops were incorporated on May 17, while in 2022-2023 and 2023-2024, plant tissues were incorporated on October 4, 2022, and August 14, 2023, respectively.

Bacterial recovery and enumeration for *Psi* survival study in summer crops

Leaves from summer crops were collected at approximately 10 DPI to assess bacterial survival in corn and pearl millet. Six fully expanded leaves were randomly selected from different plants and clipped using a disinfected pair of scissors. To minimize cross-contamination between treatments, scissors and gloves were disinfected with 70% ethanol between each sample collection. Leaf samples weighing 8 to 10 g were immersed in 30 ml of 0.1 M PBS and placed in

12 cm × 15 cm BIOREBA extraction bags (BIOREBA AG, Reinach, Switzerland). Samples were macerated for 5 mins using a homogenizer (Homex 6, BIOREBA AG). The resulting leaf extract was transferred to 50 ml Falcon tubes, and a ten-fold dilution series was carried out. From each dilution (1 to 10^{-6}), 10 µl suspension was pipetted onto the NA medium amended with rifampicin (30 µg/ml) and cycloheximide (50 µg/ml) and colonies were counted after 24-h of incubation at 28 °C.

In addition to leaf samples, bacterial survival in summer crop residue was assessed by collecting plant-tissue residue buried at a depth of 8-12 cm at various intervals following residue incorporation. Once the residue was no longer visually detectable, soil samples were collected from the same depth as the residue (8-12 cm). At each sampling point, approximately 30-50 g fresh weight of residue and an equal amount of soil were collected after residue depletion. Samples were macerated in BIOREBA extraction bags containing 40 ml of sterile PBS. In the 2021 trial, the residue was sampled four times (May 28, June 10, June 21 and July 13, 2021). In the 2022-2023 trial, sampling was conducted on October 21, November 3, November 20, December 5, 2022, and January 21, 2023. By the final sampling date in January 2023, onions had already been transplanted into microplots. In 2023-2024, residue and soil samples were collected on September 23 and October 28, 2023, with the final sampling conducted on January 5, 2024, following onion transplanting into microplots.

Bacteria were isolated from the samples on NA medium supplemented with rifampicin (30 µg/ml) and cycloheximide (50 µg/ml). Bacterial populations in leaf and residue samples were estimated by counting the colonies on the plates after 24-h incubation at 28 °C. The populations were expressed as colony-forming units per gram (CFU/g) of the sample. The

identity of the recovered colonies on the plate was confirmed as *Psi* using subspecies-specific primers 3614galE/3614galEc (Gehring et al., 2014) listed in Supplementary Table 5.1.

Onion transplanting and assessment of *Psi* survival in the rhizosphere and shoots

Onion seedlings, obtained from a local farm in Vidalia, Georgia, USA, were transplanted into microplots during the first week of December 2022-2023 and 2023-2024 trials. Seedlings were also assessed for the presence of *Psi* before transplanting into microplots. To test for *Psi* contamination in the transplants, twenty plants were arbitrarily selected from the seedling lot and clipped from the neck region. Roots and shoots were submerged with PBS in Ziploc bags separately and shaken for 2 h before plating the suspension in NA medium supplemented with rifampicin. Recovered colonies were amplified with *Psi*-specific primers as described above.

Each microplot had three rows of onion transplants, with four plants per row. The plant-to-plant and row-to-row spacing in each microplot consisted of 18 cm and 14 cm, respectively. Pathogen transmission study on onion rhizosphere and shoots was conducted only in 2022-2023 and 2023-2024 trials. In the 2022-2023 trial, approximately 70-day-old seedlings (cv. Pleothera) were transplanted in the microplot, 65 days after the incorporation of summer crops into the soil. For the 2023-2024 trial, cultivar Century was transplanted 118 days after the incorporation (DAI) of summer crops. Both the onion cultivars (Plethora and Century) are highly susceptible to *Psi*.

Pathogen presence in onion roots and shoots was monitored during both the 2022-2023 and 2023-2024 trials. In the 2022-2023 study, three onion transplants were selected arbitrarily from each plot on January 21, 2023. Roots were separated from shoots by clipping at the neck region and placed into Ziploc bags containing 150 ml of PBS. Bags were placed on beakers and shaken at 200 rpm for 2-h at 28 °C in a rotary shaker (MaxQ 4450, Thermo Fisher Scientific; Waltham, MA). The resulting root and shoot washates were transferred to 50 ml Falcon tubes

separately and centrifuged at 10,000 rpm for 2 mins. A portion of the supernatant was discarded from the top without disturbing the pellet and the remaining 30 ml of suspension containing the pellet was vortexed thoroughly for 1 min. The resulting suspension was then ten-fold serially diluted and plated onto rifampicin-supplemented NA plates. Bacterial colonies recovered on NA plates were amplified with *Psi*-specific primers to confirm their identity (Table 5.1).

In the 2023-2024 trial, shoot and root samples were collected on January 21 and April 2, 2024, and processed following the protocol for bacterial recovery as described above. Additionally, onions were visually assessed for symptoms of center rot throughout the season. Onion bulbs were also assessed for bulb rot incidence when they reached full maturity by cutting the onions in half from the neck region.

Evaluation of the role of bacterial virulence factors on *Psi* survival in crop residue and bare soil

Creation of gene deletion mutants

Gene deletion mutants of *motB*, *epsG*, *esaIR* and *hrcC* genes were generated via allelic exchange in *Psi* PNA 14-12^{Rif} to disrupt flagellar motility, exopolysaccharide secretion, quorum sensing, and pathogenicity, respectively. For each target gene, 450 base-pairs (bp) upstream and 450 bp downstream flanking regions were designed using Geneious Prime v2023.2.1 (Supplementary Table 5.2), incorporating *AvrII* restriction sites and *attB* sequences for Gateway BP recombination. The deletion construct was cloned into the suicide vector pR6KT2G (Stice et al., 2020) using the manufacturer's protocol for BP Clonase II (Invitrogen™ Gateway™ BP Clonase™ II Enzyme mix). The BP reaction mixture was desalted by placing it on an MCE membrane filter (0.025 µm) positioned on the surface of sterile distilled water before

electroporating into *E. coli* MaH1. Gentamicin-resistant *E. coli* MaH1 clones were recovered and verified via PCR and sequencing using pR6KT2G-specific primers (Supplementary Table 5.1).

The confirmed construct was transformed into *E. coli* RHO5 and conjugated into wild-type *Psi* (PNA 14-12^{Rif}) using RHO5 as the donor strain. Merodiploids were selected on gentamicin and screened on X-glucose plates, where blue-green colonies indicated single crossover events. Double crossover was induced by culturing merodiploids on a sucrose-containing medium (1 mL LB + 3 mL 1 M sucrose) at 37ºC for 24-h. A 200 µL aliquot of a 1×10^{-6} dilution was plated on X-Glucose LB agar and yellow colonies, indicative of resolution of the integrated deletion-construct plasmid via a second crossover event, were screened for mutants using flanking "out" primers (Supplementary Table 5.1). For DNA amplification, a 20 µl PCR reaction mixture was used that consisted of 10 µl of GoTaq G2 Green master mix (Promega), 0.5 µl each of forward and reverse primers (10 µM), 1 µl of DNA, and 8 µl of nuclease-free water. The PCR cycling conditions included: initial denaturation at 95°C for 2 min, 34 cycles of 30 s denaturation at 95°C, 45 s of annealing at 58°C and 2 min extension at 72°C, followed by 10 min of final extension at 72°C, before holding at 12°C indefinitely. The resulting PCR products were separated by electrophoresis on 1% agarose gels stained with GelGreen nucleic acid stain in 1× Tris-Borate-EDTA buffer for 1 h at 70 volts (V). The amplicons were observed on a Bio-Rad Gel Doc XR+ imaging system (Bio-Rad, Hercules, CA). PCR-positive clones with target gene deletion (Table 5.1 and Supplementary Figure 5.2) were further confirmed by sequencing. PNA 14-12^{Rif} *hrcC* mutant strain generated by Zhao et al., 2023, was used in this study.

Phenotypic validation of gene-deletion mutants

PNA 14-12^{Rif} mutant strains with respective gene deletions for flagellar motility (Δ*motB)*, exopolysaccharide production (Δ*epsG*), quorum sensing (Δ*esaIR*), or pathogenicity (Δ*hrcC*) were phenotypically validated in a repeated experiment. To confirm defective motility in the Δ*motB* strain, flagellar swimming motility was assessed using a soft agar motility test, as described by Shin et al. (2019). Overnight cultures of PNA 14-12 ^{Rif} Δ*motB* and the WT were adjusted to an OD_{600nm} of 0.5, and 2 µL aliquots were spot-inoculated at the center of soft agar plates (0.25% agar-LB plates). Plates were incubated at 28 °C for 48 h, and motility was evaluated by measuring the radial spread of the strains from the point of inoculation (Supplementary Figure 5.3).

Exopolysaccharide (EPS) production mutant strain was evaluated on AB minimal medium, prepared by combining AB salts (1 g/L NH_4Cl, 0.3 g/L $MgSO_4 \cdot 7H_2O$, 0.15 g/L KCl, 0.01 g/L $CaCl_2 \cdot 2H_2O$, 2.5 mg/L $FeSO_4 \cdot 7H_2O$) and AB buffer (3 g/L K_2HPO_4, 1 g/L NaH_2PO_4, 0.195 g/L MES) supplemented with 1.5% glucose. A 2 µL aliquot of overnight culture, adjusted to OD_{600nm} of 0.3, was spotted onto the AB minimal agar plates and incubated at 28°C for 24-h. Mucoid colony morphology indicated EPS production, whereas non-mucoid morphology suggested impaired EPS synthesis (Supplementary Figure 5.3).

Quorum sensing (QS) activity in Δ*esaIR* strain was evaluated using *Chromobacterium violaceum* CV026 (mutant deficient in acyl-homoserine lactone synthesis) as an acyl-homoserine lactone (AHL) biosensor (McClean et al., 1997; Shin et al., 2019). Overnight culture of CV026 was spread onto LM agar plates (10 g/L tryptone, 6 g/L yeast extract, 1.193 g/L KH_2PO_4, 0.6 g/L NaCl, 0.4 g/L $MgSO_4 \cdot 7H_2O$, 15 g agar) to form a lawn. Wells were prepared on the dried plates using circular ends of the sterile pipette tips. Bacterial cultures, grown overnight, were

centrifuged, filtered (0.22 µm syringe) and the supernatants (100 µL) were loaded into the wells to induce violacein production (Supplementary Figure 5.3).

Test for the survival of *Psi* mutants in pearl millet residue

The survival of *Psi* (PNA 14-12^{Rif}) mutant derivatives for exopolysaccharide production (Δ*epsG*), quorum sensing (Δ*luxIR*), pathogenicity (Δ*hrcc*) and flagellar motility (Δ*motB*) was assessed in pearl millet residue and compared against the survival of the wild-type (WT) strain under greenhouse conditions. Pearl millet (cv. TifGrain 102) was grown in round plastic containers (28 cm × 24 cm, diameter and depth) filled with a 1:1 mixture of field soil and Sta-green potting mixture (Spectrum Brands, Middleton, WI). Five pearl millet seedlings were transplanted into each pot and allowed to grow until full maturity before inoculating with the strains. The seedlings were maintained under greenhouse conditions at 15-25°C and 60-75% relative humidity with a light: dark cycle of 12:12 h. Osmocote smart release plant food (The Scotts Company, Marysville, Ohio) was used for periodic fertilization. At approximately 45 days post-transplanting, plants were spray-inoculated with 300 ml of the bacterial suspension (~10^5 CFU/ml) using a 32 oz hand-held sprayer. To ensure thorough coverage, suspension was applied from both the top and the sides of the plants. plants. All inoculated plants were sprayed with a fine mist of distilled water at every two-day interval until soil incorporation to facilitate pathogen survival in the foliage.

Pearl millet leaves and shoots were chopped and incorporated into the soil at 8-10 cm depth at 9 DPI. Leaf tissues and residues were sampled at 0, 4, 11, 17 and 24 DAI. At each sampling point, 15-20 g of leaf residues were suspended in 20 ml of PBS and macerated in a BIOREBA homogenizer. Bacterial isolation from the residue was carried out using the method

described above. Ten-fold serial dilutions were prepared and 10 µl from each dilution was plated onto the NA medium amended with rifampicin (30 µg/ml) and cycloheximide (50 µg/ml).

Bacterial populations were isolated on NA medium and enumerated as described above. The isolated colonies were further validated using PNA 14-12^{Rif} mutant-specific primers for each mutant strain as well as the WT (Supplementary Table 5.1). Each treatment was done in triplicate and a total of three independent experiments were conducted.

Test for the survival of *Psi* mutants in bare soil

A soil survival study was conducted in the greenhouse using *Psi* (PNA 14-12^{Rif}) WT and mutant strains (Δ*epsG*, Δ*motB*, Δ*esaIR* and Δ*hrcC*) in bare soil to better understand the role of these genes in *Psi* survival. PBS was used as a negative control. Round plastic containers of dimensions 17 cm × 16 cm (diameter × depth) were filled with soil collected from a 2 m × 2 m (length × breadth) area from a site left fallow for the past two years in HortHill farm, Tifton, GA. Soil texture, pH and organic matter content were analyzed at Waters Agricultural Laboratories Inc. (Camilla, GA) prior to the greenhouse trial. Approximately 4 g subsamples were collected from multiple points within a soil pile using a 15 ml Falcon tube. Samples were combined in Zip-loc bags and mixed thoroughly to obtain a composite sample totaling 20 g. Four independent soil samples were submitted for analysis. The soil texture ranged from sandy loam to sandy clay loam, with organic matter content below 0.84 % and the pH values ranging from 5.67 to 5.83.

The soil was drenched with the 400 ml bacterial suspensions (~10^5 CFU/ml) of *Psi* strains using a 32 oz hand-held sprayer. To assess bacterial survival, soil samples were collected just after inoculation and at different intervals between 0 to 44 DPI (i.e., 0, 0.25, 1, 2, 4, 6, 10, 12, 16, 24, 34 and 44). During each sampling point, 5 g of soil was collected from a 2-inch depth using a zig-zag sampling pattern and mixed with 10 ml of sterile PBS. Bacterial isolation,

enumeration and confirmation of the colonies were done using the protocols described for the pearl millet residue study. Briefly, 10 µl from each ten-fold serial dilution was plated onto the NA medium (supplemented with rifampicin and cycloheximide). Colonies were counted from the dilution level that yielded consistent counts between 3-30 colonies. Once the colony numbers dropped below the quantification threshold, only qualitative data were recorded to determine the presence or absence of the strains in the soil. The population counts were $\log_{10}$-transformed and an area under population decline curve (AUPDC) was calculated for each strain using only the sampling points with colony counts above the quantification threshold to minimize quantification errors. The study included two independent experiments, each with three replicates per treatment.

Statistical analysis

Treatments were arranged in a randomized complete block design (RCBD), with blocks treated as a random effect and treatments as a fixed effect. Each treatment was replicated at least three times in all trials. Colony-forming unit (CFU) data from all the bacterial population studies were transformed to $\log_{10}$ (x) before the statistical analysis. The area under population decline curve (AUPDC) was calculated based on the log-transformed data using the formula described by Shaner and Finney (1976):

$$\text{AUPDC} = \Sigma_{i=1} (Y_{i+n1} + Y_i)]/2[X_{i+1} - X_i],$$

where Y_i = bacterial population at the ith observation, X_i =time in days at the ith observation, and n = the total number of observations.

For the pathogen survival study on summer crops that had only two treatments, differences in AUPDC values were analyzed using Student's t-test (P<0.05). The AUPDC values for each strain in different experiments were analyzed independently using Analysis of Variance

(ANOVA). When the ANOVA indicated significant treatment effects ($P<0.05$), pairwise mean comparisons were performed using a linear mixed-effects model (lme). All statistical analyses were performed using R software (Version 4.2.0).

Results

1. Survival of *P. stewartii* subspecies *indologenes pv. cepacicola* (*Psi*) in corn and pearl millet leaves and residue

The survival of *Psi* was evaluated in the leaves of the summer crops (pearl millet and corn) and their residues following soil incorporation during the 2021, 2022-2023 and 2023-2024 trials. Before soil incorporation of foliar tissues, foliage of both hosts was inoculated with *Psi* and their survival was assessed at 10 DPI. *Psi* survived in the corn and pearl millet leaves at 10 DPI in all three trials. In the 2021 trial, bulk foliar sampling showed a bacterial population exceeding 3.2×10^6 CFU/g in both corn and pearl millet leaves (data not shown). During the 2022-2023 trial, corn leaves harbored mean populations slightly higher (3.4×10^5 CFU/g) than the pearl millet leaves (7.07×10^4 CFU/g) (Figure 5.1c). In the 2023-2024 trial, pearl millet leaves had higher populations (1.6×10^5 CFU/g) compared to corn (1.2×10^4 CFU/g) (Figure 5.1e).

After soil incorporation, bacterial survival was monitored on the residues of pearl millet and corn. Bacterial populations recovered from the corn and pearl millet residues at different sampling points varied across the experiments but showed a consistent decline over time. In 2021, the mean *Psi* populations at 11 DAI were 7.7×10^5 CFU/g in corn and 2.1×10^6 CFU/g in pearl millet residue. By 24 DAI, viable colonies were no longer recovered from corn residue, while pearl millet residue still harbored 2.02×10^5 CFU/g of *Psi* (Figure 5.1a). In pearl millet residue, the pathogen persisted at a population level above 3.3×10^3 CFU/g until 58 DAI.

Similarly, in the 2023-2024 trial, a sharp decline in bacterial populations was observed over the 144-day sampling period. *Psi* was not recovered from corn residue beyond 39 DAI, whereas 2.6 × 10^3 CFU/g was detected in the pearl millet residue (Figure 5.1e). By 75 DAI, the pathogen population was drastically reduced in pearl millet residue, but an estimated 5.7 × 10^1 ± 50.1 CFU/g (mean±SE) was still recoverable in NA plates. *Psi* was not recovered from any crop residue at 144 DAI. At this sampling time-point in January 2024, onion seedlings had already been transplanted in the microplot. The AUPDC value for the *Psi* population in pearl millet residue (186.7) were significantly higher as compared to corn residue (29.9) in 2021 at (P<0.05; Figure 5.1b). Similarly, in the 2023-2024 trial, the mean AUPDC was significantly higher in pearl millet leaves and residue (218.1) as compared to corn (76.1) at P<0.05 (Figure 5.1f).
In the 2022-2023 trial, *Psi* exhibited similar survival patterns in pearl millet residue as observed in other trials. The pathogen persisted in pearl millet residue up to 62 DAI at 1.3 × 10^4 CFU/g (Figure 5.1c). Across both pearl millet and corn, *Psi* populations remained above 1.9 × 10^3 CFU/g at four different sampling points between 0 DAI and 62 DAI. Notably, in corn residue, *Psi* survived for a considerably longer period as compared to other trials, with recoverable populations detected as late as 109 DAI. On January 21, 2024, 7.5 × 10^2 CFU/g was recovered on corn residue, at which point onion seedlings had already been transplanted in the microplot. The mean AUPDC for *Psi* in the leaf and residues of pearl millet (374) and corn (378) were not significantly different from each other at P<0.05 (Figure 5.1d).

The average maximum and minimum air temperature was relatively lower in 2022-2023 from the date of soil incorporation to final residue sampling as compared to 2021; maximum and minimum temperatures of 30.9 °C and 20.2 °C, respectively. Similarly, in the 2023-2024 trial, the maximum temperature was 23.8 °C and the minimum temperature was 12.8 °C. The average

rainfall during the same period was 179.6 cm in 2022-2023, which was lower than 473.4 cm and 259.4 cm in 2021 and 2023-2024, respectively (Supplementary Figure 5.1).

2. *Psi* survival test in the onion rhizosphere and shoots

In the 2022-2023 and 2023-2024 trials, onion transplants were planted into microplots containing corn or millet residue previously infested with *Psi*, to evaluate *Psi* transmission to the rhizosphere and shoots of the onions. *Psi* survival in both the rhizosphere and shoots was evaluated at different stages of plant growth. In 2022-23, onion shoots and roots were sampled on January 21, 2023, to evaluate the presence of the pathogen. *Psi* colonies were not recovered from any plant part on the NA medium. Similarly, visual inspections of the plants throughout the onion growing season did not show any center rot symptoms. In the 2023-2024 trial, *Psi* was monitored twice during the onion growing season (January 21 and April 2, 2024). In both sampling points, no pathogen was detected in the shoots or roots of onions. Moreover, similar to the 2022-2023 study, no center rot symptoms were detected in onion plants during this season. Onion bulbs tested after harvest were also found to be free of center-rot symptoms at the end of the season.

3. Survival of *Psi* mutants in pearl millet residue

The role of exopolysaccharide production, quorum sensing, flagellar motility and pathogenicity factor in the *Psi* survival on pearl millet residue was evaluated by comparing the survival of each gene-deletion mutant (i.e., Δ*epsG*, Δ*esaIR*, Δ*motB* and Δ*hrcC*) with the WT strain under greenhouse conditions.

In all three independent greenhouse experiments, the WT and its mutant derivatives maintained mean populations above 1.09×10^6 CFU/g in the pearl millet residue throughout 24-d (Supplementary Figure 5.4). The mutant strains with defective flagellar motility (Δ*motB*) and

exopolysaccharide production (Δ*epsG*) exhibited slightly lower populations in most of the sampling points compared to the WT strain in these experiments, but this difference was less than 10-fold in all sampling timepoints. The highest difference between the *motB* mutant strain (1.19×10^7 CFU/g) and the WT (8.72×10^7 CFU/g) strain was observed at the last sampling point (24 DAI) in the second experiment. Similarly, at the same time point, the Δ*epsG* mutant strain had the lowest population, 1.19×10^7 CFU/g, as compared to the WT (8.72×10^7 CFU/g).

To compare the overall persistence of the WT and mutant derivatives in the residue, Area Under the Population Decline Curve (AUPDC) values were calculated based on the $\log_{10}$-transformed populations over the 24-d sampling period. In the first experiment, only the Δ*epsG* mutant showed a significantly lower mean AUPDC value (184.5) compared to the WT (200.5) at $P<0.05$ (Figure 5.2a). However, in the second and third experiments, no significant differences in AUPDC values were observed among any of the mutant strains and the WT (Figure 5.2b and 5.2c). The AUPDC values in the second experiment varied from 183.7 to 198.3, which was similar to the third experiment, which ranged from 167.6 to 188.4.

4. Survival of *Psi* mutants in bare soil

Under bare soil conditions, PNA 14-12^{Rif} WT and its mutant derivatives survived for at least 44 days. All the strains maintained mean populations above 1.52×10^5 CFU/g throughout 2 DPI, after which the bacterial populations declined rapidly in both experiments (Supplementary Figure 5.5). No *Psi* colonies were detected from the negative control pots. Bacterial populations recovered on NA plates were quantified up to 12 DPI as the colony counts from the original suspension remained in the range of quantification threshold (3 to 30 CFU). When the recovery of the WT and PNA 14-12^{Rif} strain derivatives reduced below the quantification threshold, only

the presence or absence of the colonies for each strain was recorded. All strains, including Δ*epsG* and Δ*motB*, maintained populations above an estimated 100 CFU/g throughout 12-d.

The bacterial population decline was also consistent across strains over the 12-d period. At the last quantifiable sampling point (12 DPI), colony count for PNA 14-12^{Rif} WT was above 6.5×10^2 CFU/g in both experiments, while the population varied between an estimated 100 CFU/g and 9.3×10^2 CFU/g for Δ*esaIR*, Δ*motB*, Δ*epsG* and Δ*hrcC* mutants. Notably, all strains, including the WT and its mutant derivatives, remained viable in bare soil beyond 12 DPI (i.e, 16, 24, 34 and 44 DPI), with recoverable populations detected as late as 44 DPI (data not shown).

For each experiment, AUPDC values for each strain were calculated over the 12-d survival period to quantify cumulative survivability in bare soil and compared among the strains. No significant differences in AUPDC values were observed between the WT and the mutant strains in any of the experiments (P<0.05). The mean AUPDC values for all the strains, including the WT, ranged from 25.9 to 31.3 and were comparable across the experiments (Figure 5.3).

Discussion

Understanding the role of crop residue in the spread of bacterial diseases in onion fields is important to develop effective cultural intervention strategies, including crop rotation practices and residue management techniques. To assess the potential for summer crops to harbor and transmit onion-pathogenic *Psi*, we selected two commonly grown crops in Vidalia, GA (i.e., pearl millet and corn). A two-tiered approach was implemented to evaluate the risk of *Psi* transmission from summer crops to winter-grown onions. First, we assessed *Psi* survivability in the leaves and the decomposing residue of both crops. In the second phase, onion seedlings were transplanted into the soil containing *Psi*-infested residue to examine the ability of bacteria to infect onion

seedlings. Additionally, we explored the role of various genetic factors in *Psi* survival in pearl millet residue and bare soil.

Higher bacterial populations were recovered from the fresh corn and millet leaves prior to soil incorporation than the decomposing residue post-incorporation. In two out of three trials, *Psi* population drastically reduced in corn residue over a 40-d period; however, in 2022-2023, *Psi* persisted for over 100 DAI. Few other reports have suggested short-term survivability of *Pantoea* species in corn residue. *P. stewartii* subspecies *stewartii* (previously referred to as *Phytomonas stewartii*, *Pseudomonas stewartii* and *Erwinia stewartii*) survived for a limited period in infected corn stalks in the winter and posed low transmission risk to the spring-grown corn (Frutchey 1936). *P. ananatis* survived in corn residue for approximately 60 days (Sauer et al., 2015). In pearl millet residue, the pathogen survived consistently across all three trials for over 58 days although it did not survive beyond 75 DAI in any of the trials. The AUPDC values of *Psi* population in pearl millet was significantly higher than corn in two trials suggesting that pearl millet could be the preferential host for *Psi* survival. In fact, *Psi* can infect pearl millet but was unable to show symptoms in corn suggesting that it survives epiphytically in corn (Koirala et al., 2021).

Relatively higher bacterial populations were observed in the residue of both summer crops during the 2022-2023 trial. *Psi* survived in corn residue for the longest period (109 days) and the residues supported higher population in the first two months after incorporation as compared to other trials. Several factors can influence pathogen survival in the soil, including soil type, temperature, moisture content, microbial composition and the rate of host-tissue decomposition (Hattori 1973; Leben 1981; Júnior et al., 2012). In particular, higher temperatures and rainfall events can increase organic matter decomposition, thereby shortening the duration of pathogen survival (Lebens 1974; Schuster and Coyne, 1974; Torres et al., 2009; Junior et al., 2012). For

instance, Lana-Filho et al. (2021) recovered *Erwinia psidii* from the soil as long as the *Eucalyptus* leaf residue was present in the soil. Pathogen was not recovered after 60 days of incorporation at which point plant residue had entirely decomposed. Some *Erwinia* species can persist in the soil until the plant residue is completely decomposed (Vorokevich 1960; Schuster and Coyne, 1974). *Xanthomonas cucurbitae* populations in the debris also reduced with the decomposition of infected plant tissues (Thapa et al., 2020; Sulley et al., 2021). While we did not quantify the rate of tissue decomposition, we noted an overall reduction in the recoverable plant parts over time. Particularly, the recoverable leaf tissues gradually declined, and the leaf residue was barely detectable after 2 months in soil. Soil samples at similar depths as residue were collected after their decomposition. Since average temperature and rainfall were lower between crop incorporation and the final residue sampling in the 2022-2023 trial, we speculate that residue decomposition was also slower, thereby supporting higher *Psi* population.

The rate of residue decomposition can also be influenced by the position of the residue in the soil, as factors such as moisture, temperature and microbial compositions vary with depth (Torres et al., 2009; Sikirou and Wydra, 2004; Vega and Romero, 2016; Lanna-Filho et al., 2021). In this experiment, crop residues were buried, which may have facilitated a higher rate of decomposition. Therefore, future studies should investigate the differences between *Psi* survival in buried residues and those left on the soil surface.

More importantly, *Psi* presence in the soil overlapped with onion transplanting in the 2022-2023 trial. However, despite planting onion seedlings in soil previously infested with the pathogen, bacterial colonization was observed neither on roots, foliage or bulbs. The inability of the pathogen to colonize the roots could be due to the lower level of inoculum present in the soil during that period. We observed only lower than 100 CFU/g of inoculum in the soil with *Psi*-infested corn

residue on January 21 in 2022-2023. Consequently, no visible symptoms of center rot were observed in onions throughout the season. Therefore, our survival study indicated that *Psi* presence in cover crop residue poses minimal inoculum risk to onions if adequate intervals are kept between cover crop incorporation and onion planting. Similar to these observations, Frutchey 1936 did not recover *Pss* from soil inoculated in winter when sampled in the Spring. The authors suggested that regional temperature differences may influence the survival of *Pantoea stewartii* (Frutchey 1936). Stewart's wilt epidemic in summer is rather attributed to *Pss* harboring corn flea beetles (*Chaetocnema pulicaria*) that can overwinter and transmit it from plant to plant (Esker and Nutter, 2003). Insect vectors such as thrips cannot be ruled out from playing a role in the overwintering of *Psi* in Georgia, as thrips can carry *Pantoea* species and cause infections through feeding wounds, similar to the mechanism utilized by corn flea beetles (Dutta et al., 2016; Gitaitis et al., 2003).

Genes associated with exopolysaccharide production (*epsG*), flagellar motility (*motB*), quorum sensing (*esaIR*) and pathogenicity (*hrcC*) were investigated for their roles in *Psi* survival in pearl millet residue and bare soil. Bacterial populations of gene-deletion mutant strains of PNA 14-12^{Rif} were enumerated over 24-d and 12-d periods in pearl millet residue buried in soil and bare soil, respectively, in separate experiments. All strains maintained relatively high populations (above 10^6 CFU/g) in the pearl millet residue across the 24-d period. However, strains with defective flagellar motility (Δ*motB*) and exopolysaccharide production (Δ*epsG*) tended to have slightly lower populations at most sampling points compared to the WT strain. The Δ*motB* and Δ*epsG* mutant populations were lower than WT by 3.61 to 8.73-fold across the experiments. However, the AUPDC analysis showed that only the Δ*epsG* mutant had a significantly lower mean AUPDC value (184.5) compared to the WT (200.5) in one out of three experiments. No significant

differences were observed among any of the strains, including WT, in other trials. Similar trends were observed in the survival of PNA 14-12^{Rif} WT and all the mutant strains in bare soil, but the populations declined more rapidly in bare soil after 2 DPI. Although all strains persisted for at least 44 days, recoverable colonies remained consistent across replicates only through 12 DPI. Differences in the AUPDC values were not statistically significant among strains, including the Δ*epsG* and Δ*motB* strains, which were consistent with the results from the pearl millet residue-survival study.

The different genetic factors evaluated in this experiment are important to bacteria in various ways, ranging from effective host colonization to survival in harsh environmental conditions. However, none of these genes seemed to play a primary role in pathogen survival in bare soil and pear millet residue. Zhao et al. (2023) showed that the population of the *hrcC* mutant strain of *Psi* (PNA 14-12) was significantly reduced compared to the WT in pearl millet leaves, but the Δ*hrcC* mutant strain still maintained populations above 5 logCFU/cm^2 leaf area. The AUPDC calculations in this study were based on the cumulative population of the strain at five different time points, but we found no role of this gene in the *Psi* survival in pearl millet residue.

Exopolysaccharide and flagellar motility play a key role in biofilm development in the sister-subspecies *Pss* (Herrera et al., 2008; Koutsoudis et al., 2006; von Bodman et al., 1998; Papenfort and Bassler, 2016; Doblas-Ibanez et al., 2019). However, in only one out of three experiments, we found a significantly lower population of the Δ*epsG* strain as compared to the WT. We speculate that the role of EPS is more prominent during host colonization when the environment favors higher bacterial multiplication, as more than 10^8 CFU/ml is required for EPS production in *Pss* (Von Bodman 1998; 2003). Moreover, the role of quorum-sensing genes (*esaIR*) was also found to be non-significant. The QS mutant strain used in this study had deletions in both

AHL signal synthase (*esaI*) and cognate gene regulator (*esaR*). Reports indicate that the *esaR* gene acts as a repressor regulating the production of EPS during low cell density or the absence of AHLs (von Bodman et al., 1998; 2003). Disrupting the *esaR* gene leads to unregulated EPS production and such a strain cannot attach properly to surfaces and form amorphous biofilms (Koutsoudis et al., 2006). This could mean that our *esaIR* deletion mutant strains producing EPS constitutively may not be able to regulate EPS production under the necessary conditions. Given the established role of *eanI* (a homolog of *esaI* in *P. ananatis)* in onion pathogenicity (Morohoshi et al., 2007), it is possible that deletion of *esaI* alone in the PNA 14-12 ^{Rif} background could have distinct effects on *P. stewartii* ssp. *indologenes* survival compared to the phenotypes observed in our current experiments. Therefore, further experiments using an *esaI* single deletion mutant (*ΔesaI*) will be necessary to more precisely define the contribution of QS to bacterial survival.

Moreover, genes or their orthologs known to mediate general responses against diverse stress conditions may also play a role in the survival of *Pantoea* species. For instance, universal stress proteins (*uspA*) and their orthologs, identified in *Pss* as well as *P. ananatis* and *P. dispersa*, are regulated under different osmotic and oxidative stress and starvation conditions (Ramachandran et al., 2014; De Maayer et al., 2015; Hossain, 2024). Similarly, the *rpoS* gene responsible for stress regulation in stationary phase cells of Gram-negative bacteria is critical for survival during oxidative stress, osmotic pressure and nutrient-limiting conditions (Dodd and Aldsworth, 2002; Charoenwong et al., 2011; Suh et al., 1999; Stockwell et al., 2009; Santander et al., 2014).

Our *Psi* mutant survival study could not associate any of the tested genetic factors with *Psi* survival in bare soil. However, the notable rapid decline of *Psi* in soil compared to the residue suggested that bare soil is not a primary niche for its long-term survival. Short-term survival of

pathogens in the soil has also been documented in other bacterial species. The *Erwinia amylovora* population in the non-sterile soil reached below the detection limit after 35 days of inoculation (Hildebrand et al., 2001). Similarly, *E. psidii* only survived for 15 days in non-sterile soil (Lanna-Filho et al., 2021). Moreover, *Erwinia carotovora* subsp. *carotovora* survived for about 35-42 days in non-sterile soil (Pérombelon and Hyman, 1989; Armon et al., 1995).

Overall, our experiments demonstrated that *Psi* could survive for at least 3 months in the residue of certain summer-grown crops. Pathogen survival in the leaves, residue and soil, although transient, may influence population dynamics in onion production systems. Since the bacterium was non-detectable in onion plants, the potential for residue of the summer crops to overwinter bacterial populations for the spring-grown onions appears minimal. Nevertheless, deep incorporation of residue into soil, leaving a decomposition window of at least 3 months and finely chopping the vegetation before incorporation may allow for proper decomposition, reducing the risk of pathogen transmission. This study contributes to the existing knowledge of *Psi* survival in onion fields and its implications in disease epidemiology on onions. The inability of *Psi* to transmit to onions from the summer crop residue and their sharp decline under bare soil conditions, suggest that pathogen survival in such environments is unlikely to contribute to center rot epidemics in the summer in Vidalia, GA. Rather, the occurrence of center rot in this region is likely due to repeated reintroductions of *Pantoea* species into onion fields from multiple sources throughout the growing season. Further research is needed to elucidate additional survival mechanisms in onion fields, which may assist growers in developing effective cultural management strategies. Moreover, the genetic factors tested in this study did not significantly contribute to *Psi* survival. Genes responsible for general stress tolerance and osmoadaptation could be tested in the future for their potential role in *Psi* persistence in residue and bare soil.

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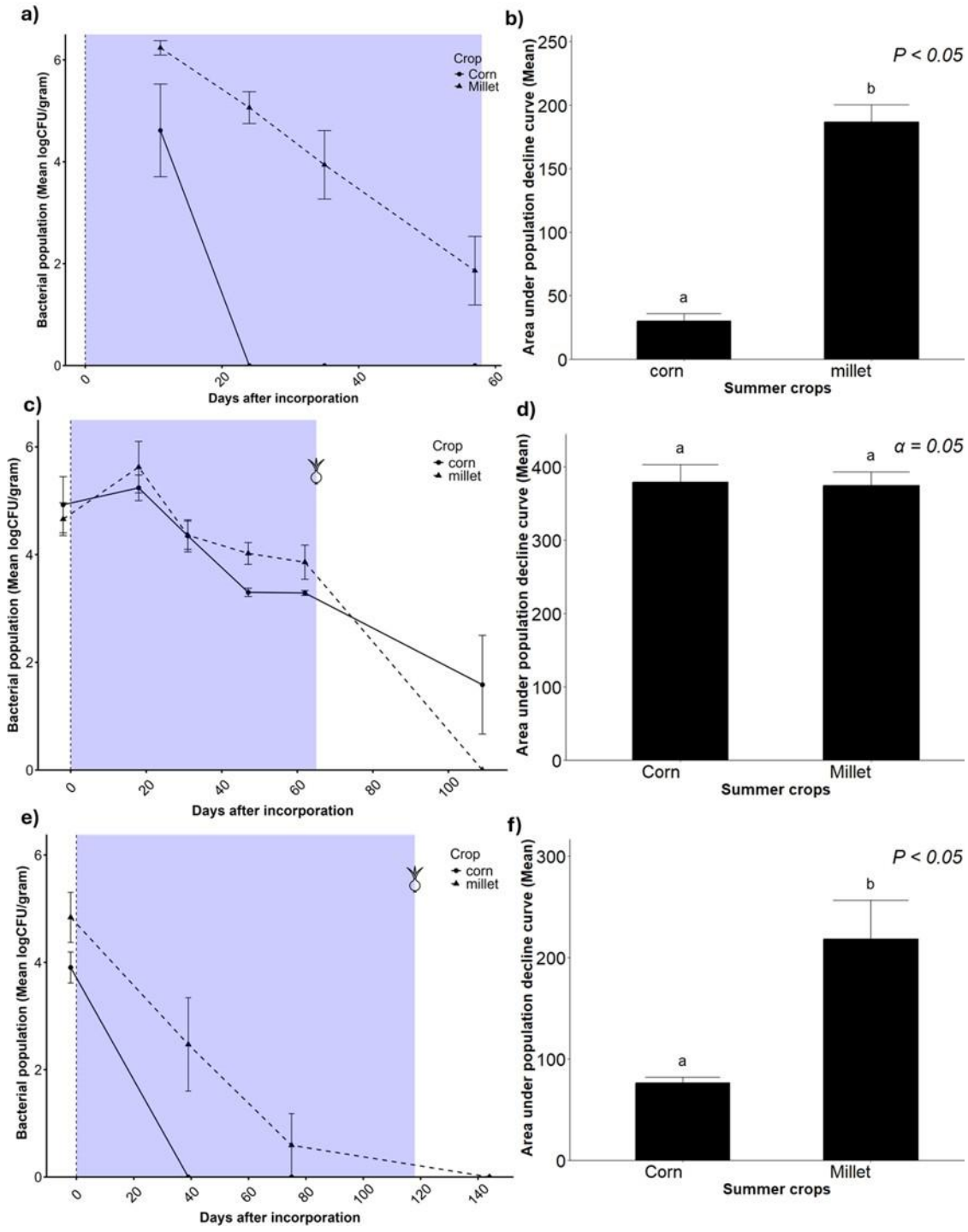


Figure 5.1. *Pantoea stewartii* subsp. *indologenes* (*Psi*) pathovar *cepacicola* (PNA 14-12^{Rif}) survival in corn (cv. DKC 68-69) and pearl millet (cv. TifGrain 102) leaves and residue. The line graph represents the population dynamics of *Psi* (expressed as mean $\log_{10}$CFU/g) surviving in a)

the residue of corn and pearl millet in 2021; c) and e) the leaves (data point left to the dashed vertical line) and the residue (all other data points right to the dashed line) of corn and pearl millet in 2022-2023 and 2023-2024 microplot trials, respectively. The dashed vertical line represents the date of summer crop incorporation into soil and the blue shaded region denotes the interval between crop incorporation and onion transplanting. A vertical bar at each sampling point in the line graph indicates the standard error (±SE) of the mean bacterial populations. Onion icons ( ) in c) and e) indicate the day of onion transplanting after crop incorporation in the microplot. Figure b) represents mean area under population decline curve (AUPDC) of *Psi* populations in corn and pearl millet residue in 2021 trial. Figures d) and f) show mean AUDPC values for *Psi* survival in corn and pearl millet leaves and residue in the 2022-2023 and 2023-2024 trials, respectively. Columns represent mean and the error bars indicate standard error (±SE). AUPDC values with different letters differ significantly according to the Student's t-test ($P<0.05$).

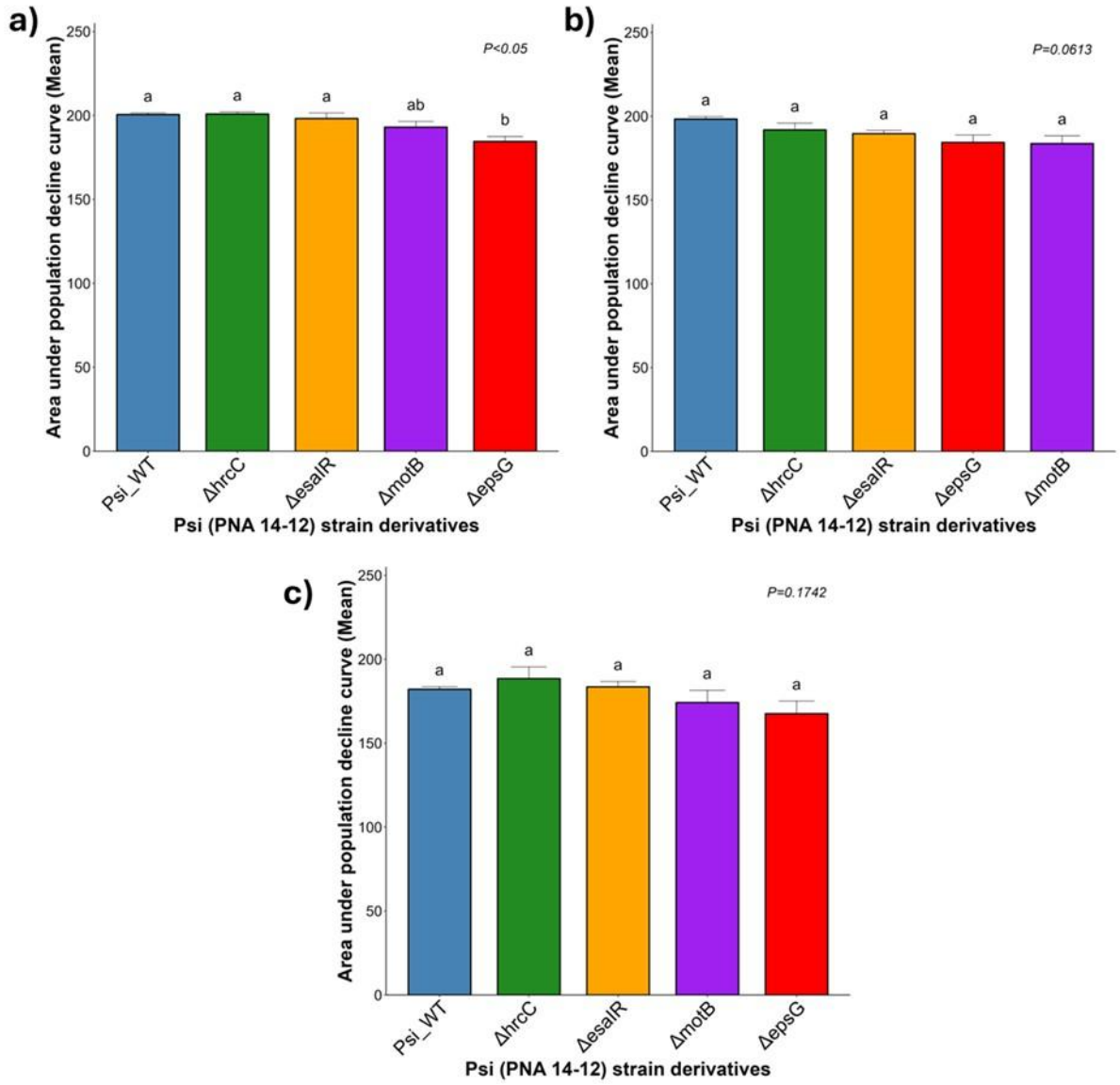


Figure 5.2. Area under population decline curve (AUPDC) of *Pantoea stewartii* subsp. *indologenes* pathovar *cepacicola* (*Psi*) mutant strains in pearl millet (cv. TifGrain 102) residue under greenhouse conditions. Gene deletion mutants of *motB*, *epsG*, and *esaIR* were generated via allelic exchange in *Psi* (PNA 14-12^{Rif}) to disrupt flagellar motility, exopolysaccharide secretion, and quorum sensing, respectively. The panels a) b) and c) show the mean AUPDC in the first, second and third experiments, respectively. The bacterial populations (CFU/g) of individual strain surviving in pearl millet residue, measured over a 24-day period, were log-

transformed before AUPDC calculation. Three replicates per treatment were used in a single experiment and a total of two independent experiments were conducted. Columns represent the mean and the error bars indicate standard error (±SE). AUPDC values with different letters differ significantly according to the linear mixed-effect model (lme) test in R (P<0.05).

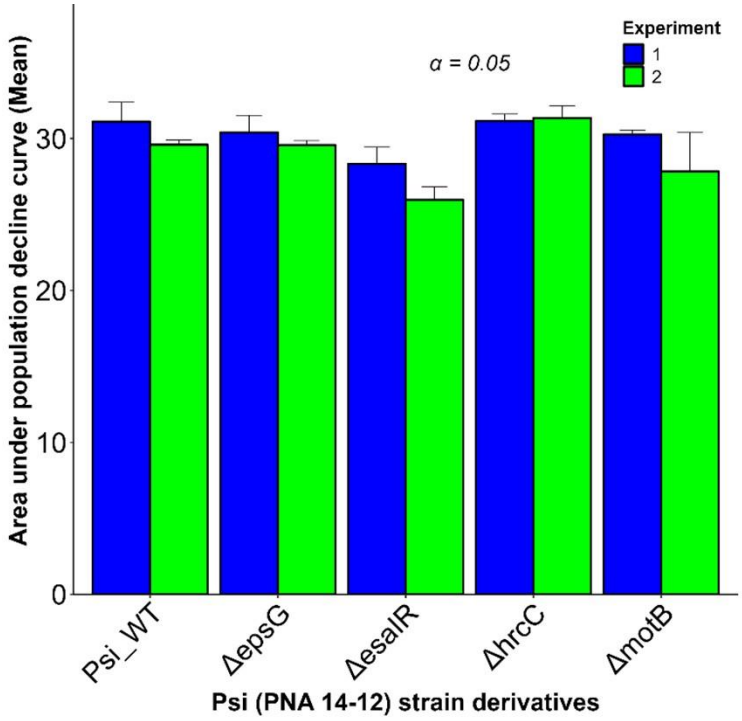


Figure 5.3. Area under population decline curve (AUPDC) of *Pantoea stewartii* subsp. *indologenes* pathovar *cepacicola* (*Psi*) mutant strains under greenhouse conditions. Gene deletion mutants of *motB*, *epsG*, and *esaIR* were generated via allelic exchange in *Psi* (PNA 14-12^{Rif}) to disrupt flagellar motility, exopolysaccharide secretion, and quorum sensing, respectively. Three replicates per treatment were used in a single experiment and a total of two independent experiments were conducted. Columns represent the mean AUPDC and the error bars in each column indicate the standard error (±SE) of the means. Bacterial population counts

(CFU/g) of each strain surviving in the bare soil over the 12-day period were log-transformed before calculating the AUPDC. For each experiment, AUPDC values were not significantly different according to the linear mixed-effect model (lme) test in R software at $P<0.05$.

Supplementary Table 5.1. Oligonucleotide primers used to create and validate knock-out mutants for exopolysaccharide production (Δ*epsG*), flagellar motility (Δ*motB*), pathogenicity (Δ*hrcC*) and quorum sensing (Δ*esaIR*) in *Pantoea stewartii* subsp. *indologenes* pathovar *cepacicola* (PNA 14-12).

Primer name	Sequence (5' ---------- 3')	WT (mutant) gene bp	Function
3614galE 3614galEc	CGACCTGTTTGCCTCTCACC CATCAGCTTGGAGGTGCCG	267	*P. stewartii* ssp. *indologenes* specific (Gehring et al., 2014)
pR6KT2G_F pR6KT2G_R	TATGCAGCGGAAAGTATACC ACAGGCTTATGTCAATTCGA	NA	Confirmation of plasmid insertion of deletion constructs
OutF_esaIR_14-12 OutR_esaIR_14-12	GGTAAGCCGAACACATCCGT CAGAGAACTTAAGCCTGGCAC	2591 (1243)	Quorum-sensing gene mutant (This study)
OutF_motB_1412 OutR_motB_1412	TGCTGAGCGTGCCAAGATTA CCAGCAAGGCATGTTATCGC	2439 (1162)	Motility gene mutant (This study)

OutF_epsG_1412 OutR_epsG_1412	CGAACTGAATTTTGGCGGCA GAGCTGTTAATGCATCGCCG	2370 (1239)	Exopolysaccharide gene mutant (This study)
Pssi-hrcCoutF2 Pssi-hrcCoutR2	CGCCCTCTTCCCTTAACTGA CGTTCTCTGGTATGCAAGGC	3863 (1829)	Zhao et al., 2023

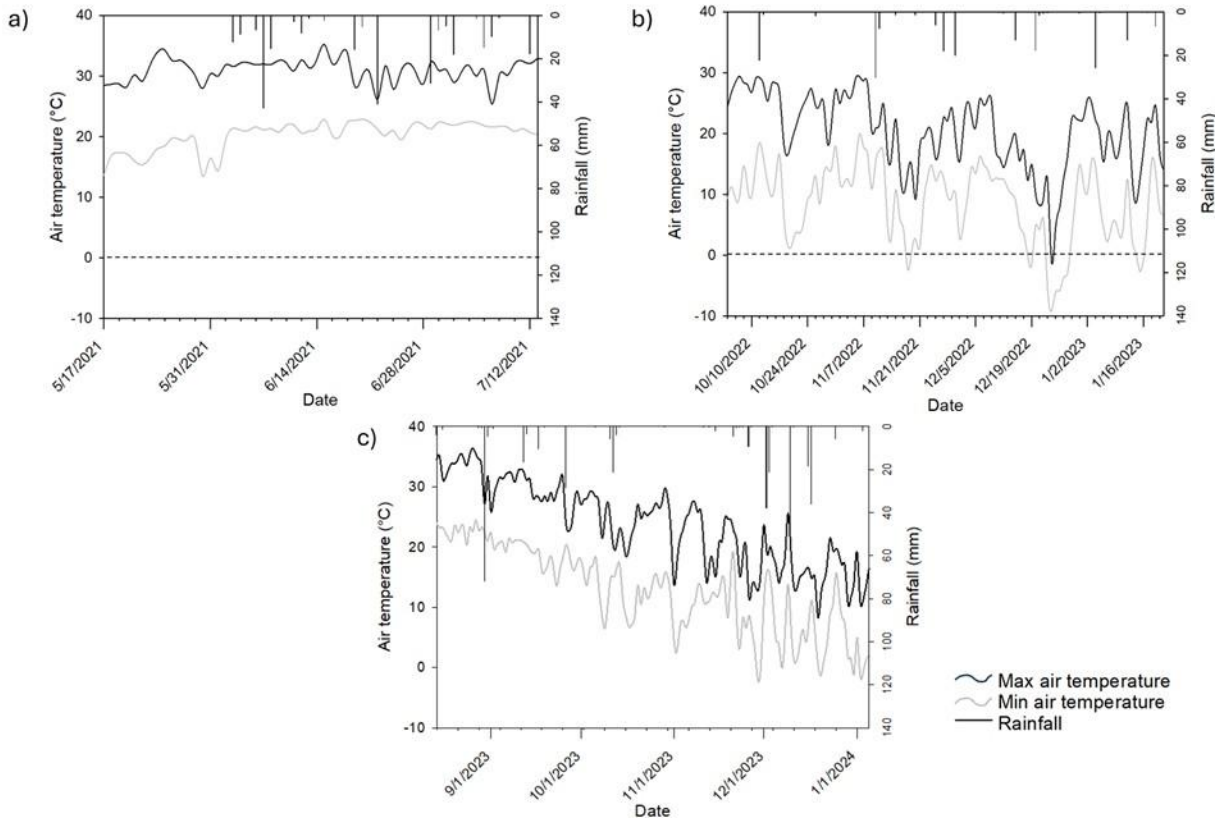
Supplementary Table 5.2. Flanking region-base pairs for synthesizing gene deletion constructs of exopolysaccharide production (Δ*epsG*), flagellar motility (Δ*motB*), and quorum sensing (Δ*esaIR*) in *Pantoea stewartii* subsp. *indologenes* pathovar *cepacicola* (PNA 14-12) using Twist Biosciences.

Name	Purpose	Sequence
esaIR_delconstruct_PNA14-12	Deleting *esaIR* in PNA 14-12	acaagtttgtacaaaaaagcaggctGGCTCAAGTATTAGTAAACTGCGCCAGGT CAACAATTACCGAAATTAACAGCCAAATTATGTTTTTTAGTTTTT TCTGTGGTTTGTGATACAGGACATATTGATTACTCTCTAATATAA AATTTTGCGCGAACCGGGGATGAAAAATAGCGATGTGCATCTGG CATTAATCTGTTAAATGCCCCTTTAGCACAAACCGACTGGGAAA ATATGCATTTTTGAAATAGTATTGTTTAGTTACTATTTGTATTGTG TTCTGCCGCGTTATGACAAATAATATCCCTTCAGGGACAGTTTTA CCGCTTGAGACGCTTTATCCTGCCCATTGACAGACAGAAAACGA CGCCATTCGCTCGAATAATGGTTAATTAACTGAACGTCATAGTGA AAAACTTGTAAAATCAGTGCAGGATAACCGCGAGGGCCGCAGTA ACTTTTAAGAGGAAATGGAcctaggTCAGGCTCCATGCTGCTTCTTT

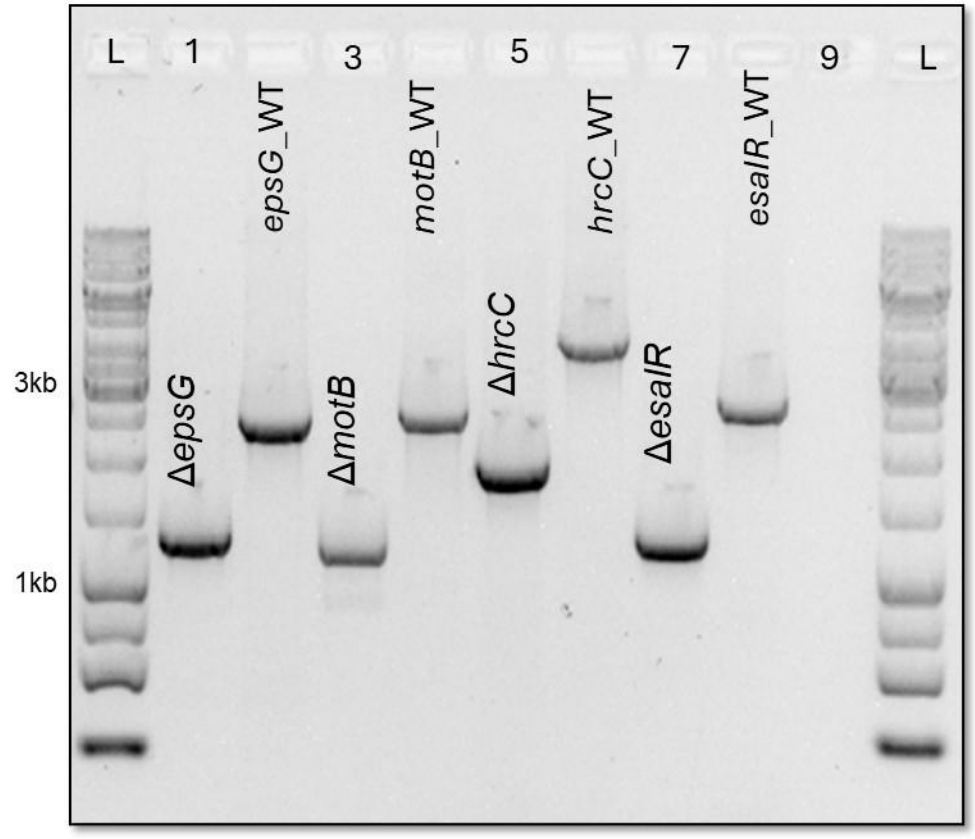
		TACTTAACGTGGACTTAACCTGCACTATAGTACAGGTAAGATGA TACTTAAGAGTAACTTACAATGAATCATTCAGAGGTTACAATGG CTTCAGTTGTTTAGCGGTAAAAAAAAACCTGACCTGTACGGCCA GGTCGCTAAAACAGCCAACACCAGGGAAAACGTCCGGCCAGAC GTCACAAAATATAAAGTGATGCTGGCCGGGACACTCATAGTAAC ATCTTTGAAACATTTGTACAAACATTCACGGTTACTCAATCTGCA GCAATTCTCCCCCTTAATGACTGTTAATACAATCTCTTATTTTCTC CCGTAATAACTGCTAGTTTCTCAGCCACTTAGGCGCTATTCCCCT TTCGTGCACGCCCTCTCTGTTTCCTGTCAGACTGGTCTGCGCCCCT GAATGCGGCCCCGCTGGCCCTATATTCacccagctttcttgtacaaagtggt
epsG_ delcon struct_ PNA1 4-12	Deleti ng *epsG* in PNA 14-12	acaagtttgtacaaaaaagcaggctTGACCTGCAGCAGCTTATCGGCGTGCCAT TCATCGTCGGCATCCAGGAAACTGACATAGTCGCCCGTGGCCAG TTCAATGCCTTTGTTACGCGCACCTGCGCCATTCAGCTTGACCTC TGACAACACCAGGTTGATGTCCAGGTCCTGGTAACGCTCGCTGC GAACCACCTCGGCGAGCGCGGCGGCATCCGCCGATTTGTCATCA ACGATGATGACCTCGAAATGACGATACGTCTGTGCTTTAACGCA ATCCAGCGTCGTGACAATCGATTCTGACGCGTTATAAGCCGGAA TTACGATGGAGAAACGAATGTCCTTCATTGCCAGCACCTCATTAA AATAGTTCAAAAATAAACAATTCAATCGACAAAGGTCGAAGAAA AACCTGTGTTCTGACTGGCAACCGCCCCTGCGTGACGCCTTTCAC GGGAGGACGATCGCCGGGTTGAcctaggGGTGTGATTCCTTAAATC CACAAGTTCACATGCCAACAGTGTCCAGCGCTGATGCCCTTGCA GGGGGCTGTCTCAGCGGGTCTGCGTGAAAGACCAGGGCATGTTG

		ATGATGAATTCTGATGATTGACGTGAGATCGTCGCTTATTGTGCG TTCTATCCAGAATCATCTGTGCAAATCGTTGCGTGTCGTCATTGC AAGCCAGCGATCAAAAGGGGCGGTGAAACCCGCCCCTTCTCACT TAGCGCTTAGCTTTTCGTGGGTTTACCGTATTCGTAGTCGTAGTA GTCGTAGCCGTAACCATAAGAGTTAGCGGCTTTGCGTACCACGG CGTTCAGAATCACCCCTTTAATTTCGATACCGTTCTGGGCAAAGC GTTTGAAGCTGACATCCACTTCTTTCGTGGTGTTAGTTTCGAAAC GCGCCACCATCAGCGACGTACCGGCCAGTTTacccagctttcttgtacaaagtg gt
motB_ delcon struct_ PNA1 4-12	Deleti ng *motB* in PNA 14-12	acaagtttgtacaaaaaagcaggctGACTGACAGGCGTTTCAAGCGCAACCACA ACCGGTGCTTCAACCGGCTGACCTTTCGCCTCTAACGCCAGCTGG CGTAATGCTTCACAGATATACTTGAAGCTTTCCGCGTTAGGCTCT TGCGCGGTTTTATAAGCATCTAGCTGTTCCTGCATAATATCTTTA GTTTCCAAAAACAGATTGATGATGTCGGTGCTGAGCTGCATCTCG CCGCGACGAGCACCATCCAGAATGTTTTCCAGGATGTGGGTTGTT TCCTGTAAAACCGTAAAACCAAAGGTTCCGGCTCCGCCTTTAATC GAGTGGGCGGCGCGGAAGATGGCGTTAAGCTGTTCAGAGTCTGG CTCCTGGGGATCGAGTCCCAACAGGTGTTGCTCCATATCCGCTAA CAGCTCATCGGCTTCATCAAAAAACGTCTGGTAAAAATCGCTGA TGTCCATGCTCACGGGAAcctaggGAGGTCTGATCCGAAGTCTGTTT AGCCGGGTTCTTCGCGTTGCGAACGTGTTCTTCCAGTTCGCTGAA CGATGGACGCTCAGCTGAATAAAGCGTTTTACGACCAAACTCGA CGGCGATCTGCGGTGCATAACCGTTCAGGCTGGAAAGCAATGTC

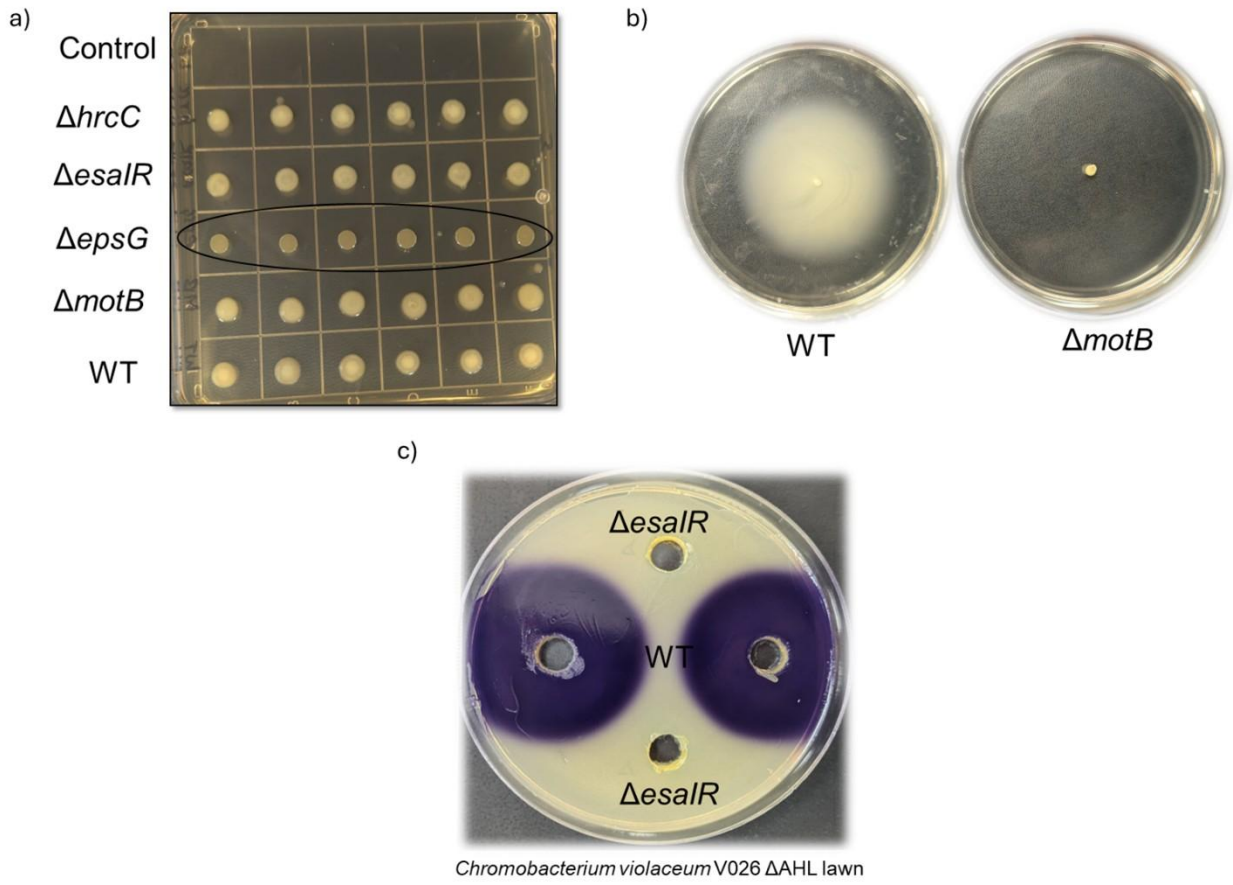
		ACTTTCACGCACTGCATCATCTTGGTCGTTTCAGCACACTTCTGA CGTAATACCGACGCCAGAGGCGAAATAAAACCGTATGCCAGTAA AATCCCGAGGAACGTTCCCACCATGGCATGTGCCACCAGTGCAC CCAGCTCGGCGGCTGGACGGTCAGCTGACGCCAGGGCATGAACC ACGCCCATTACCGCCGCAACGATACCGAAAGCGGGCAGGCCATC GCCGACGGCAGCGATACTTTGTGCCGGCACTTCACACTCGTGTTC ATAGGTTTCAATCTCTTCATCCATCAATacccagctttcttgtacaaagtggt



Supplementary Figure 5.1: Maximum and minimum air temperature and the average rainfall during *Pantoea stewartii* subspecies *indologenes* pv. *cepacicola* survival studies conducted under field conditions in a) 2021, b) 2022-2023 and c) 2023-2024. For each graph, the timeframe on the X-axis ranges from the day of soil incorporation of summer crops to the last residue sampling point, i.e., from 05/17/2021 to 07/13/2021 in 2021; from 10/04/2022 to 01/21/2023 in 2022-2023 and from 08/14/2023 to 01/05/2024 in 2023-2024 study, respectively. Data retrieved from the Georgia Automated Environmental Monitoring Network at the University of Georgia, Tifton, GA.

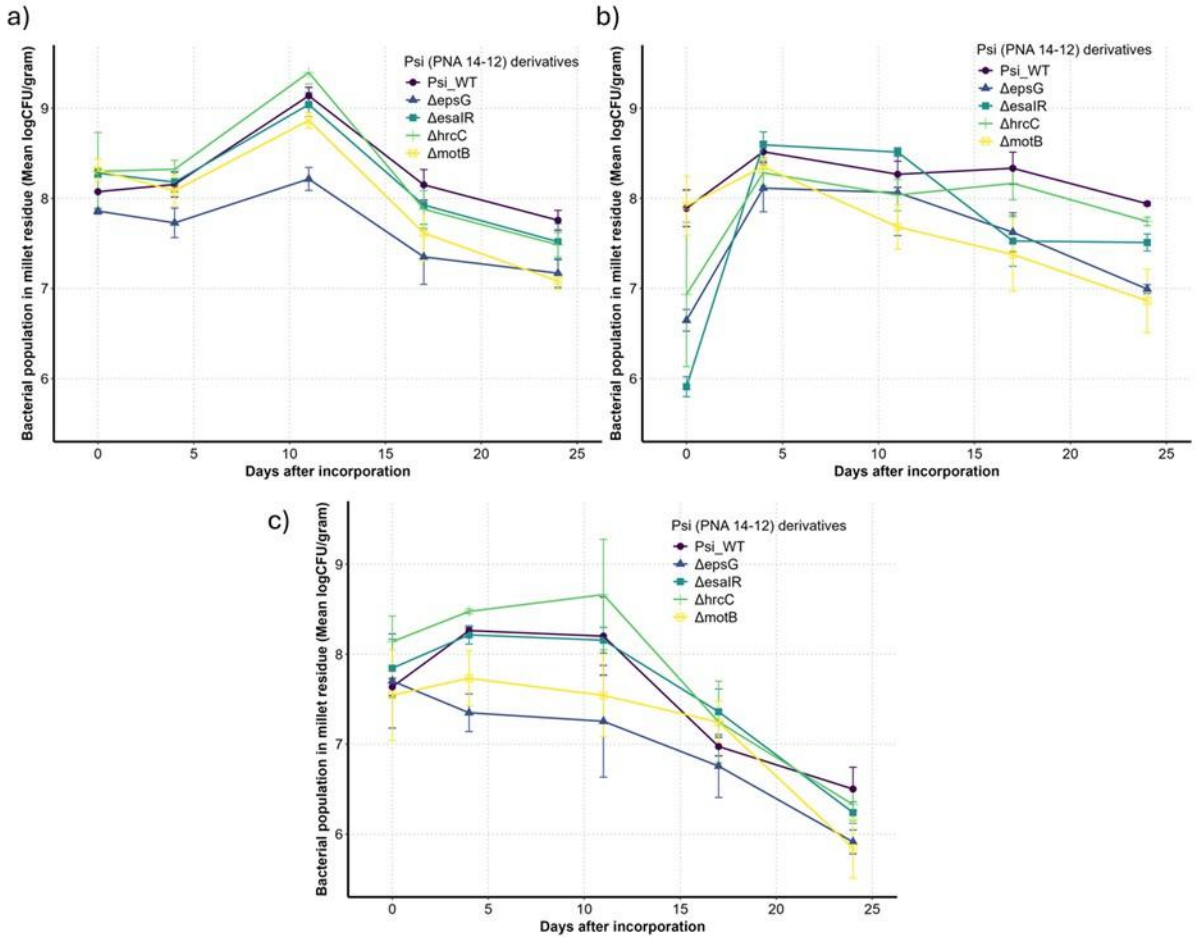


Supplementary Figure 5.2: Agarose gel image as a representative for the PCR verification of knock-out mutations of exopolysaccharide (*epsG*), flagellar motility (*motB*), pathogenicity (*hrcC*) and quorum-sensing (*esaIR*) genes in *Pantoea stewartii* subsp. *indologenes* pathovar *cepacicola* (PNA 14-12 strain). Lane 1, 3, 5 and 7 show DNA fragments of the knock-out strains for Δ*epsG* (1239 bp), Δ*motB* (1162 bp), Δ*hrcC* (1829 bp) and Δ*esaIR* (1243 bp) genes, respectively. The DNA fragments of the PNA 14-12 wild-type strain for *epsG* (2370 bp), *motB* (2439 bp), *hrcC* (3863 bp) and *esaIR* (2591 bp) are shown adjacent to each mutant strain derivative. Genes were deleted using the primer pairs shown in Supplementary Table 5.1. The DNA ladder is represented by L on the first and the last lane of the agarose gel and the corresponding sizes of the bands 1 and 3 kilobases (GeneRuler 1 kb plus DNA ladder; Thermo Fisher Scientific, Vilnius, Lithuania) are indicated on the left.



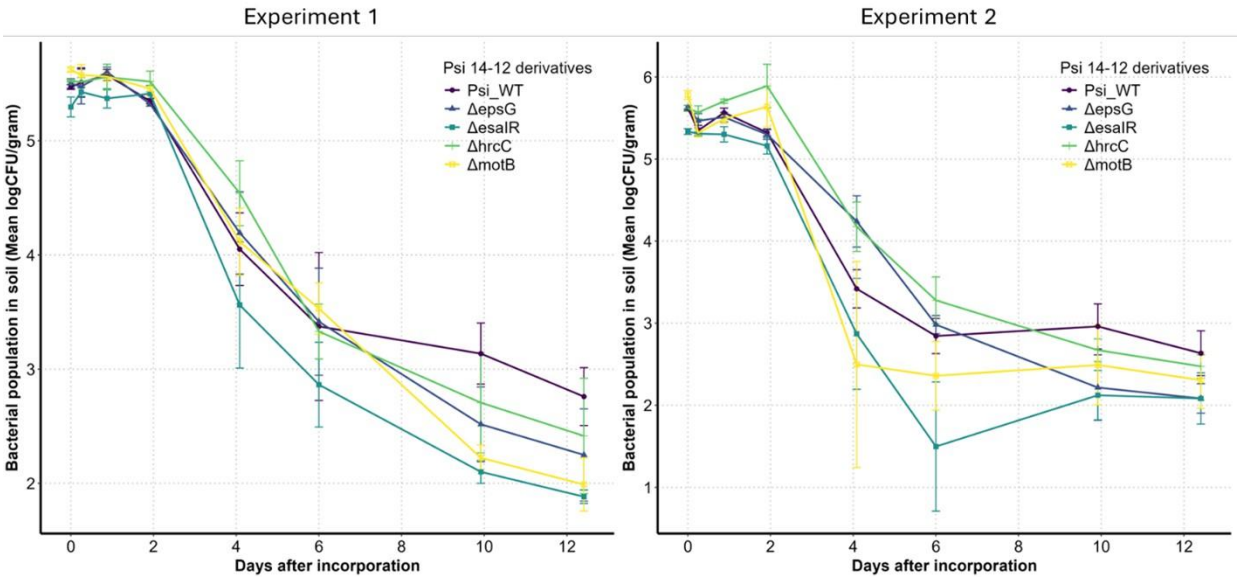
Supplementary Figure 5.3. Phenotypic validation of gene deletion mutants for exopolysaccharide production, flagellar motility and quorum sensing in *Pantoea stewartii* subsp. *indologenes* pathovar *cepacicola*. a) Reduced exopolysaccharide (EPS) production of the gene-deletion mutant strain (Δ*epsG*) on AB minimal agar media after incubation of a 2 µl aliquot for 24 h at 28 °C. The wild-type (WT; PNA 14-12) and the mutant strains for pathogenicity factor (Δ*hrcC*), flagellar motility (Δ*motB*) and quorum sensing (Δ*esaIR*) produced highly mucoid slimy colony morphology as compared to the Δ*epsG* strain, indicating EPS production; b) Impaired motility of the motility gene mutant (Δ*motB*) in 0.25% soft nutrient agar as compared to the WT. A 10 µl aliquot of each strain was spot inoculated at the center of the plate and incubated for 24 h at 28 °C. The WT strain showed extensive radial outward growth as compared to the Δ*motB* strain. c)

Quorum sensing mutant (Δ*esaIR*) defective in producing autoinducers (acyl-homoserine lactones), unable to produce purple pigment (violacein) in *Chromobacterium violaceum* CV026 ΔAHL lawn (a *Chromobacterium violaceum* strain lacking the ability to produce AHLs for violacein production) as compared to the WT strain complementing the CV026 ΔAHL lawn with AHLs enabling it to produce violacein.



Supplementary Figure 5.4. Survival of *Pantoea stewartii* subspecies *indologenes* pv. *cepacicola* (*Psi*) in pearl millet residue under greenhouse conditions. Line graphs in a), b) and c) show the

population (mean log_{10}CFU/gram) of *Psi* (PNA 14-12^{Rif}) in the millet residue during the 24-day period. Error bar at each sampling point indicates standard error (±SE).



Supplementary Figure 5.5. Survival of *Pantoea stewartii* subspecies *indologenes* pv. *cepacicola* (*Psi*) in bare soil under greenhouse conditions. Line graphs in experiments 1 and 2 show the population (mean log_{10}CFU/gram) of *Psi* (PNA 14-12^{Rif}) in the bare soil during the 12-day period. Error bar at each sampling point indicates standard error (±SE).

CHAPTER 6

CONCLUSIONS

Pantoea stewartii subspecies *indologenes* (*Psi*), known to cause disease in several Poaceae hosts, including pearl millet (*Pennisetum glaucum*) (Mergaert et al., 1993), was recently known to cause foliar and bulb symptoms characteristic of center rot in onions (Stumpf et al., 2018). Seventeen *Psi* isolates were tested for their pathogenicity on multiple hosts belonging to the *Allium* or Poaceae family, including onions and millets. All *Psi* isolates were pathogenic to millets; however, only a few caused symptoms in onions characteristic of center rot. Based on their distinctive pathogenicity profiles, we proposed two pathovars within this subspecies; *Psi* pathovar *cepacicola*, which infects *Allium* species [onion (*Allium cepa*), leek (*Allium porrum*), chive (*Allium schoenoprasum*) and Japanese bunching onion (*Allium fistulosum*)], and *Psi* pv. *setariae* that does not infect *Alliums* (Koirala et al., 2021). We also proposed that the Type strain of *P. stewartii* subsp. *indologenes* (LMG 2632^{T}) be designated as the pathotype strain of *Psi* pv. *setariae* and 'PNA 14-12' be designated as the pathotype strain of *Psi* pv. *cepacicola*. Zhao et al., 2023, later identified the involvement of two distinct phosphonate bio-synthetic gene clusters (namely HiVir and halophos), which produced phosphonate toxins, in *Psi* pv. *cepacicola* that enables it to cause hallmark lesions in onion leaves and bulbs (Asselin et al., 2018, Polidore et al., 2021, Zhao et al., 2023). Similarly, the same study implicated the Type-III secretion system (T3SS) in *Psi* as the primary virulence factor for pathogenicity on the foliage of pearl millet. The distribution of these pathovars in onion fields across the onion-producing states in USA and their impact on the center rot pathosystem is yet to be determined.

Numerous modes of survival and transmission of *Pantoea* species, including weeds and thrips and seeds, have already been described (Gitaitis et al., 2002; 2003; Walcott et al., 2002; Dutta et al., 2016), but we identified summer crop residue as another potential inoculum source of *Psi*. Our microplot study that evaluated the survival of *Psi* in pearl millet and corn showed that both corn and millet leaves can support the growth of *Psi* pathovar *cepacicola*. Notably, the presence of the pathogen in soil with the crop residue coincided with onion transplanting. However, despite planting onion seedlings in *Psi*-infested soil, no bacterial colonization was observed on the roots and foliage of onions. Similarly, no visible symptoms of center rot were observed in onion bulbs. Our *Psi* transmission study indicated that the presence of *Psi* in summer crop residue poses minimal inoculum risk to onions. Residue decomposition rate may impact the bacterial survival in soil as diminishing residue supported lower *Psi* inoculum in our study. Adequate intervals should be left between crop incorporation and onion planting, and the crop residues should be finely chopped before incorporation to allow proper decomposition, which may further reduce the risk of pathogen transmission. Incorporated crop residues may degrade more rapidly than the residues left on the surface due to the exposure to diverse soil microorganisms. Since residues were incorporated in our experiment, future studies should investigate the differences between *Psi* survival in buried residues and those left on the soil surface.

We also explored the potential role of exopolysaccharide production (*epsG*), pathogenicity (*hrcC*), flagellar motility (*motB*) and quorum sensing (*esaIR*) genes in *Psi* survivability by analyzing the survival and growth of the specific gene-deletion mutant strains in pearl millet residue and bare soil. Our findings could not implicate any of these genes for their role in contributing to bacterial survival. Genes responsible for general stress-tolerance and

osmoadaptation, such as *uspA* and *rpoS,* could be studied for their potential role in *Psi* persistence in soil.

Findings from our experiment that aimed at improving the ability of biological control agents' (BCAs) colonization suggested that peroxygen did not play a significant role in improving the efficacy of the subsequently applied BCAs. The foliar disease severity of onions in plots pre-treated with peroxygen before BCA was similar to that of plots treated only with BCA. Similarly, peroxygen pre-treated plots had a comparable level of bulb rot incidence to that of peroxygen non-treated plots, irrespective of the BCAs applied (Koirala et al., 2023). Analysis of the microbial population dynamics in *P. fluorescens*-treated plots showed some traces of *P. fluorescens* in peroxygen pre-treated leaves just after its application. However, *P. fluorescens* was drastically reduced and virtually non-existent at the end of the growing season, irrespective of the peroxygen pre-treatment. We noted that *Pantoea* species were predominant in onion leaves throughout the season. These observations suggested that *Pantoea* species, being native residents of onion leaves, can outcompete the growth of introduced BCAs. Future studies should probe into novel BCAs capable of effectively outcompeting *Pantoea* species.

Our study on the prospect of combining BCAs with copper in spray programs could help develop a durable disease management strategy against center rot. Under low disease pressure, similar levels of foliar and bulb disease control were achieved even by extending the rotation interval of BCA and copper treatment from 5 to 15 days (Koirala et al., 2024). This implied that two to three copper applications could be eliminated from the current spray program without compromising disease control. In onion fields with low disease pressure, *Bacillus* species can be alternately treated with copper at 10-14-day intervals to manage center rot. On the other hand, combining copper and BCAs provided slightly better disease suppression than standalone BCA

treatment, but did not outperform standalone copper treatment. The commercially available *Bacillus* species used in our study (*B. subtilis* QST 713 and *B. mycoides* isolate J) were sensitive to copper sulfate pentahydrate ($CuSO_4.5H_20$) at concentrations exceeding 250 ppm, which is below the typical application rates recommended for commercial copper products. This incompatibility between copper and *Bacillus* species likely explains why adding BCAs to copper did not improve the efficacy of standalone copper. Overall, copper alone or its tank mix application with BCAs at optimum intervals provided greater effectiveness than their sporadic applications or lack thereof. Standalone BCA applications, however, may not be as effective as copper. Therefore, copper remains integral for bacterial disease management in onions but mixing it with BCAs requires prior knowledge of their compatibility. Alternative plant defense activators and non-synthetic compounds that may not be affected by copper could be evaluated in the future for their synergistic effect in center rot management in organic and conventional onion production systems.

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