Genome, transmission, and quantification studies of *Xanthomonas translucens*, the pathogen causing bacterial leaf streak in small grains

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Kristi Ellen Ledman

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Dedication

In loving memory of my grandparents Garald and Mary Ellen Ledman and LeRoy and Kathleen Holmes, my beloved dogs Miss Mina and SunnyD, and our family dog Vesper. May you all rest in peace.

This body of work is also dedicated to my living family. To my parents David and Lisa Ledman and my brother Justin Ledman, thank you for all your love and support.

Abstract

Bacterial leaf streak (BLS) of wheat and barley is an economically important disease in most small grain growing regions worldwide and is primarily caused by *Xanthomonas translucens* pv. *undulosa* and *X. translucens* pv. *translucens*, respectively. BLS is generally described as a seedborne disease, however, the pathogen may overwinter in a range of host plants. *X. translucens* pv. *undulosa* has a broad host range and infects several cultivated small grains along with perennial and annual weedy grasses. The advancement of high-throughput sequencing has facilitated a growing database of whole genome sequences of *X. translucens* strains. In this study we sequenced and assembled the complete genomes of five *X. translucens* pv. *undulosa* strains originating from wheat, cultivated wild rice, and three weedy grasses. This study details the first *X. translucens* pv. *undulosa* genomes isolated from non-wheat hosts. We compared these genomes to publicly available strains and found all the *X. translucens* pv. *undulosa* were closely related, sharing ≥99.25% average nucleotide identity, but non-clonal. Genome structures and the presence of Type III secreted effectors varied among strains, but no unique features were identified to differentiate those originating from wheat and non-wheat hosts. Despite being considered a seedborne disease, the records of seed transmission are circumstantial and/or inconsistent. In this study, wheat and barley seed were inoculated with a rifampicin-resistant strain of *X. translucens* pv. *undulosa* and *X. translucens* pv. *translucens*, respectively, with the aim of tracking the

movement of the pathogens from the host's seed into developing plant tissues. Rifampicin-resistant strains were recovered from both wheat and barley at multiple growth stages through maturity. Under mist-irrigated field conditions, pathogen recovery occurred from all sampled tissue from the third leaf to spikes at soft dough, in both hosts. Recovery was reduced under dryland conditions, especially from wheat. Quantitative PCR can be used to detect and quantify *X. translucens* from wheat and barley seed but does not distinguish living cells from dead cells. We tested a protocol for conducting a viability PCR assay, which quantifies viable *X. translucens* cells, and then used the assay to examine pathogen populations on different wheat seed lots. We found that the viable populations of *X. translucens* cells on wheat seed decreased over one year, although the final concentration of viable cells exceeded 1×10^3 CFU/g, the reported threshold needed to cause BLS. The genome, transmission, and quantification studies of *X. translucens* contribute to our understanding of pathogen diversity and BLS epidemiology and may inform the development of management strategies.

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CHAPTER 1: Epidemiology, host resistance, and genomics of the small grain cereals pathogen *Xanthomonas translucens*: New advances and future prospects

Ledman, K. E.[1]*, Osdaghi, E.[2], Curland, R. D.[1], Liu, Z.[3], and Dill-Macky, R.[1]

[1]Department of Plant Pathology, University of Minnesota, St. Paul, MN, USA

[2]Department of Plant Protection, University of Tehran, Karaj, Iran

[3]Department of Plant Pathology, North Dakota State University, Fargo, ND, USA

My contributions to Chapter 1:

- Lead author of the following sections: Abstract, Introduction, Bacteriological Features of the Pathogen, Etiology and Epidemiology, Taxonomic History, Management, and Pathogen Virulence,
- Secondary author on the host resistance section

Co-author contributions to Chapter 1:

- Osdaghi, E. was the lead author on the Economic Impact and Current Distribution and Diagnostics sections
- Liu, Z. was the lead author on the Host Resistance and Prospective Research
- All authors reviewed and edited the entire document

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1.1 Abstract

Bacterial leaf streak (BLS) of barley and wheat is mainly caused by the pathogens *Xanthomonas translucens* pv. *translucens* and *X. translucens* pv. *undulosa*, respectively. BLS is distributed globally and poses a risk to food security and the supply of malting barley. *X. translucens* pv. *cerealis* can infect both wheat and barley but is rarely isolated from these hosts in natural infections. These pathogens have undergone a confusing taxonomic history, and the biology has been poorly understood, making it difficult to develop effective control measures. Recent advancements in the ability and accessibility to sequence bacterial genomes have shed light on phylogenetic relationships between strains and identified genes that may play a role in virulence, such as those that encode Type III effectors. In addition, sources of resistance to BLS have been identified in barley and wheat lines and ongoing efforts are being made to map these genes and evaluate germplasm. While there are still gaps in BLS research, progress has been made in recent years to further understand epidemiology, diagnostics, pathogen virulence, and host resistance.

1.2 Introduction

Epidemics of BLS in the Northern Great Plains region of the United States taking place for more than a decade have brought to light the need for effective management strategies to reduce losses due to BLS (Adhikari et al. 2012; Curland et al. 2018; Kandel et al. 2012). Currently, there are no highly effective chemical control or biocontrol measures available and host resistance is likely to

be the most promising method of mitigation. Gaps in the understanding of the biology of *X. translucens*, the BLS disease cycle, pathogen virulence, and sources of host resistance have hindered the development of effective management strategies and resistant cultivars.

A manual published in 1997, *The Bacterial Diseases of Wheat: Concepts and Methods of Disease Management*, included the first comprehensive overview of BLS and black chaff caused by *X. translucens* (Duveiller et al. 1997). More recently, Sapkota et al. (2020) has published the only other review focused on the pathogens that cause BLS in small grains. Since the last review, BLS research has progressed in the areas of epidemiology, diagnostics, pathogen virulence, and host resistance. Therefore, a new review is warranted.

At the time of the review by Sapkota et al. (2020), complete genome sequences of one *X. translucens* pv. *translucens* and two *X. translucens* pv. *undulosa* strains were publicly available. Within the last two years, more genome sequence resources have been made available from NCBI GenBank for *X. translucens* including strains from diverse geographic locations worldwide. Currently available complete genome sequences with full annotations from strains that cause BLS on wheat and barley include: two from *X. translucens* pv. *cerealis*, 11 from *X. translucens* pv. *translucens*, and 11 from *X. translucens* pv. *undulosa* (Charkhabi et al. 2017; Clavijo et al. 2022; Goettelmann et al. 2022; Heiden et al. 2022; Jaenicke et al. 2016; Peng et al. 2016; Roman-Reyna et al. 2020; Schachterle et al. 2022; Shah et al. 2019, 2021). The increase in genome

resources has expanded our understanding of *X. translucens* diversity, effector content, and aided in the improvement of diagnostic tools.

This review provides a general overview and present status of BLS, focusing primarily on the economically important pathogens *X. translucens* pv. *translucens* and *X. translucens* pv. *undulosa*. We highlight new research and current understanding of BLS epidemiology, diagnostic tools, pathogen virulence, and host resistance. This review also identifies areas where knowledge is limited and provides an outline for future research directions with the goal of reducing the impact of BLS on small grains.

1.3 Bacteriological Features of the Pathogen

Xanthomonas translucens is Gram-negative, non-spore-forming, and motile by a single polar flagellum. The bacterium is rod-shaped with rounded edges and is 0.5 to 0.8 x 1.0 to 2.5 µm in size (Dowson 1939; Jones et al. 1917). *X. translucens* is strictly aerobic and produces acid from glucose under aerobic conditions (Duveiller et al. 1997; Jones et al. 1917). Kovac's oxidase test is negative and production of nitrite from nitrate does not occur (Duveiller et al. 1997; Dye 1962). Starch is not hydrolyzed, and lactose is not used. *X. translucens* produces a hypersensitive response on tobacco (Duveiller et al. 1997). Metabolic activity on several carbon substrates can distinguish *X. translucens* from other *Xanthomonas* spp., including dextrin, maltose, D-psicose, D-trehalose, alaninamide, L-alahyglycine, and L-glutamic acid (Vauterin et al. 1995). When grown on solid media, round, convex, yellow viscous colonies

producing extracellular polysaccharides are formed. The yellow color comes from xanthomonadin pigments which are unique to *Xanthomonas* species, as compared to other yellow colored bacteria which do not contain xanthomonadins (Schaad & Stall 1988; Starr et al. 1977). Favorable temperatures for growth of *X. translucens* are 25 to 27°C, however the pathogen is able to multiply at a wide temperature range between 7 and 40°C (Bamberg 1936; Dye & Lelliott 1974).

1.4 Etiology and Epidemiology

BLS and black chaff, caused by *X. translucens* pathovars, are diseases that affect several host species within the *Poaceae* family. While BLS and black chaff are the most recognized names, synonyms of the disease include: bacterial-blight, bacterial streak, and cereal leaf streak (Jones et al. 1917; Langlois et al. 2017; Smith et al. 1919; Vauterin et al. 1995). Symptoms and signs are similar between hosts, although some variation in disease development and severity may be evident. Factors that can influence symptom progression include environmental conditions, host species, host susceptibility, pathogen virulence, and interactions with other pathogens and microorganisms (Bamberg, 1936; Boosalis, 1952; Wallin, 1946a). Early symptoms appear as small water-soaked lesions primarily on leaf tissue (Figure 1.1a). These initial symptoms are often overlooked in young seedlings (Bamberg 1936; Jones et al. 1917). Water-soaked lesions expand and progress parallel with the leaf veins, becoming chlorotic but with a greasy appearance (Figure 1.1b and c). Noticeable lesions typically begin mid-leaf where the leaf blade naturally bends creating a platform

for dew and rainfall to settle providing a favorable environment for infection (Duveiller 1994b). The disease progresses from the infection point towards the leaf tip and down towards the leaf sheath. Streaked lesions will coalesce, to expand across the width of the leaf blade, eventually becoming necrotic (Figure 1.1c) (Bamberg 1936; Jones et al. 1917). When the disease is observed in the spike, the glumes develop dark-purplish longitudinal streaks, and a characteristic banding pattern of dark discoloration alternating with healthy tissue on the awns. The symptoms on the spike are referred to as black chaff (Figure 1.1d) (Bamberg 1936; Smith et al. 1919). Under humid or moist conditions bacterial ooze may be present in and around leaf lesions. The initial signs of the pathogen appear as milky droplets and later as yellow resinous beads (Figure 1.1b), or the ooze may spread over the leaf surface and dry as a greyish, thin flaky layer (Bamberg 1936; Jones et al. 1917). Although not as common as on the leaves, bacterial ooze may be present on the glumes of infected spikes (Smith et al. 1919).

A disease cycle for *X. translucens* pv. *undulosa* has been proposed by Duveiller et al. (1997) and, although described for BLS on wheat, provides a general disease cycle for all BLS-causing pathogens. In a recent review, Sapkota et al. (2020) acknowledged that there are still many aspects of the BLS disease cycle that remain unclear and further research is warranted to elucidate all stages and differences in the life cycle between the various hosts. The pathogen enters the plant through stomata, hydathodes, or wounds (Bamberg 1936). As disease develops, movement of bacteria up the plant occurs by aid of rain splash

and leaf-to-leaf contact. Similarly, spread of disease within a field is facilitated by rain, wind, and plant to plant contact (Duveiller et al. 1997). The ability of the pathogen to overwinter, and thus the source(s) of primary inoculum, remain largely unanswered questions in BLS epidemiology.

Seed is generally considered an important source of primary inoculum. However, there are conflicting reports on *X. translucens* seed transmission and there are several reports of low transmission from wheat seeds to seedlings (Bamberg 1936; Boosalis 1952; Braun 1920; Tubajika et al. 1998). In addition, reports of seed transmission in barley, as compared to wheat, are limited (Boosalis 1952; Fourest et al. 1990; Wallin 1946b); yet both *X. translucens* pv. *translucens* and *X. translucens* pv. *undulosa* are considered seed-borne pathogens. Storage conditions of seed as well as length of storage, host resistance, and field environmental conditions (before harvest and following planting) have been suggested to influence the capacity of seed to support primary inoculum (Boosalis 1952; Duveiller et al. 1997; Forster & Schaad 1989; Milus & Mirlohi 1995). More research is needed to determine the conditions necessary for BLS transmission via seed and thus the significance of seed in the epidemiology of BLS.

Weeds are also considered a potential host for the pathogen to survive between growing seasons. The grass species referred to here are described as weeds in the context of growing small grains, however in other circumstances they may be grown as cultivated grasses for pasture, seed production, or other

intentional purposes. To our knowledge cultivated grasses have not been studied as a source of survival for the *X. translucens* pathovars that cause BLS on small grains. Perennial grasses, such as smooth brome (*Bromus inermis*), timothy (*Phleum pratense*), and quackgrass (*Elymus repens*) have been shown to support survival of the bacteria in senesced leaf tissue, and symptom development was observed in new growth the following spring (Boosalis 1952; Wallin 1946b). *X. translucens* pv. *undulosa* and *X. translucens* pv. *cerealis* have been isolated from both perennial and annual weedy grasses near infected wheat fields. Two known hosts, smooth brome and quackgrass exhibited typical BLS symptoms, while other weedy grasses identified, though asymptomatic, are reported to harbor epiphytic populations, including species in the genera *Avena, Festuca, Hordeum, Lolium, Poa,* and *Setaria* (Ledman et al. 2021; Thompson et al. 1989). Epidemiological studies showing the transmission of *X. translucens* from a weedy host to a crop host have not been conducted. The wide host range of *X. translucens* pv. *undulosa* suggest that weedy grasses may serve as reservoirs for the pathogen within wheat fields. By contrast, weeds may not play an important role in survival and transmission of *X. translucens* pv. *translucens*, which has a narrower host range, and studies of the pathogen in weeds growing in and around infected barley fields, that may answer this question, have not been pursued. Additional hosts, such as broadleaf weeds and non-Poaceous crops, have not been readily included in studies looking to identify where *X. translucens* may survive. In one study, Thompson et al. (1989) reported

epiphytic populations of *X. translucens* on alfalfa (*Medicago sativa*), demonstrating the potential for broadleaf plants to play a role in the survival of *X. translucens*.

Soil and crop residues are not considered important sources of inoculum. *X. translucens* may survive for a short period of time in soil and crop residues, but do not likely survive between cropping seasons (Boosalis 1952; Duveiller et al. 1997; Milus & Mirlohi 1995). In addition, the bacterial load on residues decreased when mixed in with soil and *X. translucens* does not survive in decomposing debris (Boosalis 1952; Duveiller et al. 1997).

Environmental conditions for BLS development in the field have not been well studied. BLS is considered a sporadic disease, and the exact conducive conditions are largely unknown. Observational circumstances have suggested that high humidity and warm temperatures promote BLS epidemics. Both in the laboratory and the field, *X. translucens* grows between 15 and 30°C and most rapidly at temperatures at or above 26°C (Bamberg 1936; Duveiller et al. 1997). In one study, temperature was indicated as the major cause for BLS epidemics (Duveiller & Maraite 1995). Moisture aids in the colonization and infection process and supports the movement of the bacterium on a leaf, within a plant, and across a field. In addition, it was noted that moisture is needed to release bacteria from infected seed, suggesting that there is a pathway to the colonization of seedlings (Duveiller et al. 1997). Clearly, moisture and

temperature are important in disease development, but it is not clear how they interact to influence disease severity.

1.5 Taxonomic History

Xanthomonas translucens has had a complicated history undergoing multiple taxonomic revisions. First isolated from barley, the bacterium causing BLS was named *Bacterium translucens* by Jones et al. (1917) to reflect the translucent symptoms that developed on the leaves. Host range studies including barley, oat, rye, and wheat revealed that this pathogen only caused disease on barley (Jones et al. 1917). A morphologically similar bacterium was isolated from wheat leaf tissues exhibiting leaf streak symptoms. The wheat pathogen caused disease in a broader host range including barley, rye, and wheat, indicating a biological differentiation from the barley pathogen (Smith et al. 1919). Smith et al. (1919) named this organism *B. translucens* var. *undulosum*. In 1924, Reddy et al. described a similar pathogen that caused BLS on rye (*Secale cereale*), though host range studies on barley, oat (*Avena sativa*), rye, and wheat indicated that the host range was limited to rye and named this variant *B. translucens* var. *secalis*. It appears that only one strain with this limited host range was isolated by Reddy et al. (1924). Previously, strains isolated from rye were identified as *B. translucens* var. *undulosum* based on their pathogenicity on barley, rye, and wheat (Jones et al. 1916; Reddy et al. 1924).

The genus *Bacterium* was used to describe non-sporing rod-like bacteria; this vague definition incorporated all Gram-negative plant pathogens that had

been described by the 1930's. Depending on the classification system used, other synonyms for the pathogen were used during the 1920s and 1930s including *Phytomonas translucens* and *Pseudomonas translucens* (Dowson 1939; Hagborg 1936). Recognizing a need for more consistent and descriptive taxonomic groups for plant pathogens, Dowson (1939) proposed the genus *Xanthomonas* to include bacteria that are Gram-negative, non-sporing, rod shaped, with polar flagellum, and exhibit a slimy-yellow appearance in culture. At this time, *B. translucens* was reclassified as *X. translucens* (Dowson 1939).

After the acceptance of *Xanthomonas* as the recognized genus for the organisms causing BLS on small grains, Hagborg (1942) recommended the use of *forma specialis* (f. sp.) instead of variant (var.) to differentiate *X. translucens* strains based on their observed hosts of origin and inoculation tests. Five *formae speciales* were proposed after pathogenicity tests on barley, oat, rye, and wheat were performed: *X. translucens* f. sp. *hordei*, naturally found on barley and pathogenic only on barley; *X. translucens* f. sp. *undulosa*, naturally found on wheat and pathogenic on barley, rye, and wheat; *X. translucens* f. sp. *secalis*, naturally found on rye and pathogenic only on rye; *X. translucens* f. sp. *hordei-avenae*, naturally found on barley and pathogenic on barley and oat; and *X. translucens* f. sp. *cerealis* naturally found on wheat and pathogenic on barley, rye, oat, and wheat. Fang et al. (1950) generally agreed with Hagborg's *formae speciales* classifications but could not reproduce infection on several oat varieties by *X. translucens* f. sp. *hordei-avenae* strains and therefore proposed that taxon

should be considered synonymous with *X. translucens* f. sp. *hordei.* Fang et al. (1950) also argued that Hagborg's definition of *X. translucens* f. sp. *cerealis* was synonymous with *X. translucens* f. sp. *undulosa*, in that they both occurred naturally on wheat and could infect barley, rye, and wheat, and redefined *X. translucens* f. sp. *cerealis* as having smooth brome and quackgrass as the natural hosts and being pathogenic on barley, rye, wheat, smooth brome, and quackgrass. Interestingly, they maintained the *X. translucens* f. sp. *secalis* nomenclature, although the strain from rye in their study showed to be pathogenic on barley, rye, and wheat, like the *X. translucens* f. sp. *undulosa* strains from wheat.

The designation of *forma specialis*, typically used with fungal organisms to describe an adaptation to a specific host, was later thought to be too limiting for bacterial pathogens that have a unique but potentially broad host range (Young et al. 1978). The use of the term pathovar thus replaced *forma specialis*. A pathovar is defined as an infrasubspecific level of classification that describes the entirety of the host range, which might include multiple hosts (Dye et al. 1980).

In the past, the conventional method for naming new species in the genus *Xanthomonas* was based on their host of isolation, where a new species would be assigned if a bacterium fitting the description of *Xanthomonas* was isolated from a newly reported host. This led to an abundance of *Xanthomonas* species that could only be differentiated by host and no other characteristics (Vauterin et al. 1995). Dye and Lelliott (1974) found this practice to be unmanageable and

reclassified the genus *Xanthomonas* into five species, in which all strains that cause BLS were considered pathovars of *X. campestris*. After this reclassification, the five recognized pathovars of *X. campestris* were; *cerealis, hordei, secalis, translucens,* and *undulosa* (Young et al. 1978). Bradbury (1986) proposed that *X. campestris* pv. *hordei* should be considered the same as *X. campestris* pv. *translucens*. Today, a major source of confusion in the taxonomy of this time is the use of *X. campestris* pv. *translucens*. This name was used to describe the pathogens of barley (the organism the current nomenclature recognizes as *X. translucens* pv. *translucens*) and wheat (*X. translucens* pv. *undulosa*), making it unclear if the authors were referring to one of these, or if they were referring more broadly to all pathogens that cause BLS in small grains.

The addition of new pathovars to the species *X. campestris* eventually resulted in the species including more than 140 pathovars; recreating the problem Dye and Lelliott (1974) intended to eliminate with their reclassification (Vauterin et al. 1995, 1992). Recent technological advancements, including DNA-DNA hybridization, have allowed for more in-depth biochemical and molecular analyses of genomes, leading to another reclassification of the genus *Xanthomonas*, grouping strains that cause disease on both small grains and grasses as their own species, *X. translucens*. Two groups within *X. translucens* were identified, the ‘translucens’ group which causes BLS of small grains and the ‘graminis’ group which causes bacterial blight of forage grasses (Vauterin et al. 1995, 1992). Within the identified ‘translucens’ group, Bragard et al. (1997)

identified three pathogenicity groups: *X. translucens* pv. *cerealis, X. translucens* pv. *translucens*, and *X. translucens* pv. *undulosa*. They noted that the *X. translucens* pv. *secalis* strain used in their study grouped with the *X. translucens* pv. *undulosa* pathogenicity type.

Currently, the accepted pathovars for BLS pathogens in the 'translucens' group are *X. translucens* pv. *cerealis, X. translucens* pv. *secalis, X. translucens* pv. *translucens,* and *X. translucens* pv. *undulosa* (Bull et al. 2010). Phylogenies based on multilocus sequence analyses of housekeeping genes as well as whole genome sequences corroborate the results of Bragard et al. (1997) where three distinct groups; *X. translucens* pv. *cerealis, X. translucens* pv. *translucens,* and *X. translucens* pv. *undulosa*, are identified (Curland et al. 2018; Goettelmann et al. 2022; Ledman et al. 2021; Peng et al. 2016). These studies were unable to distinguish *X. translucens* pv. *secalis* from *X. translucens* pv. *undulosa*. Although no formal proposition has been made, it appears reasonable to consider *X. translucens* pv. *secalis* synonymous with *X. translucens* pv. *undulosa*.

1.6 Economic Impact and Current Distribution

BLS has a wide geographic distribution and significant history of causing yield losses on barley and wheat (Osdaghi et al. 2023; Sapkota et al. 2020). During the past decade, economic losses due to BLS outbreaks were primarily recorded on wheat and caused by *X. translucens* pv. *undulosa* (Adhikari et al. 2011; Curland et al. 2018; Khojasteh et al. 2019). This may be because worldwide production of wheat is greater than the production of barley (USDA

2022). The economic impact of *X. translucens* pv. *cerealis* has not yet been measured in any small grain crop (Bragard et al. 1997; Langlois et al. 2017; Rademaker et al. 2006; Shah et al. 2023). The economic importance of BLS varies among regions depending on the level of variety susceptibility and environmental conditions. Grain yield losses due to BLS can range from insignificant to sixty percent on susceptible wheat cultivars (Friskop et al. 2022; Forster 1982; Lux et al. 2020). Yield losses occur from a reduction of grain test weight and/or the number of grains per spike (Duveiller et al. 1997; Waldron 1929). In addition, infection on the head may lead to discoloration of kernels, thus reducing grain quality in wheat and barley (Lux et al. 2020). The disease is also capable of reducing the grain quality by altering protein content, which is problematic for malting barley (Shane et al. 1987). Generally, yield loss is correlated with the disease severity on the flag leaf. In Minnesota, Shane et al. (1987) demonstrated that 50% disease severity on the flag leaf reduced kernel weight by 8 to 13%, and 100% disease severity on the flag leaf resulted in a 13 to 34% grain weight loss in spring wheat. A recent field study in North Dakota demonstrated that BLS can cause yield loss up to 60% in highly susceptible varieties and up to $8 million U.S. dollars in economic losses were estimated (Friskop et al. 2022).

BLS is widespread in the United States, Canada, and Mexico while the disease is generally absent in Western Europe (Paul & Smith 1989). In Australia, BLS has been recorded on wheat and rye in New South Wales (EPPO 2022). In

South America, BLS occurs in Argentina, Bolivia, Brazil, Paraguay, Peru and Uruguay (Duveiller et al. 1991; Mehta 1990; Mohan & Mehta 1985). The disease has been reported on wheat in China (Sun & He 1986), Pakistan (Akhtar & Aslam 1986), Iran (Khojasteh et al. 2019), and India (Richardson & Waller 1974). In the Near and Middle East, BLS has been recorded in Syria (Mamluk et al. 1990), Israel, Turkey (Demir & Üstün 1992), and Yemen (Bragard et al. 1995). In Africa, the disease has been reported in Ethiopia, Kenya, Libya, Madagascar, Morocco, South Africa, Tanzania, and Zambia (Bradbury 1986; Bragard et al. 1997, 1995; Korobko et al. 1985; Sands & Fourrest 1989).

The increasing worldwide distribution of *X. translucens* is strongly driven by the global expansion of international trades of plant materials, as well as by the multifaceted impact of climate change on plant-pathogen interaction and conducive weather conditions (Khojasteh et al. 2020; Sapkota et al. 2020). Despite the similarities in morphological features and biochemical characteristics, the three pathovars in the translucens group are considered independent quarantine pathogens around the world (CABI 2022; EPPO 2022). The exact distribution of *X. translucens* pathovars has not yet been determined because of the challenges posed by the taxonomic complexities within the species. Prior to the reclassification of the species by Vauterin et al. (1995), BLS was sometimes considered a disease complex and specific pathovars of *X. translucens* were not identified in outbreaks. Even after the reclassification of the species, many descriptions of the pathogens in areas with no history of the disease did not

accurately identify the pathovar (CABI 2022; Shah et al. 2021). *X. translucens* is one of the very few quarantined bacterial pathogens for which no exact distribution map is proposed by the European and Mediterranean Plant Protection Organization (EPPO) global database. It appears that the distribution maps for the BLS pathogens are grouped together under the taxon *X. translucens* pv. *translucens* (https://gd.eppo.int/taxon/XANTTR/distribution), with the following note: “Due to the complex taxonomy of this bacterium and its changes over time, it is difficult to establish an accurate world distribution.” This emphasizes an urgent need for action to accurately identify the pathogens causing BLS on small grains and determine their global distribution (Alvandi et al. 2023). Appropriate identification and global distribution is also important for quarantine policy.

1.7 Diagnostics

Isolation of *X. translucens* can be achieved by plating on general or semi-selective culture media. *X. translucens* is not likely to be confused with other bacterial pathogens of small grains on culture media, though it resembles other *Xanthomonas* species or yellow-pigmented saprophytic bacteria commonly associated with plants or seeds (Duveiller & Bragard 2017). Therefore, colony morphology on semi-selective media is not a reliable diagnostic tool.

DNA-based methods, such as polymerase chain reaction (PCR), multilocus sequence analysis (MLSA), and loop-mediated isothermal amplification (LAMP) have been used to develop diagnostic tools for

X. translucens. Several PCR protocols have been developed to identify *Xanthomonas* species, but are not species specific (Adriko et al. 2014; Maes 1993; Parkinson et al. 2009). Maes et al. (1996) developed a PCR protocol for detection and discrimination of *X. translucens*, and two quantitative PCR (qPCR) protocols have been developed to detect and quantify *X. translucens* (Clavijo et al. 2022; Hong et al. 2023; Sarkes et al. 2022). However, these methods do not differentiate the *X. translucens* pathovars.

Until recently, no pathovar specific PCR primers had been available for detection and identification of *X. translucens*. Alvandi et al. (2023) and Hong et al. (2023), in separate studies, developed DNA markers that specifically identified *X. translucens* pv. *undulosa* from *X. translucens* pv. *translucens* from plant materials. A multiplex PCR protocol, using a set of six primers, was developed based on whole genome differences and was designed to detect and distinguish *X. translucens* pv. *translucens* and *X. translucens* pv. *undulosa* (Roman-Reyna et al. 2022). These new PCR methods are a step toward the precise detection and identification of BLS pathogens. However, the definition of a pathovar, according to the rules of the International Society for Plant Pathology, requires in planta assays for identification (Dye et al. 1980).

MLSA using partial sequences of four housekeeping genes (*rpoD, dnaK, fyuA,* and *gyrB*) is another method that has been used, in corroboration with in planta host range studies, to identify *X. translucens* strains to pathovar (Clavijo et al. 2022; Curland et al. 2018, 2020a; Khojasteh et al. 2019; Ledman et al. 2021).

This method is highly reproducible and effective in predicting pathovar designation, however it requires sequencing and analyzing multiple genes and is more time consuming than conducting a diagnostic PCR or qPCR.

LAMP is a rapid (60 to 70 minutes), DNA-based diagnostic tool that amplifies targeted nucleotide sequences at a constant temperature, eliminating the need for specialized equipment (Fischbach et al. 2015; Notomi et al. 2000). Diagnostic LAMP primers have been developed to distinguish *X. translucens* pathovars in the 'translucens' group from those in the 'graminis' group, though it cannot differentiate *X. translucens* pv. *translucens* from *X. translucens* pv. *undulosa* (Langlois et al. 2017). Enormous progress has been made in the ability to diagnose BLS pathogens, but there are still challenges in reconciling the use of modern molecular tools with taxonomic groupings defined by classical methods.

1.8 Management

Control strategies for the management of BLS in cereal crops are limited. Crop rotation is thought to be ineffective as the pathogen is reported to not survive for long in crop debris or soil (Boosalis 1952; Duveiller et al. 1997; Milus & Mirlohi 1995). No effective means of chemical control have been identified, although these studies have been limited (Sapkota et al. 2020). Effective biocontrol options are not currently available, however recent advancements in this subject have identified several potential candidates to control *X. translucens* using endophytic bacteria (Taheri et al. 2022), bacteriophages (Erdrich et al.

2022), and antimicrobials or plant extracts (Mačionienė et al. 2022). Silva et al. (2010) reported that a silicon soil ammendment reduced BLS severity in wheat. These biocontrol and mineral nutrient studies were not conducted under field conditions and more studies are needed to evaluate their efficacy in the natural environment.

Because seed is considered a primary source of inoculum, it is thought that planting pathogen-free seed is an effective way to limit disease (Duveiller et al. 1997). Rapid and effective molecular assays have been developed to detect *X. translucens* in seed, however these DNA-based methods cannot distinguish between living versus non-living cells (Hong et al. 2023; Langlois et al. 2017; Maes et al. 1996; Roman-Reyna et al. 2022). It is reported that *X. translucens* pv. *undulosa* at a concentration ≤1,000 CFU/g of seed is unlikely to cause severe disease in the field (Forster & Schaad 1987), so having the ability to quantify the living bacteria associated with seed is important when testing for seed contamination.

Currently, the only non-destructive means to detect the presence of viable bacteria in seed lots is to wash whole seeds and plate the seed-wash onto a semi-selective media. Counting the yellow *Xanthomonas*-like colonies can be used to estimate the pathogen concentration in association with the seed sample (Duveiller et al. 1997). There are several limitations for using a seed-wash protocol to detect and quantify living bacterial cells. No selective medium has been developed specifically for *X. translucens*, therefore when plating seed

washes other organisms may grow, including other *Xanthomonas* spp. and yellow saprophytic bacteria, thus the identification of the pathogen may be challenging. Dilution plating with semi-selective media is also time consuming and it takes several days for the bacteria to grow. In addition, further diagnostic tools must be used to confirm pathogen identification from dilution plating, such as in planta, PCR, or LAMP assays (Duveiller & Bragard 2017; Langlois et al. 2017). Another limitation to using a non-destructive seed wash, is that the location of bacteria on or in the seed is not determined. Cells living within the seed may not be recovered by a seed wash without destructive processing to expose the internal tissues of the seed. Further, it is unknown if the location in seed differs between the wheat pathogen, *X. translucens* pv. *undulosa*, the barley pathogen, *X. translucens* pv. *translucens*, or indeed other pathovars.

There are inconsistent reports of chemical and heat treatments being effective in disinfecting *Xanthomonas*-infested seed and none have been reported to be entirely effective (Duveiller et al. 1997). Copper compounds have been used to manage other *Xanthomonas* pathogens, as both seed and leaf protectants, but the use of copper poses a number of risks including; phytotoxicity, altering the soil microbiota, and the development of copper-resistant strains (Lamichhane et al. 2018). In a review on copper use in agriculture, Lamichhane et al. (2018) identified 24 reports of copper resistance in plant pathogenic *Xanthomonas* species. Several antibiotics and copper seed treatments have been shown to reduce *X. translucens* transmission compared to

non-treated plots, but some disease developed in all treatments and under favorable conditions BLS still developed and spread throughout the field (Sands et al. 1981). Dry heat (72°C) for seven days was found to be effective in reducing the bacterial load in barley seed (Fourest et al. 1990). The same method was conducted on wheat seed and found the effectiveness declined as seed samples exceeded 100 g (Duveiller et al. 1997). Given these findings, dry heat may be more useful for breeders using small seed lots as opposed to growers. Another concern for both chemical and heat seed treatments is seed viability, as in both cases the treatment that kills the bacteria may also kill the embryo (Fourest et al. 1990; Sands et al. 1981).

The lack of effective cultural and chemical controls to reduce BLS development, and the limitations of the chemical and physical control methods available, to reduce primary inoculum from seed and in the field, highlight the importance of host resistance in the control of BLS. Breeding varieties of small-grained cereals with improved resistance to *X. translucens* appears to offer the best approach to disease management and will be discussed later in this review.

1.9 Pathogen Virulence

In Gram-negative bacteria, the Type III secretion system (T3SS) and Type III effector proteins (T3Es) are key components for bacterial pathogenicity and virulence. The T3SS is a protein delivery apparatus with a needle-like structure that injects T3Es into host cells to promote infection (Büttner & Bonas 2002, 2010). An *hrp* gene cluster encodes the T3SS in plant-pathogenic bacteria and is

highly conserved in *X. translucens* (Büttner & Bonas 2010; Goettelmann et al. 2022; Pesce et al. 2017). The core *hrp* cluster in *X*. *translucens* consists of 23 genes including 11 *hrc* genes, 8 *hrp* genes and 4 *hpa* genes (Goettelmann et al., 2022). Mutations of the *hrp* cluster in both *X. translucens* pv. *translucens* and *X. translucens* pv. *undulosa* strains demonstrated that the T3SS is essential in disease development in barley and wheat, respectively (Peng et al. 2016; Pesce et al. 2017).

In Xanthomonadaceae, T3Es are further classified into transcription activator-like effectors (TALEs) and non-transcription activator-like effectors (non-TALEs). *Xanthomonas* outer proteins (Xops) are an important group of non-TAL effectors delivered to the host by the T3SS (Büttner & Bonas 2010; White et al. 2009). White et al. (2009) documented 53 families of Xops in the genus *Xanthomonas*, designated into family names from XopA to XopBA. The advancement and feasibility of genome sequencing and analysis has allowed the prediction of many Xop effectors in *X. translucens*, revealing 21 to 36 classes of Xop effectors in a given strain (Gardiner et al. 2014; Goettelmann et al. 2022; Heiden et al. 2022; Peng et al. 2016; Shah et al. 2021). Comparative genomics have identified a core set of Xops in *X. translucens* species, including XopF, XopK, XopN, XopP, XopQ, XopR and XopX. In contrast, a few Xop effectors were found to exist exclusively in certain strains or pathovars, for example XopAJ, XopAL1, XopE3 and XopM were specific to *X. translucens* pv. *translucens* (Goettelmann et al. 2022; Shah et al. 2021). Functional analyses

have shown that some Xop effectors contribute to virulence in other *Xanthomonas* pathosystems, however no such analyses have tested the functionality of *X. translucens* Xop effectors in BLS development (Büttner & Bonas 2010; Goettelmann et al. 2022; White et al. 2009).

TALEs are proteins found in many, but not all, species and pathovars of plant-pathogenic *Xanthomonas.* TALEs are modular in structure consisting of three domains. The N-terminus domain is essential for secretion and translocation of the protein into the host cell, and the C-terminus domain is required for nuclear localization and transcriptional activation. The central repeat domain consists of tandem repeats of 33 to 35 amino acids that are generally conserved across all the repeats except at amino acids 12 and 13. These two residues are called the repeat variable di-residues (RVDs) and bind to specific nucleotides in host DNA inducing gene expression that promotes disease (Bogdanove et al. 2010; Schornack et al. 2013).

TALEs have been identified in all *X. translucens* pathovars that cause BLS in small grains, with the number of TALEs particular to each pathovar. *X. translucens* pv. *undulosa* is generally reported to have seven to eight TALEs (Charkhabi et al. 2017; Goettelmann et al. 2022; Khojasteh et al. 2020; Peng et al. 2016, 2019; Shah et al. 2021). *X. translucens* pv. *translucens* typically contains four to eight TALEs (Heiden et al. 2022; Roman-Reyna et al. 2020; Schachterle et al. 2022; Shah et al. 2021); however, Khojasteh et al. (2020) also identified one *X. translucens* pv. *translucens* strain with no TALEs (XtKm7) and

one with 12 (XtKm33). The TALEs for *X. translucens* pv. *cerealis* have only been described for two strains, but in each case, two TALEs were identified (Goettelmann et al. 2022; Shah et al. 2019). Although each pathovar contains a typical number of TALEs, the TALEs are not identical from strain to strain. Khojasteh et al. (2020) identified eight different TALE profiles for *X. translucens* pv. *undulosa* and five for *X. translucens* pv. *translucens* from 57 and 8 strains, respectively, originating from Iran. Similarly, a genome comparison of eight *X. translucens* pv. *translucens* strains showed that a few TALEs were conserved among all or most strains, but each of the eight strains had a unique TALE repertoire based on their RVD sequences (Heiden et al. 2022). The number of complete genomes published for *X. translucens* is still limited, but as new sequences become available our understanding of the diversity of TALEs will also likely increase. Genome comparisons between strains will also indicate which TALEs are highly conserved within a pathovar and which may be unique to a strain, host of origin, or geographical region, and thus which can best inform functional analyses of these proteins.

To date, four TALEs, which have arbitrary names, have been demonstrated to play an important role in BLS development. Tal2 and Tal4b from *X. translucens* pv. *undulosa* strain ICMP11055 and Tal8 from strain XT4699 were shown to individually contribute to virulence in wheat (Charkhabi et al. 2017; Peng et al. 2019). Tal1 from *X. translucens* pv. *cerealis* strain NXtc01 was shown to contribute to virulence in wheat, though it was not tested on barley or its grass

host of origin, smooth brome (Shah et al. 2019). Tal8 (XT4699) was demonstrated to promote disease susceptibility by inducing the expression of *TaNCED* and modulating ABA pathways in wheat (Peng et al. 2019). The exact mechanisms to which Tal2 (ICMP11055), Tal4b (ICMP11055), or Tal1 (NXtc01) contribute to virulence have not yet been identified. Currently, no TALEs from *X. translucens* pv. *translucens* have been tested for their role in virulence.

There has been some confusion in the naming of TALEs, where TALEs have been given arbitrary names that are strain specific. This can lead to naming TALEs that share the same RVD patterns with different names or naming unique TALEs from different strains with the same name. Grau et al. (2016) have developed a software package (AnnoTALE, http://www.jstacs.de/index.php/AnnoTALE), that classifies TALEs into classes based on RVD similarities and proposes a unified method to name TALEs to eliminate the inconsistencies that currently exist.

The Type II secretion system (T2SS) is also reported to play a role in virulence for some bacterial pathogens by exporting toxins or cell wall degrading enzymes to the host apoplast (Jha et al. 2005). Two types of gene clusters, *xps* and *xcs*, were found to encode T2SS in *Xanthomonas* species with the *xps* cluster being highly conserved (Szczesny et al. 2010). Goettelmann et al (2022) identified the *xps* cluster in *X. translucens* strains, but the *xcs* cluster was not present in these strains. No study has currently been conducted to functionally

characterize the role of the *xps* gene cluster of the T2SS in disease development.

Xanthomonas translucens pv. *undulosa* colonizes mesophyll tissue as shown by electronic microscopic images of bacterial cells residing within this tissue after spray inoculation on wheat (Sapkota et al. 2020). Spray inoculations of *X. translucens* pv. *translucens* on barley resulted in little to no infection, whereas leaf clippings and dip inoculation promoted infection. These observations suggested a difference in tissue specificity between these two BLS pathovars (Pesce et al. 2017; Sapkota et al. 2020). Pathovars of other *Xanthomonas* spp. are known to have either non-vascular or vascular lifestyles, such as *X. oryzae* pv. *oryzicola* and *X. oryzae* pv. *oryzae* as well as *X. campestris* pv. *raphani* and *X. campestris* pv. *campestris*, respectively (Bogdanove et al. 2011). A recent study identified a Type II secreted cell wall-degrading cellobiohydrolase, CbsA, that contributes to vascular pathogenicity in *X. translucens* pv. *translucens*, which contains an intact copy of *cbsA,* the gene encoding this protein*. X. translucens* pv. *undulosa* does not have an intact copy of *cbsA*, as it is disrupted at the 5' end by a transposon and does not colonize the vascular tissue. Expression of *cbsA* in *X. translucens* pv. *undulosa* promoted vascular infection and deletion of *cbsA* in *X. translucens* pv. *translucens* reduced vascular symptoms and promoted non-vascular symptoms, supporting the hypothesis of two different pathogenic lifestyles (Gluck-Thaler et al. 2020).

1.10 Host Resistance

Host resistance is the most effective and economical control option for bacterial diseases, including BLS of wheat and barley. Disease screening to identify sources of resistance has been conducted on diverse wheat and barley germplasm under field or greenhouse conditions, including commercially grown varieties, breeding lines, landraces, and related species, as well as some alien species introgression lines (Adhikari et al. 2011; Akhtar & Aslam 1986; Alizadeh et al. 1994; Cunfer & Scolari 1982; Duveiller et al. 1993; El Attari et al. 1996; Hagborg 1974; Kandel et al. 2012; Milus et al. 1996; Ramakrishnan et al. 2019; Ritzinger et al. 2023; Sapkota et al. 2018; Tillman et al. 1996). From these screening efforts, several wheat and barley lines have been identified that have a sufficient level of resistance to BLS, to serve as potential sources of resistance for breeding programs. For example, Ramakrishnan et al. (2019) identified 10 genotypes with BLS resistance, both in greenhouse and field conditions, from 299 accessions of hard winter wheat lines from the United States. Recently, Ritzinger et al. (2023) screened 2,094 accessions of barley germplasm under field conditions and found 32 (1.5%) that exhibited a consistently high level of resistance across locations and years. Disease evaluations from a regional coordinated effort in the Upper Midwest of the United States showed that ‘Boost’ had the best BLS resistance among over 100 hard red spring wheat cultivars that have been tested (Curland, R. D., Liu, Z, Ali S., and Dill-Macky, R., *unpublished results*). Several triticale (x *Triticosecale*) lines have also been shown to have

high levels of resistance to BLS (Cunfer & Scolari 1982; Johnson et al. 1987; Sapkota et al. 2018).

In most cases, BLS resistance has been shown to be a quantitative trait controlled by multiple genes (Adhikari et al. 2012; Duveiller et al. 1993; El Attari et al. 1996; Kandel et al. 2015; Ramakrishnan et al. 2019; Tillman & Harrison 1996). Quantitative trait locus (QTL) analysis and genome-wide association mapping efforts have located BLS resistance genes to various wheat and barley chromosomes (Adhikari et al. 2012; El Attari et al. 1996; Kandel et al. 2015; Ramakrishnan et al. 2019). Resistance to BLS has been reported across the wheat genome comprising of the chromosomes or arms 1A, 4A, 4B, 6B, and 7D (Adhikari et al. 2012); 2A and 6B (Kandel et al. 2015), 1AL, 1BS, 3AL, 4AL and 7AS (Ramakrishnan et al. 2019). Barley chromosomes harboring BLS resistance included 3H and 7H (El Attari et al. 1996). By contrast, Duveiller et al. (1993) identified five major genes (*Bls1* to *Bls5*) that contributed to partial resistance with *Bls1* having the largest effect. Resistance in some triticale accessions has been shown to be controlled by a major gene (Johnson et al. 1987; Wen et al. 2018). One of the major genes, *Xct1* has been mapped to chromosome 5RS indicating a novel type of resistance different from the QTL identified in wheat. Genetic and mapping studies should be conducted for more wheat or barley lines with partial or high levels of resistance, which can provide us with better understanding of BLS resistance and the tools for breeding programs.

Several inoculation and rating methods have been used in disease evaluation for resistance screening and genetic studies. A common method used in greenhouse assays was to infiltrate bacterial cells into leaf issue using a needless syringe and then rate the development of water-soaked lesions several days after inoculation (Adhikari et al. 2011, 2012; Curland et al. 2018; Milus & Chalkley 1994; Ramakrishnan et al. 2019; Sapkota et al. 2018). This method allows for acceptable disease development, but it is difficult to control the volume of bacterial inoculum infiltrated into the tissue. By marking the length of the initial infiltration area, differences in lesion length that expands beyond the initial inoculated length can be compared across treatments (Ramakrishnan et al. 2019). Using the needless syringe method, inconsistencies can be observed, and more replications are needed. However, this method has been effectively used to characterize host reactions in a qualitative manner by describing the following character states: water-soaking, chlorosis, and no reaction, where water-soaking is considered a pathogenic response (Curland et al. 2020a; Ledman et al. 2021). The second common method used in the greenhouse was the direct spraying of bacterial cells on leaf surface followed by estimating the percent of leaf area that developed water-soaking (Akhtar & Aslam 1986; El Attari et al. 1996; Wen et al. 2018). This method mimics the pathogen infection process in nature, which is believed to provide a more accurate assessment. However, optimal conditions in the greenhouse are critical for symptom development.

Field evaluations have been conducted by rating disease symptoms that developed either from natural or artificial inoculations (Ramakrishnan et al. 2019; Ritzinger et al. 2023; Tillman et al. 1996). Several methods of disease assessment have been applied in the field. The percent area of disease on flag leaves has been used to rate disease severity and a pictorial guide was developed by Duveiller (1994a) to estimate percent area of diseased leaf tissue on various small grain hosts (Ramakrishnan et al. 2019; Tillman et al. 1996). This method can be time consuming depending on the size of the trial and may require a trained eye. Saari and Prescott (1975) developed a rapid method of assessment based on the height of disease in the canopy and can be used to assess individual plants or a whole experimental plot. It uses a 0 to 9 scale where 0 is no disease, 5 indicates disease is present in 50% of the plant height, and 9 indicates disease to the top of the plant. This method can be applied to screen a large trial relatively quickly and provides information about disease spread within the canopy but does not account for disease severity (Saari & Prescott 1975). Similarly, a 0 to 9 scale has been used to evaluate disease severity based on percent of leaf tissue affected in an experimental plot, but this rating does not account for spread upwards in the canopy (Ritzinger et al. 2023). An adaptation of Saari and Prescott's standardized rating system was developed by Kandel et al. (2012) which uses a double-digit scale from 00 to 99. The first digit indicates height of disease development in the plant canopy as described above and the second digit indicates severity based on percent of diseased leaf

area, where 0 is no disease, 5 is 50%, and 9 is 90%. This double-digit system can be used to calculate an overall percent disease severity score or when multiple assessments are made over time can be used to calculate the area under the disease progress curve (Kandel et al. 2012). Field conditions are difficult to control, so evaluations at multiple locations across several years are needed to obtain confident data. In many cases, field evaluations encounter the problem of the close association of disease reactions with plant maturity and/or plant height (Ritzinger et al. 2023; Tillman et al. 1996). Utilizing both greenhouse and field data provides a more robust assessment when evaluating host resistance and genetics.

1.11 Prospective Research

Xanthomonas translucens pv. *undulosa* and *X. translucens* pv. *translucens* pose a great threat to global food security and adequate malting barley supply. BLS has been reported as a disease of economic importance to wheat and barley production in many regions of the world. Unfortunately, we lack effective management tools to mitigate BLS, although progress has been made in our overall understanding of the disease systems. We suggest that the following research areas need to be initiated or strengthened in the coming years to meet the challenge posed by these pathosystems.

First, rapid and reliable diagnostic tools need to be developed that can identify viable *X. translucens* cells and differentiate pathovars. Pathovar-specific primers for *X. translucens* have been recently developed, and there is an

opportunity to utilize these protocols to innovate methods to detect viable cells (Hong et al. 2023; Roman-Reyna et al. 2022). The ability to identify and quantify living cells would be useful in investigating sources of primary inoculum, as well as improve the monitoring of infested seed lots.

Second, the sources of primary inoculum and their possible role(s) in disease epidemics need to be investigated. Seed, crop residues, and alternative hosts are all believed to be sources of primary inoculum, but little research has been conducted to examine to what extent they contribute to BLS development in the field or to identify additional sources. Further knowledge is also needed to understand the pathways whereby seed, crop residues, and alternative hosts can initiate disease.

Third, functional analyses of *X. translucens* effectors need to be conducted to identify virulence factors contributing to disease incidence and severity. Genome sequencing of bacterial strains has generated rich sources of data informing biological characteristics of bacterial pathogens, particularly in respect to identifying T3Es. However, the majority of T3Es (TALEs and non-TALEs) have not been studied for their functions and roles in disease development. In many *Xanthomonas* plant pathogens, T3Es enter plant cells to target a specific host factor for disease development and susceptibility. Host targets can be modified to obtain high levels of resistance through genome editing or traditional breeding. The wheat and barley targets of *X. translucens*

effectors should also be investigated and eliminated for improving BLS resistance.

Fourth, the wheat and barley lines identified having high levels of resistance should be confirmed in additional environments (locations, years) prior to their use in breeding programs. Due to the quantitative nature of BLS resistance, DNA markers linked to the resistance QTLs are highly desired for breeding programs. Understanding the molecular mechanisms underlying resistance genes/QTLs would help us breed resistant cultivars, utilizing multiple additive QTLs to increase both the efficacy and durability of resistance. Given that wheat cv. 'Boost' has demonstrated reliable resistance to BLS, efforts should be placed to map and characterize resistance in this variety. The resistance gene *Xct1* in triticale may be conferred by a different resistance mechanism to 'Boost' and thus it may be beneficial to transfer this gene to wheat. However, this process might take a long time and there is a risk of problematic unwanted linkage drag. In addition, standardized and effective disease evaluation protocols for greenhouse and field experiments will be very useful in the identification of reliable sources of resistance and breeding of resistant cultivars. For this purpose, field nurseries can be established at a regional level with multiple locations that implement the same protocol at all locations.

Overall, advancements have been made in the understanding of BLS and the pathogens causing this disease. Although there are still many questions to be answered regarding significant sources of primary inoculum, we now understand

that *X. translucens* pv. *undulosa* is present on multiple weed species that grow in and around wheat fields which could play a role in the epidemiology of BLS (Ledman et al. 2021). The development of species and pathovar specific primers for diagnostics can be utilized to develop tools that can also identify and quantify living bacterial cells (Clavijo et al. 2022; Hong et al. 2023; Langlois et al. 2017; Roman-Reyna et al. 2020). The increase in genome sequencing of *X. translucens* have permitted the identification of potential virulence factors, including non-TALE and TALE T3Es (Goettelmann et al. 2022; Heiden et al. 2022; Roman-Reyna et al. 2020; Shah et al. 2021). However, functional characterization of these effectors is still limited. Sources of host resistance have been identified in barley, wheat, and triticale, but more work is needed to utilize these genes in breeding programs.

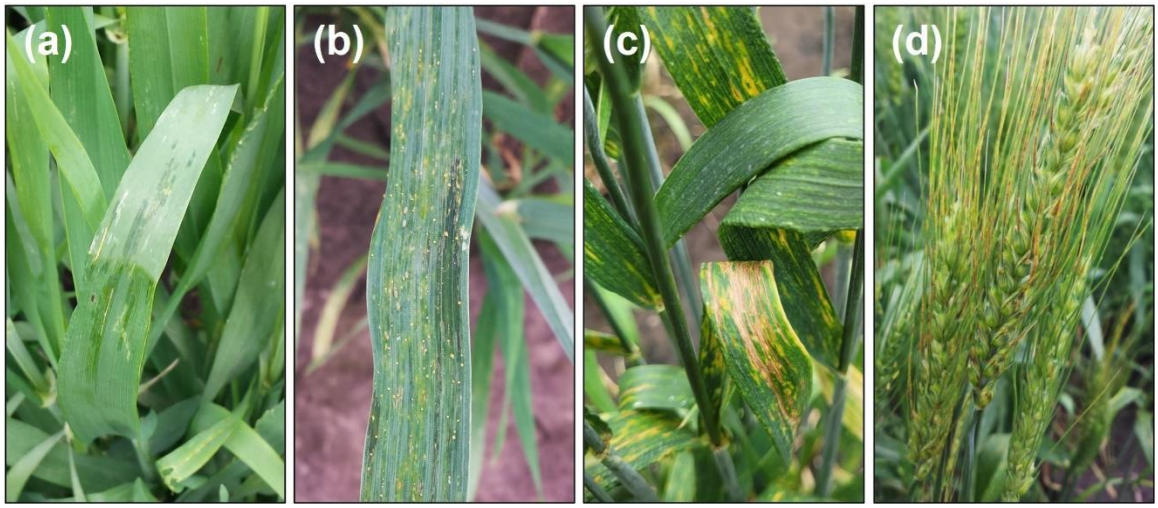

Figure 1.1 Bacterial leaf streak symptoms and signs caused by *Xanthomonas translucens*: (a) water-soaked lesions on the mid-leaf of barley; (b) yellow resinous bacterial ooze and water-soaking on wheat; (c) chlorotic and necrotic leaf streak symptoms on wheat; and (d) black chaff symptoms on wheat spike.

CHAPTER 2: Comparative genomics of *Xanthomonas translucens* pv. *undulosa* strains isolated from weedy grasses and cultivated wild rice

Kristi E. Ledman[1]*, Veronica Roman-Reyna[2,3], Rebecca D. Curland[1], Nathaniel Heiden[2,3], Jonathan M. Jacobs[2,3], and Ruth Dill-Macky[1]

[1]Department of Plant Pathology, University of Minnesota, St. Paul, MN

[2]Plant Pathology Department, The Ohio State University, Columbus, OH

[3]Infectious Diseases Institute, The Ohio State University, Columbus, OH

My contributions to Chapter 2:

- Was the lead author of the manuscript
- Ran the comparative analyses with the guidance of Roman-Reyna, V.
- Conducted in planta assays
- Generated the figures and tables

Co-authors contributions:

- Roman-Reyna, V. led and mentored me in the genome assemblies and the comparative analyses; providing the scripts and choosing programs to use. Also was the secondary author especially in the material and methods section
- Heiden, N. sequenced the strains
- All authors reviewed the entire document

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2.1 Abstract

Bacterial leaf streak (BLS) of wheat, caused by *Xanthomonas translucens* pv. *undulosa*, is a disease of major concern in the Northern Great Plains. The host range for *X. translucens* pv. *undulosa* is relatively broad, including several small grains and perennial grasses. In Minnesota, *X. translucens* pv. *undulosa* was isolated from weedy grasses in and around wheat (*Triticum aestivum*) fields that exhibited BLS symptoms and from cultivated wild rice (*Zizania palustris*) with symptomatic leaf tissue. Currently, no genomic resources are available for *X. translucens* pv. *undulosa* strains isolated from non-wheat hosts. In this study, we sequenced and assembled the complete genomes of five strains isolated from the weedy grass hosts; foxtail barley (*Hordeum jubatum*), green foxtail (*Setaria viridis*) and wild oat (*Avena fatua*), and from cultivated wild rice and wheat. These five genomes were compared to the publicly available genomes of seven *X. translucens* pv. *undulosa* strains originating from wheat, and one genome of *X. translucens* pv. *secalis* strain originating from rye (*Secale cereale*). Global alignments of the genomes revealed little variation in genome structures. Average nucleotide identity-based phylogeny and life identification numbers revealed that the strains share ≥99.25% identity. We noted differences in the presence of Type III secreted effectors, including transcription activator-like effectors. Despite differences between strains, we did not identify unique features distinguishing strains isolated from wheat and non-wheat hosts. This study contributes to the availability of genomic data for *X. translucens* pv. *undulosa*

from non-wheat hosts, thus increasing our understanding of the diversity within the pathogen population.

2.2 Introduction

Bacterial leaf streak (BLS) of wheat (*Triticum aestivum*), caused by *Xanthomonas translucens* pv. *undulosa* (Smith et al. 1919), is a disease found worldwide in wheat growing regions (Sapkota et al. 2020). BLS has been reported to cause up to 40% yield loss in wheat production (Forster 1982). In the United States, BLS has become a major concern in the Northern Great Plains (Adhikari et al. 2012; Curland et al. 2018; Kandel et al. 2012). Seed is considered a primary source of inoculum, however reports on transmission of *X. translucens* pv. *undulosa* from wheat seed are conflicting and several indicate that transmission rates may be low (Bamberg 1936; Boosalis 1952; Braun 1920; Tubajika et al. 1998). Weeds are also considered a potential source of inoculum as the pathogen has been isolated from several perennial and annual grasses. However, transmission from weedy hosts to crop hosts has not yet been demonstrated (Boosalis 1952; Ledman et al. 2021; Thompson et al. 1989). Currently, there are no chemical controls available to effectively mitigate BLS so understanding where the pathogen is surviving is important in developing management strategies.

Xanthomonas translucens pv. *undulosa* primarily infects wheat but has a relatively wide host range causing BLS on barley (*Hordeum vulgare*), rye (*Secale cereale*), intermediate wheatgrass (*Thinopyrum intermedium*), cultivated wild rice

(*Zizania palustris*), smooth brome (*Bromus inermis*), and quackgrass (*Elymus repens*) (Curland et al. 2020b; 2021; Fang et al. 1950; Jones et al. 1917; Smith et al. 1919). In Minnesota, *X. translucens* pv. *undulosa* was isolated from several annual and perennial weedy grasses growing adjacent to fields of wheat with apparent BLS symptoms. The hosts examined included wild oat (*Avena fatua*), smooth brome, quackgrass, foxtail barley (*Hordeum jubatum*), perennial ryegrass (*Lolium perenne*), and green foxtail (*Setaria viridis*). Of these hosts, smooth brome, quackgrass, and perennial ryegrass exhibited typical BLS symptoms. In contrast, BLS symptoms were not observed on wild oat, foxtail barley, or green foxtail (Ledman et al. 2021). Ledman et al. (2021) demonstrated that strains originating from weedy grasses were pathogenic on wheat and barley facilitating pathovar identification but this study did not determine pathogenicity on the weedy grass hosts, speculating that some of these hosts may not develop BLS symptoms but rather serve as hosts for epiphytic populations of *X. translucens* pv. *undulosa*.

Advances in high-throughput sequencing technologies have increased our ability to generate whole-genome sequences of bacterial pathogens. Whole-genome sequences can be used to understand phylogenetic relationships and to predict genes that inform strain specific virulence factors and behavior, such as host adaptation. For example, comparative genomics revealed that a single cellobiohydrolase gene is a major determinant for whether *X. translucens* infects vascular tissue (Gluck-Thaler et al. 2020). An important virulence factor for

Gram-negative bacterial pathogens is the Type III secretion system which enables the pathogen to secrete effector proteins directly into the host plants cells. For *Xanthomonas* species, many of these effectors are named *Xanthomonas* outer proteins (Xop) (Büttner and Bonas 2010). Another group of Type III effectors are the transcription activator-like effectors (TALEs), which have been shown to be important for *X. translucens* pv. *undulosa* virulence (Charkhabi et al. 2017; Peng et al. 2019). Carbohydrate active enzymes (CAZymes) enable a bacterium to gain carbon from the complex nutrient environment of a plant host and can be used as determinants of how *X. translucens* behaves within its host plant (Zhang et al. 2018). At the time of this study, there were 10 publicly available whole-genome sequences for *X. translucens* pv. *undulosa*, all of which originated from wheat (Charkhabi et al. 2017; Clavijo et al. 2022; Goettelmann et al. 2022; Jaenicke et al. 2016; Peng et al. 2016; Shah et al. 2021).

In this study, we used the whole-genomes of five *X. translucens* pv. *undulosa* strains isolated in Minnesota, USA. The strains were originally isolated and previously characterized from five hosts: wheat, green foxtail, wild oat, foxtail barley, and cultivated wild rice (Curland et al. 2018, 2021; Ledman et al. 2021). Utilizing the publicly available *X. translucens* genomes, we generated an average nucleotide identity (ANI) based phylogeny to compare these closely related strains and performed comparative analyses to determine if unique features of the genomes may play a role in virulence and/or host adaptation.

2.3 Materials and Methods

2.3.1 Bacterial strains

Five *X. translucens* pv. *undulosa* strains were sequenced and tested for pathogenicity in this study (CIX40, CIX162, CIX207, CIX282, and CIX303). These strains have been previously described and were chosen because they originated from different hosts, including small grain crops and perennial and annual weedy grasses found growing near wheat fields in Minnesota (Table 2.1). In addition, the five strains have different sequence types based on multilocus sequence typing of the four housekeeping genes *rpoD, dnaK, fyuA,* and *gyrB* (Table 2.1) (Curland et al. 2018; Ledman 2019; Ledman et al. 2021).

Whole-genome sequences of nine additional *X. translucens* strains were acquired from the NCBI GenBank database on March 8, 2022, and were used as comparative strains. Seven of the nine strains were *X. translucens* pv. *undulosa* originating from wheat. One strain, described as *X. translucens* pv. *secalis* presumably because it was isolated from rye (Reddy et al. 1924), was subsequently reported to group genetically within the *X. translucens* pv. *undulosa* clade in multiple studies (Curland et al. 2018; Goettelmann et al. 2022; Ledman et al. 2021). One *X. translucens* pv. *translucens* strain, isolated from barley, was included as an outgroup strain (Table 2.2).

2.3.2 Sequencing

The genomic DNA (gDNA) of CIX40 was extracted with the Genomic DNA Buffer Set and Genomic-tip 100/G (QIAGEN, Germantown, MD). The gDNA of

the other four strains, CIX162, CIX207, CIX282, and CIX303, were extracted using MasterPure Gram Positive DNA Purification Kit (Lucigen, Middleton, WI) following the manufacture's protocol. DNA quality was checked using gel electrophoresis.

The library for strain CIX40 was prepared with 20-kb SMRTbell template (PacBio, Menlo Park, CA) and was sequenced by Psomagen (Rockville, MD) with PacBio RSII (P6-C4). The sequencing libraries for CIX162, CIX207, CIX282, and CIX303 were prepared with the Rapid Barcoding Kit (SQK-RBK004, Oxford Nanopore Technologies, Oxford, U.K.) based on the manufacturer's protocol. The four libraries were pooled together, and samples were sequenced on a MinION R9.4 flow cell (Oxford Nanopore Technologies). Raw reads were demultiplexed and base called with the program Guppy 4.4 using the config file dna_r9.4.1_450bps_hac.cfg. In addition to long read sequencing, the gDNA for all five strains were sequenced with Illumina NextSeq 2000 (Illumina, San Diego, CA) by the Infectious Diseases Institute at The Ohio State University. The library preparations and base calling were also performed by the Infectious Diseases Institute.

2.3.3 Long-read assembly with short-read polishing

The genomes were assembled based on the Trycyler pipeline (Wick et al. 2021) described on their GitHub website (https://github.com/rrwick/Trycycler/wiki/Guide-to-bacterial-genome-assembly). Briefly, the fastq files were assembled with the software Flye version 2.9-b1768

(Kolmogorov et al. 2019) and the assembled genomes were polished with the Illumina short reads in four steps. First, long reads were polished with Medaka using only the Nanopore raw reads (Oxford Nanopore Technologies). Second, the Illumina raw reads were trimmed and cleaned with Fastp (Chen et al. 2018). Third, a new assembly was generated using a Nanopore assembled genome as a reference and the Illumina clean raw reads with Burrows-Wheeler Alignment tool (Li and Durbin 2009). Fourth, the assembled genomes were polished with Polypolish (Wick and Holt 2022). Finally, the genomes were polished with Homopolish-v0.0.1 (Huang et al. 2021). The genomes were rotated to the *dnaA* gene using a script shared by Ralf Koebnik (Institute of Research for Development, Marseille, France).

To assess the completeness of the genome assemblies, we obtained benchmarking universal single-copy orthologs (BUSCO) scores using the BUSCO software with *Xanthomonadaceae* designated as the linage (Manni et al. 2021).

2.3.4 Data availability

The five complete genomes were uploaded to the NCBI Sequence Read Archive and GenBank under BioProject PRJNA814270 with consecutive accession numbers from SAMN21323344 to SAMN21323348.

2.3.5 Phylogeny

To define the genomic relationships among strains the average nucleotide identity (ANI) of all strains was calculated using the webtools Enveomics and a

phylogenetic tree was generated using Ward's hierarchical clustering method (Rodriguez-R & Konstantinidis 2016). Life Identification Numbers (LINs), which groups prokaryotes based on the similarities between genome sequences, were assigned to each strain through the LINbase web server (Tian et al. 2020). A core-genome phylogeny was also constructed using Parsnp (Treangen et al. 2014).

2.3.6 Genomic structures

Mauve (v2.4.0, 2015-02-25) was used to assess genome structure and rearrangements of the five novel and nine comparative *X. translucens* pv. *undulosa* strains (Darling et al. 2004). ProgressiveMauve default alignment options were selected and pathotype strain CFBP 2055 was used as the reference genome.

2.3.7 Type II, III, and IV effector identification

Type III effectors included TALEs and non-TALEs. Non-TAL secreted effectors were identified using the BLAST 2.8.1+ blastx algorithm (Camacho et al. 2009) using *X. translucens* pv. *undulosa* genomes as queries and a database of known *Xanthomonas* Type III secreted effectors (https://euroxanth.ipn.pt/doku.php?id=bacteria:t3e:t3e). Threshold values used to predict non-TALE Type III effectors were set at ≥60% identity and ≥65% coverage to reference effectors. TALEs were predicted and classified using the AnnoTALE suite (Grau et al. 2016). Differences among repeat variable diresidues (RVDs) were analyzed with FuncTAL version 1.1 in the QueTAL suite

(Pérez-Quintero et al. 2015). The gene clusters that encode the Type II and Type IV secretion machinery were identified using BLAST 2.8.1+ blastx algorithm, with the genes reported for *Xanthomonas*.

2.3.8 CAZymes

CAZymes were predicted for the *X. translucens* pv. *undulosa* strains using the web server dbCAN2 (https://bcb.unl.edu/dbCAN2/blast.php) and the combined tools HMMER (E-value < 1e-15, coverage > 0.35), DIAMOND (E-value < 1e-102), and Hotpep (frequency > 2.6, hits > 6) (Zhang et al. 2018).

2.3.9 Greenhouse in planta assays

Pathogenicity assays of the five *X. translucens* pv. *undulosa* strains sequenced in this study were conducted in the greenhouse on each of the five hosts of origin. Wheat (variety Mayville), green foxtail, wild oat, foxtail barley, and cultivated wild rice (variety Dovetail) seedlings at the 3- to 4-leaf stage were inoculated with each of the five strains (CIX40, CIX162, CIX207, CIX282, and CIX303) by leaf infiltration with an inoculum concentration of approximately 1×10^7 CFU/ml (OD_{600} = 0.01). Each host was also mock-inoculated with 0.85% NaCl (wt/vol), serving as a negative control. A completely randomized design with three replications was used, two leaves were inoculated per replication, and the trial was repeated. Growing conditions and inoculation methods were previously described by Ledman et al. (2021). The character states 'water-soaking', 'chlorosis', or 'no response' were assessed visually at 6 days post-inoculation and used to determine pathogenicity (Curland et al. 2020a). Isolations

were performed from the tissue of inoculated regions, after the character states were assessed, to evaluate pathogen viability and fulfill Koch's postulates.

2.4 Results

2.4.1 Whole-genome assemblies

Each of the five genomes sequenced and assembled in this study consisted of a single circular chromosome. All but one (CFBP 2055) of the comparative strains consisted of a single circular chromosome as indicated by assembly data on the NCBI database. In addition to a singular circular chromosome, CFBP 2055 also has an unnamed plasmid. Coverage of CIX40 was 174× using the PacBio platform, while the coverage of the four strains (CIX162, CIX207, CIX282, CIX303) sequenced with the Oxford Nanopore MinION ranged from 12× to 54×. The genome sizes ranged from 4.55 to 4.74 Mbp. BUSCO scores for genome completeness were between 98.8% and 99.0% (Table 2.3).

2.4.2 Phylogeny

The ANI-based phylogeny showed that the strains originating from weedy grasses (CIX162, CIX207, and CIX282) and cultivated wild rice (CIX303) grouped among the wheat strains and did not form a distinct clade. The *X. translucens* pv. *secalis* strain (CFBP 2539) and the 12 *X. translucens* pv. *undulosa* strains were 97% identical to the *X. translucens* pv. *translucens* outgroup strain CFBP 2054. The *X. translucens* pv. *secalis* strain grouped within the *X. translucens* pv. *undulosa* clade; these 13 strains shared ≥99.25% identity.

Each of these closely related strains had a unique LIN, and the ANI phylogeny showed that these strains were not clonal (Figure 2.1). The core-genome phylogeny supported the ANI results that *X. translucens* pv. *undulosa* is monophyletic regardless of host of origin (Supplementary Figure 2.1).

2.4.3 Comparative analyses

Structural comparison of *X. translucens* pv. *undulosa* showed 30-34 local collinear blocks (LCBs) (Figure 2.2). Several genome rearrangements were identified between the strains. The two main regions of rearrangement also showed an inversion where the genes are encoded on the opposite strand compared to the reference genome (CFBP 2055). Strain ICMP11055 had the greatest number of rearrangements compared to the other *X. translucens* pv. *undulosa* strains.

All *X. translucens* strains contained an *xps* Type II secretion system and a Type III secretion system, while only CFBP 2054 (*X. translucens* pv. *translucens*) contained a Type IV secretion system. Fifteen non-TAL Type III secreted effectors were identified across the *X. translucens* pv. *undulosa* strains. Twelve of these effectors were conserved among these strains. There was variation in the presence or absence of the XopAF1, XopJ5, and XopX effectors among the *X. translucens* pv. *undulosa* strains (Figure 2.3). The effectors XopAJ, XopAL1, and XopE5 were present only in the *X. translucens* pv. *translucens* outgroup strain CFBP 2054, whereas XopE4 was present in all strains, except CFBP 2054.

The *X. translucens* pv. *undulosa* strains were predicted to have either seven or eight TALEs belonging to classes that have been previously described based on RVD sequences (Grau et al. 2016) (Table 2.3, Supplementary Table 2.1). A total of 11 classes of TALEs were described (TalCT, TalCZ, TalDA, TalDB, TalDC, TalDD, TalDE, TalDF, TalHM, TalHN, and TalJA). Four TALE classes (TalCZ, TalDA, TalDD, and TalDF) were conserved among all 13 strains. TalCT was conserved among all strains except CIX282. TalHM3 and TalHN2 were unique to strain ICMP11055 and TalJA5 was unique to strain CIX303. Two copies of a TALE were sometimes present in a strain and found at different locations (Figure 2.3, Supplementary Table 2.1, and Supplementary Figure 2.2).

The number of secreted CAZymes for *X. translucens* strains ranged from 48 to 52 and the number of non-secreted CAZymes ranged from 66 to 73 (Table 2.3). At least one CAZyme for each strain was identified from each of the six classes currently included in the CAZy database (http://www.cazy.org/). The most abundant number of CAZymes belong to the glycoside hydrolase and the glycosyl transferase families. Between 46 and 48 glycoside hydrolases were secreted CAZymes and 23 to 26 were non-secreted. Although there are 35 to 36 glycosyl transferases, none of these enzymes are secreted (Supplementary Table 2.2).

2.4.4 Greenhouse in planta assays

The pathogenicity of a given strain was defined by the production of a water-soaked lesion in the inoculated leaves. Non-pathogenic responses

included character states of chlorosis, which was restricted to the inoculated region, and no response to the inoculation (Curland et al. 2018; Ledman et al. 2021) (Figure 2.4). All strains tested were pathogenic on wheat and cultivated wild rice, producing symptoms of water-soaking. No strain was considered pathogenic on wild oat, foxtail barley, or green foxtail. All strains typically caused non-expanding chlorotic lesions on wild oat (Table 2.4). The strain CIX282, originating from foxtail barley, produced a reddish coloration within the inoculated region on foxtail barley. Reactions on green foxtail varied, many of the plants produced necrotic lesions which appeared to be the result of mechanical damage in the first trial. Otherwise, the green foxtail produced a mixed response, between chlorosis and no response (Table 2.4). Typical yellow *Xanthomonas*-like colonies were isolated from all host-strain combinations.

2.5 Discussion

In this study, we have made publicly available the whole-genome sequences of five *X. translucens* pv. *undulosa* strains originating from various small grain and weedy grass hosts. These are the first complete *X. translucens* genomes that were isolated from hosts other than wheat.

The ANI among 13 genomes, including 12 *X. translucens* pv. *undulosa* strains and 1 *X. translucens* pv. *secalis* strain, were ≥99.25%. This study concurred with previous studies that indicate that *X. translucens* pv. *secalis* is genetically similar to *X. translucens* pv. *undulosa*. An overlapping host range between the two pathovars may indicate that *X. translucens* pv. *secalis* is

synonymous with *X. translucens* pv. *undulosa* (Curland et al. 2018; Goettelmann et al. 2022; Ledman et al. 2021). Even within this closely related *X. translucens* pv. *undulosa* cluster, the phylogeny and comparative analyses demonstrated that there was diversity among the strains examined in this study. All the strains had a unique LIN, showing that there are no clonal strains.

There were few genome rearrangements among the *X. translucens* pv. *undulosa* genomes. There was no obvious correlation between genome rearrangements and host of origin, for example wheat versus non-wheat hosts. Strain ICMP11055, originating from Iran, had the most structural variations. A multilocus sequence typing study reported higher genetic diversity in Iranian *X. translucens* pv. *undulosa* strains compared to North American strains (Khojasteh et al. 2019). Higher diversity among strains originating from this region may be consistent with the domestication of wheat that is believed to have occurred in the Iranian Plateau. In contrast, strain XtKm12 which also originated from Iran had a genome structure similar to the North American strains. A more in-depth genome comparison focusing on geographical origin would be needed to evaluate diversity in genome structures.

Fifteen non-TAL Type III secreted effectors for *X. translucens* pv. *undulosa* strains were identified in this study, with 12 being conserved. Consistent with our results, a genome analysis of Iranian strains predicted XopAF1 and XopX to be present in *X. translucens* pv. *undulosa* strains and did not identify XopAJ, XopAL1, or XopE5 (Shah et al. 2021). *X. translucens* non-

TAL Type III effectors have not been tested for functionality, however variations of Xop proteins among strains and/or pathovars may play a role in host specificity (Goettelmann et al. 2022).

Xanthomonas translucens strains analyzed in this study had small variations among CAZymes classes. This suggests that there is a conserved repertoire of secreted and non-secreted enzymes possessed by *X. translucens* pv. *undulosa* that neither varies according to host nor prevents metabolic viability on alternative hosts.

All strains contained seven or eight TALEs, which is consistent with previous genome analyses (Charkhabi et al. 2017; Peng et al. 2016). Each TALE class predicted had been previously identified (Grau et al. 2016). Currently, three TALEs from *X. translucens* pv. *undulosa* have been characterized as having a role in virulence on wheat. Of those, Tal2 and Tal4b from ICMP11055 belong to class TalDD and TalHM, respectively, and Tal8 from Xtu 4699 belong to class TalDC (Charkhabi et al. 2017; Peng et al. 2019). Strains containing TALEs belonging to these classes may play an important role in virulence as they have similar RVD sequences to previously characterized TALEs. The conserved and unique TALEs predicted in this study opens the door for further exploration of their specific role in virulence. More TALEs need to be functionally characterized to understand their role in host-pathogen interactions, including TALEs that are strain or host specific.

Pathogenicity assays demonstrated that the strains used in this study caused a pathogenic response of water-soaking on wheat and cultivated wild rice, which are previously described hosts of *X. translucens* pv. *undulosa.* The weedy grass hosts (foxtail barley, green foxtail, and wild oat) did not produce a pathogenic response of water-soaking when inoculated with any of the five strains tested. On all hosts, the pathogen was recovered from leaf tissue post-inoculation. These findings support previous hypotheses that *X. translucens* pv. *undulosa* can cause BLS on a wide host range, but can also maintain asymptomatic epiphytic populations on weedy grass hosts (Ledman et al. 2021; Thompson et al. 1989). However, bacterial population studies were not conducted in this study and highlights the need for more research to understand the epiphytic nature of *X. translucens* pv. *undulosa* on weedy grasses.

In this study, whole-genome comparisons of strains originating from non-wheat hosts were not differentiated from those originating from wheat. The closely related strains of *X. translucens* pv. *undulosa* had unique features at a strain level, but the genome comparisons provided no insight about features that were specific to strains isolated from weedy grass hosts versus cultivated wild rice or wheat. While four of the *X. translucens* pv. *undulosa* strains in this study did not originate from wheat, each strain was isolated from a different host. Future genome studies including several *X. translucens* pv. *undulosa* strains originating from the same non-wheat host may identify host specific characteristics.

The genome of *X. translucens* pv. *undulosa* is highly conserved compared to *X. translucens* pv. *translucens* (Heiden et al. 2022). Unlike *X. translucens* pv. *translucens*, *X. translucens* pv. *undulosa* is reported to be restricted to nonvascular tissue and it is unknown if this nonvascular lifestyle is linked to host range expansion decreasing host-specific behaviors and metabolism (Gluck-Thaler et al. 2020). Compared to *X. translucens pv. translucens*, the genome of *X. translucens* pv. *undulosa* may contain unique genes, deletions, or structural features that allow this pathovar to colonize a wide range of poaceous hosts.

The genome sequences published in this study provide a valuable contribution to the *X. translucens* research community by increasing the number of publicly whole-genomes sequences available for *X. translucens* pv. *undulosa* strains. Specifically, the five genomes published represent strains isolated from diverse poaceous hosts, whereas previously published *X. translucens* pv. *undulosa* genomes represent only strains isolated from wheat. As genome sequencing becomes more affordable and accessible, a robust repository of *X. translucens* pv. *undulosa* genomes can be created to include strains from diverse locations, isolation dates, and host plants. Such a resource will allow researchers to engage questions about global diversity, evolution, virulence, and host adaptations in *X. translucens* pv. *undulosa*.

Table 2.1 Host of origin and sequence type of the five *Xanthomonas translucens* pv. *undulosa* strains sequenced in this study.

Strain	Host of origin	Host common name	Year of isolation	Sequence type[a]	Reference
CIX40	*Triticum aestivum*	Wheat	2010	36	Curland et al. 2018
CIX162	*Setaria viridis*	Green foxtail	2017	56	Ledman et al. 2021
CIX207	*Avena fatua*	Wild oat	2017	34	Ledman et al. 2021
CIX282	*Hordeum jubatum*	Foxtail barley	2016	29	Ledman et al. 2021
CIX303	*Zizania palustris*	Cultivated wild rice	2016	30	Ledman et al. 2019

[a] Sequence types were previously assigned based on the unique concatenations of four housekeeping genes (*rpoD*, *dnaK*, *fyuA*, and *gyrB*).

Table 2.2 Accession information for the nine *Xanthomonas translucens* strains used as comparative strains in whole-genome sequence analyses.

NCBI BioSample	Strain[a]	Pathovar designation	Host of origin	Geographic origin[b]	Year of isolation
SAMEA4358958	CFBP 2054^{T}	*translucens*	*Hordeum vulgare*	USA (MN)	1933
SAMN18753780	CFBP 2055^{PT}	*undulosa*	*Triticum turgidum*	Canada	1966
SAMN18753387	CFBP 2539^{PT}	*secalis*	*Secale cereale*	Canada	1966
SAMN03122553	ICMP11055	*undulosa*	*Triticum aestivum*	Iran	1983
SAMN10823694	LW16	*undulosa*	*Triticum aestivum*	USA (ND)	2009
SAMN23719974	MAI5034	*undulosa*	*Triticum aestivum*	Uruguay	2018
SAMN10823695	P3	*undulosa*	*Triticum aestivum*	USA (ND)	2009
SAMN16598361	XtKm12	*undulosa*	*Triticum aestivum*	Iran	2015
SAMN02797519	Xtu 4699	*undulosa*	*Triticum aestivum*	USA (KS)	1999

[a] Superscript “T” and “PT” indicate type and pathotype strains, respectively.
[b] U.S. states: KS, Kansas; MN, Minnesota; and ND, North Dakota.

Table 2.3 Genome summaries, including genome size (bp), total number of genes detected, sequencing coverage, and benchmarking universal single-copy ortholog (BUSCO) scores of 13 *Xanthomonas translucens* pv. *undulosa* strains, number of transcription activator-like effectors (TALEs), and number of secreted and non-secreted carbohydrate-active enzymes (CAZymes).

						CAZymes	
Strain[a]	Genome size (bp)	Total genes	Genome coverage (x)	BUSCO[b] score (%)	TALEs	Secreted	Non-Secreted
CIX40	4,551,101	3,915	174	99.5	7	51	67
CIX162	4,631,958	3,990	12	99.0	8	50	67
CIX207	4,742,492	4,076	23	98.8	8	51	70
CIX282	4,594,915	4,005	20	99.0	8	51	66
CIX303	4,645,091	4,037	54	99.0	8	49	70
CFBP 2055	4,607,252	4,052	379	NA	7	52	66
CFBP 2539	4,565,955	3,948	186	NA	7	52	66
ICMP11055	4,761,583	4,149	118	NA	7	48	71
LW16	4,746,074	4,051	90	NA	8	52	68
MAI5034	4,625,916	4,001	139	NA	7	50	66
P3	4,618,583	4,004	90	NA	8	51	68
XtKm12	4,581,137	3,954	313	NA	7	50	68
Xtu 4699	4,561,137	3,908	60	NA	8	51	66

[a] Strains sequenced and assembled in this study are indicated in bold. Genome size, genes, and coverage for comparative strains were available on NCBI.
[b] BUSCO scores were only provided for strains used in this study.

Table 2.4 Character states based on host reactions to five *Xanthomonas translucens* pv. *undulosa* strains[a]

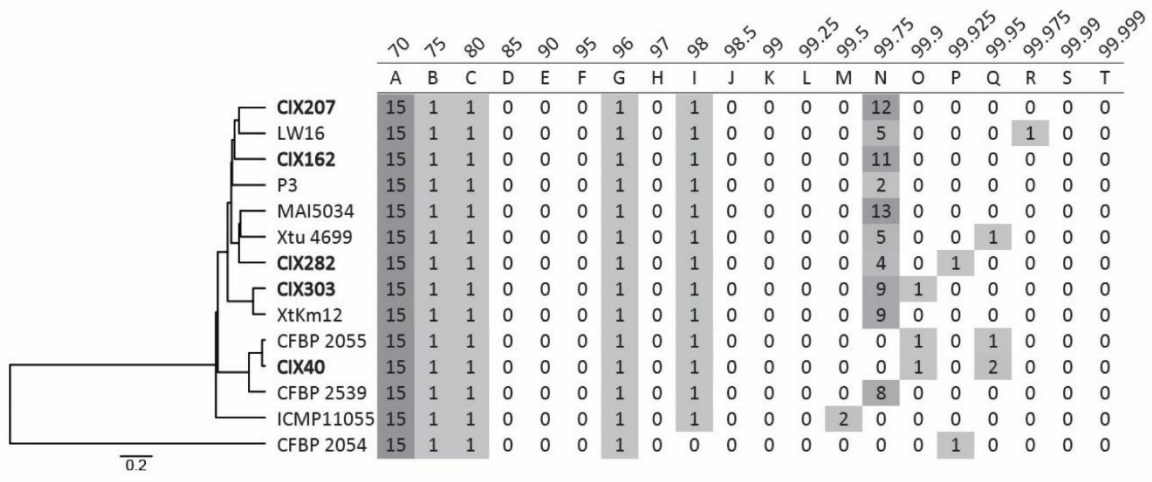
A

	Character State[a]																													
	Avena fatua						*Hordeum jubatum*						*Setaria viridis*						*Triticum aestivum*						*Zizania palustris*					
	Rep 1		Rep 2		Rep 3		Rep 1		Rep 2		Rep 3		Rep 1		Rep 2		Rep 3		Rep 1		Rep 2		Rep 3		Rep 1		Rep 2		Rep 3	
Strain	L1	L2	L1	L2	L1	L2	L1	L2	L1	L2	L1	L2	L1	L2	L1	L2	L1	L2	L1	L2	L1	L2	L1	L2	L1	L2	L1	L2	L1	L2
0.85% NaCl	-	-	-	-	-	-	-	-	-	-	-	-	N	N	-	-	N	-	-	-	-	-	-	-	-	-	-	-	-	-
CIX40	C	C	C	C	C	C	C	-	C	-	-	-	CN	CN	N	N	N	N	W	W	W	W	W	W	W	W	W	W	W	W
CIX162	C	C	C	C	C	-	C	C	C	-	-	-	N	N	N	N	N	N	W	W	W	W	W	W	W	W	W	W	W	W
CIX207	C	-	C	C	C	-	C	-	C	-	-	-	N	N	N	N	N	-	W	W	W	W	W	W	W	W	W	W	W	W
CIX282	C	C	C	C	C	C	R	R	CR	CR	CR	R	CN	CN	CN	CN	N	N	W	W	W	W	W	W	W	W	W	W	W	W
CIX303	C	-	C	C	C	C	C	-	-	-	C	-	C	C	C	CN	CN	N	W	W	W	W	W	W	W	W	W	W	W	W

B

	Character State																													
	Avena fatua						*Hordeum jubatum*						*Setaria viridis*						*Triticum aestivum*						*Zizania palustris*					
	Rep 1		Rep 2		Rep 3		Rep 1		Rep 2		Rep 3		Rep 1		Rep 2		Rep 3		Rep 1		Rep 2		Rep 3		Rep 1		Rep 2		Rep 3	
Strain	L1	L2	L1	L2	L1	L2	L1	L2	L1	L2	L1	L2	L1	L2	L1	L2	L1	L2	L1	L2	L1	L2	L1	L2	L1	L2	L1	L2	L1	L2
0.85% NaCl	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CIX40	C	C	C	C	C	C	C	-	C	-	-	-	-	-	C	-	C	C	W	W	W	W	W	W	W	W	W	W	W	W
CIX162	C	-	C	-	C	-	-	-	C	C	C	C	C	C	C	-	C	-	W	W	W	W	W	W	W	W	W	W	W	W
CIX207	C	-	C	C	C	C	C	C	C	-	C	C	C	-	C	-	C	C	W	W	W	W	W	W	W	W	W	W	W	W
CIX282	C	C	C	-	C	-	CR	CR	CR	CR	R	R	C	-	C	-	C	C	W	W	W	W	W	W	W	W	W	W	W	W
CIX303	C	-	C	C	C	C	C	C	C	-	C	-	C	-	C	-	C	-	W	W	W	W	W	W	W	W	W	W	W	W

[a] Seedlings of five weedy grass and small grain species were inoculated in the greenhouse by leaf infiltration. A 0.85% NaCl (wt/vol) buffer was used as a negative control. For each host, two leaves (L1 and L2) were inoculated per replication (Rep). The entire trial was conducted twice: trial one (**A**) and trial two (**B**). Character states: -, no response; C, chlorosis; N, necrosis; W, water-soaking; and R, reddish coloration.



	70	75	80	85	90	95	96	97	98	98.5	99	99.25	99.5	99.75	99.9	99.925	99.95	99.975	99.99	99.999
	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T
CIX207	15	1	1	0	0	0	1	0	1	0	0	0	0	12	0	0	0	0	0	0
LW16	15	1	1	0	0	0	1	0	1	0	0	0	0	5	0	0	0	1	0	0
CIX162	15	1	1	0	0	0	1	0	1	0	0	0	0	11	0	0	0	0	0	0
P3	15	1	1	0	0	0	1	0	1	0	0	0	0	2	0	0	0	0	0	0
MAI5034	15	1	1	0	0	0	1	0	1	0	0	0	0	13	0	0	0	0	0	0
Xtu 4699	15	1	1	0	0	0	1	0	1	0	0	0	0	5	0	0	1	0	0	0
CIX282	15	1	1	0	0	0	1	0	1	0	0	0	0	4	0	1	0	0	0	0
CIX303	15	1	1	0	0	0	1	0	1	0	0	0	0	9	1	0	0	0	0	0
XtKm12	15	1	1	0	0	0	1	0	1	0	0	0	0	9	0	0	0	0	0	0
CFBP 2055	15	1	1	0	0	0	1	0	1	0	0	0	0	0	1	0	1	0	0	0
CIX40	15	1	1	0	0	0	1	0	1	0	0	0	0	0	1	0	2	0	0	0
CFBP 2539	15	1	1	0	0	0	1	0	1	0	0	0	0	8	0	0	0	0	0	0
ICMP11055	15	1	1	0	0	0	1	0	1	0	0	0	2	0	0	0	0	0	0	0
CFBP 2054	15	1	1	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0

Figure 2.1 Whole-genome comparison among *Xanthomonas translucens* pv. *undulosa* strains. Left panel is an average nucleotide identity (ANI)-based phylogenetic tree constructed with Enveomics online tools (Rodriguez-R & Konstantinidis 2016) using Ward's hierarchical clustering method. Strain CFBP 2054 (*X. translucens* pv. *translucens*) was used as an outgroup. Strains sequenced in this study are indicated in bold. The right panel is a matrix based on life identification numbers (LIN). LINs were assigned using the LINbase web server (Tian et al. 2020). Each LIN positions (A-T) represents a different ANI threshold, ranging from 70% at position A to 99.999% at position T. The more similar two genomes are, the further to the right their LINs overlap. Column F (95%) is the commonly accepted threshold for species.

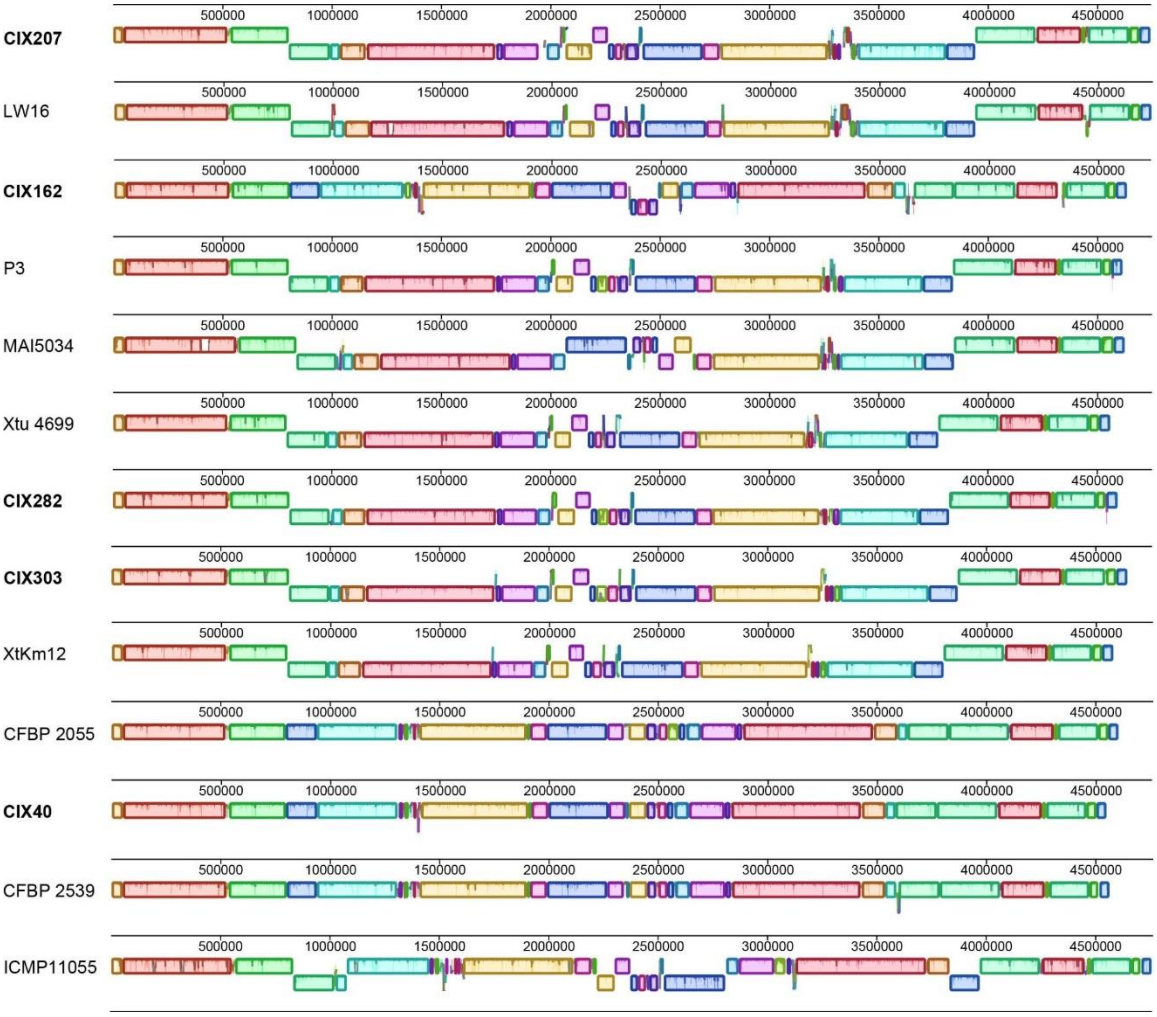


Figure 2.2 Global alignments of *Xanthomonas translucens* pv. *undulosa* genomes was constructed using Mauve (Darling et al. 2004). The order of the strains follows the phylogeny shown in Figure 2.1 and novel strains from this study are indicated in bold. The colors represent locally collinear blocks (LCBs), indicating conserved regions among all genomes. LCBs presented immediately below each genome structure represent an inverted region compared with the reference genome. Pathotype strain CFBP 2055 was used as the reference genome for all comparisons.

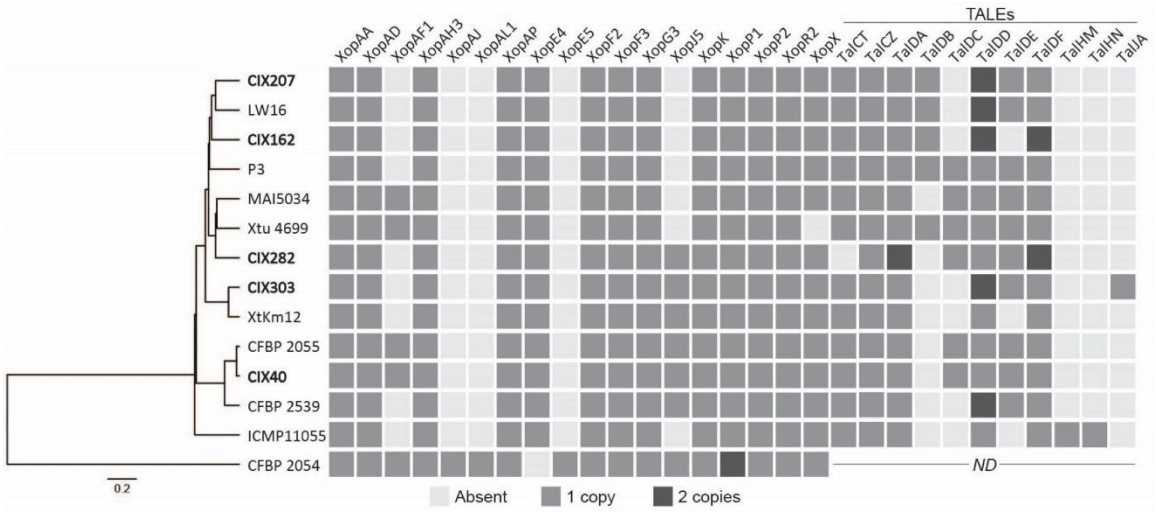


Figure 2.3 Presence of Type III secreted effectors (T3SEs) for *Xanthomonas translucens* pv. *undulosa* strains, including transcription activator-like effectors (TALEs). CFBP 2054 (*X. translucens* pv. *translucens*) was used as the outgroup and TALEs were not described (ND) for this strain. Strains sequenced in this study are indicated in bold. Gray shaded boxes indicate the presence of and copy number of T3SEs. Xop effectors were identified using the genome as a query with the blastx algorithm. Threshold values included an e-value of 0.01, percentage of identity ≥60, and percent coverage ≥65. TALE classes were identified using AnnoTALE (Grau et al. 2016).

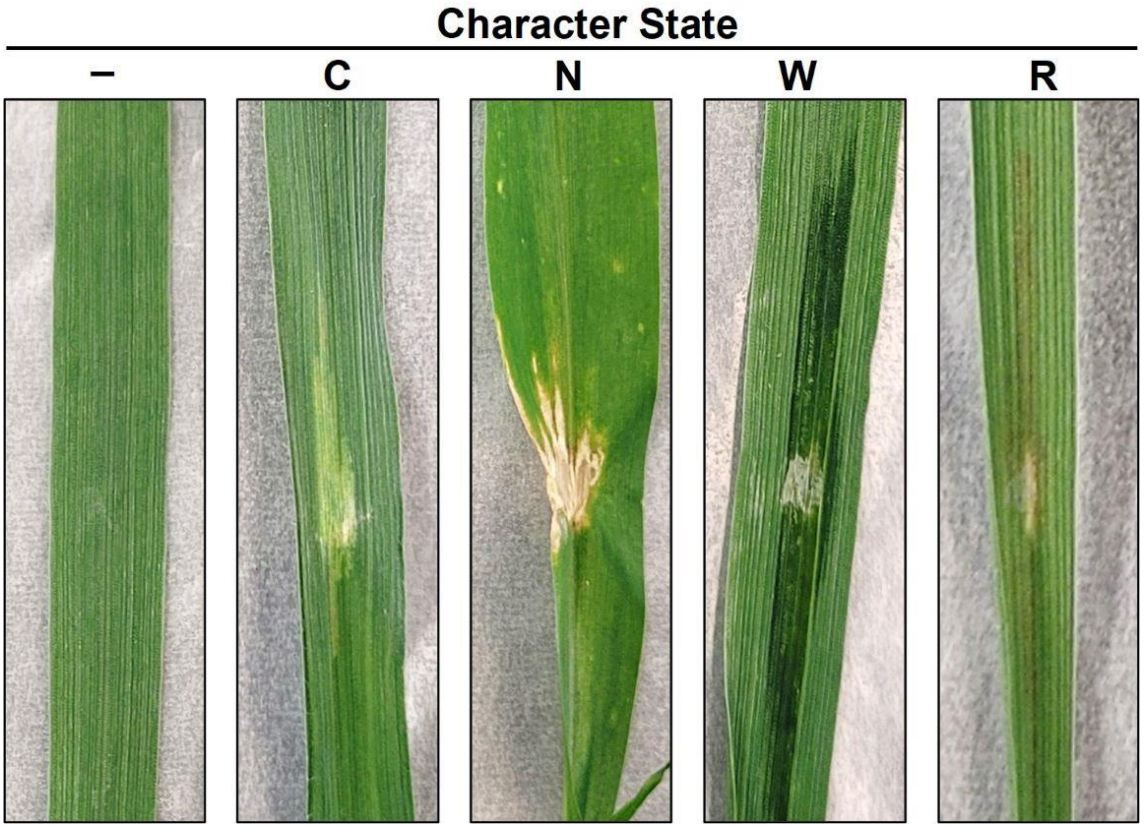


Figure 2.4 Representative character states observed at 6-days post inoculation when seedling leaves of Poaceous hosts were infiltrated with *Xanthomonas translucens* pv. *undulosa* strains. Character states included: no response (-); chlorosis (C); necrosis (N); water-soaking (W), and a reddish coloration (R). Water-soaking was the only character state to be considered a pathogenic response on the host.

Supplementary Table 2.1 Repeat variable di-residue (RVD) sequences of transcription activator-like effectors (TALEs) identified in the novel *Xanthomonas translucens* pv. *undulosa* strains sequenced in this study.

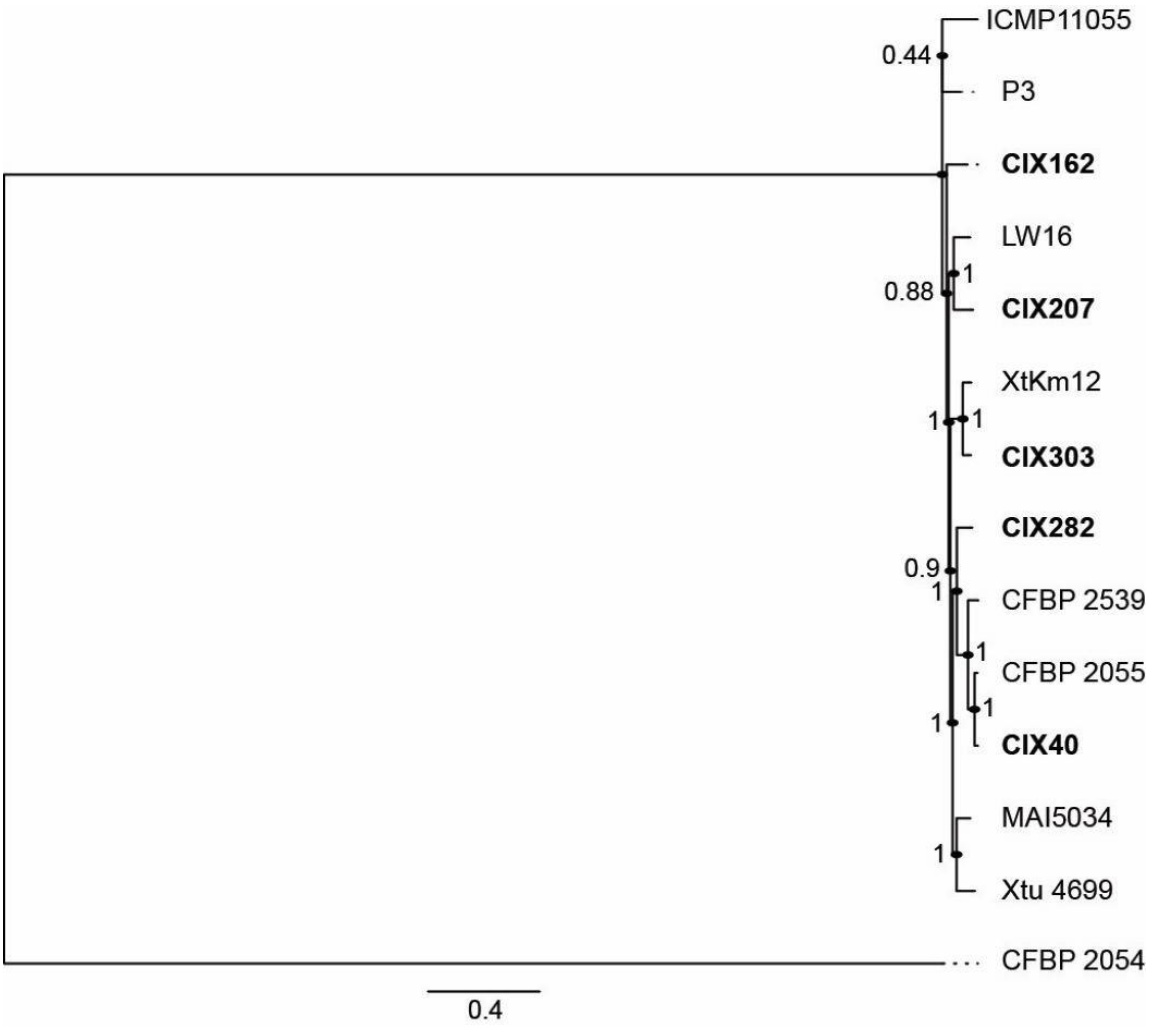
TALE class[a]	Strain	RVD sequence	Genome location:strand
TalCT	CIX40	HN-HD-HD-HD-NI-NI-NI-HN-HD-HD-NN-NN-NI-NN-HD	1324387-1327612:1
	CIX162	NN-HD-HD-HD-NI-NI-NI-HN-HD-HD-NN-NN-NI-NN-HD	414434-417665:1
	CIX207	NN-HD-HD-HD-NI-NI-NI-HD-HD-HD-NN-NN-NI-NN-HD	308717-311948:1
	CIX303	NN-HD-HD-HD-NI-NI-NI-HN-HD-HD-NN-NN-NI-NN-HD	476261-479492:1
TalCZ	CIX40	NH-NN-HD-NN-HD-NH-HD-YK-NG-NH-Y*-HD-NN-NI-NG-QD	1921057-1924393:-1
	CIX162	NH-NN-HD-NN-HD-NH-HD-YK-NG-NH-Y*-HD-NN-NI-NG-QD	1012830-1016172:-1
	CIX207	NH-NN-HD-NN-HD-NH-HD-YK-NG-NH-Y*-HD-NN-NI-NG-QD	758780-762539:1
	CIX282	NH-NN-HD-NN-HD-NH-HD-YK-NG-NH-Y*-HD-NN-NI-NG-QD	1850433-1853769:-1
	CIX303	NH-NN-HD-NN-HD-NH-HD-YK-NG-NH-Y*-HD-NN-NI-NG-QD	1056750-1060086:-1
TalDA	CIX40	HD-YD-NI-NG-NG-NN-YK-NG-HD-NG-NG-ND-NG-QD-NH-HD	593155-596335:1
	CIX162	HD-YD-NI-NG-NG-NN-YK-NG-HD-NG-NG-ND-NG-QD-NH-QD	308687-311873:-1
	CIX207	HD-YD-NI-NG-NG-NN-YK-NG-HD-NG-NG-ND-NG-QD-NH-HD	452728-455914:-1
	CIX282	HD-YD-NI-NG-NG-NN-YK-NG-HD-NG-NG-ND-NG-QD-NH-HD	1283327-1286507:1
	CIX282	HD-YD-NI-NG-NG-NN-YK-NG-HD-NG-NG-ND-NG-QD-NH-HD	598561-601741:1
	CIX303	HD-YD-NI-NG-NG-NN-YK-NG-HD-NG-NG-ND-NG-QD-NH-HD	593439-596619:1
TalDB	CIX162	NN-HD-NG-HD-HD-HN-NF-NI-NN-HD-HD-HD-HN-NN-HD	44001-47229:1
	CIX207	NN-HD-KG-HD-HD-HN-NF-NI-NN-HD-HD-HD-HN-HN-HD	860758-863989:-1
TalDC	CIX40	NN-NG-HD-HD-HD-KG-NN-Y*-NG-HD-HD-QD-HN	1321034-1324049:1
	CIX282	NN-NG-HD-HD-HD-KG-NN-Y*-NG-HD-HD-QD-HN	1279833-1282845:1
TalDD	CIX40	NN-HD-NG-NN-HN-KG-NI-HD-NI-NN-HD-HN-HD-HD-NI-HN-HD-QD	612523-616063:1
	CIX162	NN-HD-NG-NH-HN-NG-NI-HD-NI-NN-HD-HD-NN-NN-NI-NN-HD	410655-414096:1
	CIX162	NN-HD-NG-NN-HN-KG-NI-HD-NI-NN-HD-HN-HD-HD-NI-HN-HD-QD	288957-292497:-1
	CIX207	NN-HD-NG-NN-HN-NG-NI-HD-NI-NN-HD-HD-NN-NN-NI-HN-HD	304938-308379:1
	CIX207	NN-HD-NG-NN-HN-KG-NI-HD-NI-NN-HD-HN-HD-HD-NI-HN-HD-QD	431870-435410:-1
	CIX282	NN-HD-NG-NN-HN-KG-NI-HD-NI-NN-HD-HN-HD-HD-NI-HN-HD-HD	617934-621474:1

Supplementary Table 2.1. *Continued from previous page.*

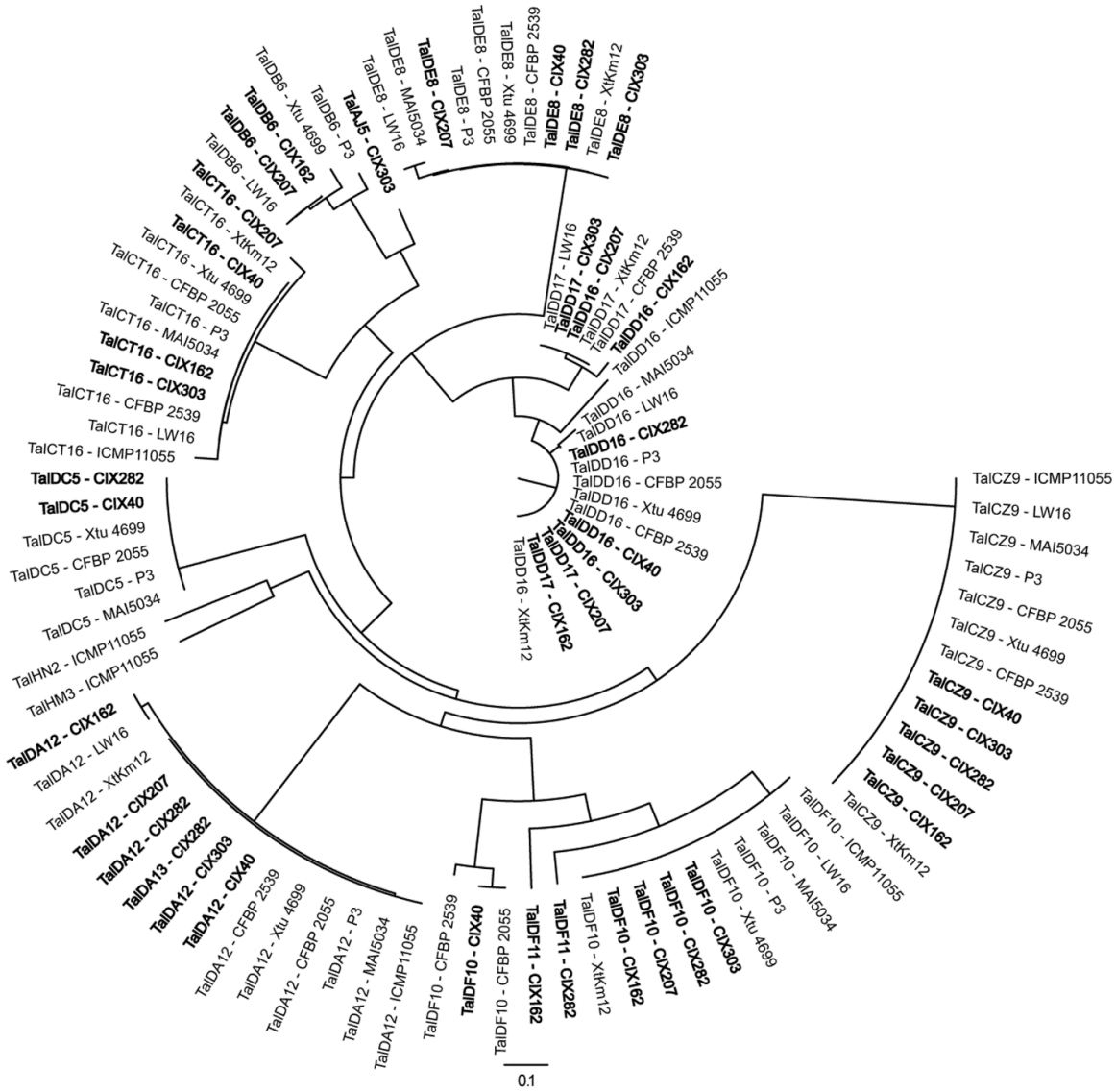
	CIX303	NN-HD-NG-NN-HN-KG-NI-HD-NI-NN-HD-HN-HD-HD-NI-HN-HD-QD	613963-617506:1
	CIX303	NN-HD-NG-NN-HN-NG-NI-HD-NI-NN-HD-HD-NN-NN-NI-HN-HD	472482-475923:1
TalDE	CIX40	NN-HD-NG-NN-HN-HN-NI-NI-NI-NH-NN-HD-NN-NH-HD-HD	1721207-1724546:1
	CIX207	NN-HD-NG-NN-HN-HN-NI-NI-NI-NH-NN-HD-NN-NH-HD-HD	946016-949355:-1
	CIX282	NN-HD-NG-NN-HN-HN-NI-NI-NI-NH-NN-HD-NN-NH-HD-HD	1662994-1666333:1
	CIX303	NN-HD-NG-NN-HN-HN-NI-NI-NI-NH-NN-HD-HN-NH-HD-HD	870286-873625:1
TalDF	CIX40	HD-HN-HN-HD-NH-NH-HG-HD-KG-NN-Y*-NG-HD-NI-NH-NG-HD-HN	1716293-1719830:1
	CIX162	HD-HN-HN-HD-NH-NH-HG-HD-KG-NN-Y*-NG-HD-HD-HN	807106-810325:1
	CIX162	NN-HD-NG-NN-HN-HN-HD-NH-NH-HG-HD-KG-NN-Y*-NG-HD-HD-HN	811702-815233:1
	CIX207	HD-HN-HN-HD-NH-NH-HG-HD-KG-NN-Y*-NG-HD-HD-HN	950732-953951:-1
	CIX282	HD-HN-HN-HD-NH-NH-HG-HD-KG-NN-Y*-NG-HD-HD-HN	1658398-1661617:1
	CIX282	HD-NG-HN-HD-NH-NH-HF-HD-KG-NN-Y*-NG-HD-HD-HN	2585170-2588386:1
	CIX303	HD-HN-HN-HD-NH-NH-HG-HD-KG-NN-Y*-NG-HD-HD-HN	865690-868909:1
TalJA	CIX303	NN-HD-KG-HD-HD-HN-NF-NI-NN-HD-HD-NN-NN-NI-HN-HD	1827403-1830739:-1

Supplementary Table 2.2 Number of secreted and non-secreted carbohydrate-active enzymes (CAZymes) in six classes of CAZymes of 14 *Xanthomonas translucens* strains predicted by dbCAN meta server (https://bcb.unl.edu/dbCAN2/blast.php).

	Auxiliary activities		Carbohydrate-binding modules		Carbohydrate esterases		Glycoside hydrolases		Glycosyl transferases		Polysaccharide lyases	
Strain	Secreted	Non-Secreted	Secreted	Non-Secreted	Secreted	Non-Secreted	Secreted	Non-Secreted	Secreted	Non-Secreted	Secreted	Non-Secreted
CIX40	0	1	2	4	1	3	47	24	0	35	1	0
CIX162	0	1	2	4	1	3	46	24	0	35	1	0
CIX207	0	1	2	4	1	3	47	26	0	36	1	0
CIX282	0	1	2	4	1	3	47	23	0	35	1	0
CIX303	0	1	1	5	1	3	46	25	0	36	1	0
CFBP 2054	0	1	3	4	1	3	46	25	0	36	0	1
CFBP 2055	0	1	2	4	1	3	48	23	0	35	1	0
CFBP 2539	0	1	2	4	1	3	48	23	0	35	1	0
ICMP11055	0	1	2	4	1	3	48	23	0	35	1	0
LW16	0	1	2	4	1	3	48	25	0	35	1	0
MAI5034	0	1	1	4	1	3	47	23	0	35	1	0
P3	0	1	2	4	1	3	47	24	0	36	1	0
XtKm12	0	1	2	4	1	3	46	25	0	36	1	0
Xtu 4699	0	1	2	4	1	3	47	23	0	35	1	0



Supplementary Figure 2.1 Core-genome phylogeny of *Xanthomonas translucens* pv*. undulosa* strains was constructed using Parsnp (Treangen et al. 2014). Bootstrap values are labelled at nodes, with a maximum bootstrap value of 1.0. CFBP 2054 (X. translucens pv. translucens) was used as an outgroup. Strains shown in bold were sequenced in this study.



Supplementary Figure 2.2 A phylogeny of transcription activator-like effectors (TALEs) of 13 strains of *Xanthomonas translucens* pv. *undulosa*. Strains sequenced in this study are indicated in bold. TALEs were predicted using AnnoTALE (Grau et al. 2016). The output was generated using FuncTAL in the QueTAL suite (Pérez-Quintero et al. 2015).

CHAPTER 3: Transmission of *Xanthomonas translucens* associated with seed in wheat and barley

3.1 Introduction

Bacterial leaf streak (BLS) is a disease that causes economic losses of wheat (*Triticum aestivum*) and barley (*Hordeum vulgare*) (Osdaghi et al. 2023; Sapkota et al. 2020). *Xanthomonas translucens* pv. *undulosa* is the primary pathogen that causes BLS on wheat, and *X. translucens* pv. *translucens* is the primary pathogen of barley (Curland et al. 2018; Jones et al. 1917; Khojasteh et al. 2019; Smith et al. 1919). Symptoms begin as water-soaked lesions, becoming chlorotic and greasy in appearance. The lesions develop into chlorotic and necrotic streaks, initially delineated by the leaf veins, though they will coalesce over time, expanding to the width of the leaf blade. Under favorable conditions, the infection spreads upwards through the plant canopy and symptoms may ultimately develop in the spikes. In the spike, dark-purplish streaks will be observed on the glumes and awns and these symptoms are referred to as black chaff (Bamberg 1936; Duveiller et al. 1997; Jones et al. 1917; Smith et al. 1919).

Seed has long been considered a primary source of inoculum. However, there is an equally long history of inconsistencies regarding the importance of infested seed in the development of BLS. The initial description of *X. translucens* pv. *undulosa* by Smith et al. (1919) described circumstantial evidence of transmission from infected wheat seed. In later studies, it was demonstrated that planting infected wheat seed resulted in subsequent disease in the field (Braun

1920; Tubajika et al. 1998). However, Bamburg (1936) found no evidence of seed transmission of BLS on wheat in either the field or the greenhouse. In another study, transmission was observed from barley seed in the field and greenhouse, but transmission from wheat seed was not evident (Boosalis 1952). Milus and Mirlohi (1995) used a rifampicin-resistant strain of *X. translucens* to inoculate winter wheat seed and recovered the rifampicin-resistant strain from seed samples at harvest. This demonstrated that the specific *X. translucens* strain used to inoculate seed was able to colonize the wheat plant, including the spikes, and survive throughout the growing season.

In the present study, rifampicin-resistant strains of *X. translucens* pv. *undulosa* and *X. translucens* pv. *translucens* were used to inoculate seed of susceptible cultivars of hard red spring wheat ('Mayville') and two-row spring barley ('Pinnacle'), respectively. The seeds were planted in controlled and field conditions. Tissues sampled from the plants at different growth stages were tested for the presence of the rifampicin-resistant strains, with the objective of tracking the movement of *X. translucens* from seed into the vegetative and reproductive tissues of the plant.

3.2 Materials and Methods

3.2.1 Rifampicin-resistant strains

Rifampicin-resistant CIX480^{Rif} (*X. translucens* pv. *undulosa*) and CIX485^{Rif} (*X. translucens* pv. *translucens*) were selected from the previously described wild-type strains CIX40 and CIX95, respectively (Curland et al. 2018).

Rifampicin-resistant strains were selected by dispensing 100 µl of *X. translucens* at a concentration of approximately 1 $\times 10^9$ CFU/ml (OD_{600} = 1.0) onto each of five plates of Wilbrink's agar (WBA); containing 75 mg/L cycloheximide (Sands et al. 1986) and amended with 50 mg/L rifampicin (WBA-R). The plates were incubated in the dark at 28°C for three days. Colonies that grew were sub-cultured onto WBA-R plates. The stability of the strains was maintained after three sequential transfers. Strains were stored at -80°C in nutrient broth with yeast extract (NBY) (Vidaver 1967) containing 10% glycerol.

3.2.2 Seed inoculations

Seeds of wheat (hard red spring wheat variety 'Mayville') and barley (spring two-rowed barley variety 'Pinnacle') were surface disinfested by soaking in 2% NaOCl for 5 minutes. Surface disinfested seeds were then rinsed thoroughly three times in deionized water and air dried on filter paper in a laminar flow hood. Seed samples were divided into two groups to be used for inoculated and non-inoculated treatments.

The two strains, CIX480^{Rif} and CIX485^{Rif}, were grown from freezer stock for 4 days and sub-cultured as bacterial lawns on WBA-R and incubated in the dark for 3 days at 28°C. Cells from the lawns were suspended in 0.85% NaCl (wt/vol) solution to obtain an approximate concentration of 1 $\times$ 10^8 CFU/ml (OD_{600} = 0.1). Wheat seeds were inoculated with CIX480^{Rif}, and barley seeds were inoculated with CIX485^{Rif}. To inoculate, seeds were submerged into the respective bacterial cell suspension at a 1:2 ratio of seed weight (g) to bacterial

cell suspension volume (ml), and a vacuum was applied for 5 minutes. The cell suspension was poured off and the seeds were dried on filter paper in a laminar flow hood. Inoculated seed was planted within 1 day of inoculation.

3.2.3 Tissue culture box assays

Transmission of *X. translucens* from wheat and barley seed to leaf tissue was assessed by growing the inoculated seed in tissue culture boxes. Treatments (n = 4) included wheat seed inoculated with CIX480^{Rif}, non-inoculated wheat seed, barley seed inoculated with CIX485^{Rif}, and non-inoculated barley seed. Fifteen seeds were placed into each sterile clear plastic tissue culture box (11 × 11 × 9.5 cm; l × w × h) containing 150 ml of 1% water agar. Tissue culture boxes were placed in growth chambers with a daytime temperature set at 25°C and a nighttime temperature at 15°C, with a 16 h photoperiod. Tissue types and time points assessed were: seed at 1 day post inoculation (dpi); coleoptile at 4 dpi; primary leaf at 10 dpi; primary leaf at 17 dpi, and second leaf at 17 dpi. A completely randomized design, with three replications (boxes) was used. The entire experiment was repeated.

At each assessment, 10 samples of the designated tissue type (e.g., 10 seeds, coleoptiles, or leaves) were bulked together, weighed, then chopped into approximately 0.5 cm-length pieces. Prior to chopping the leaf tissue, incidence of water-soaking symptoms was noted for each leaf. Seed and coleoptile tissue samples were placed into 1 ml 0.85% NaCl and primary and second leaf tissues were placed into 3 ml 0.85% NaCl. The tissue samples were then shaken for 20

minutes. Ten-fold serial dilutions of the tissue washes were performed and 100 µl of the 10^{-3} to 10^{-5} dilutions were plated onto WBA-R and incubated at 28°C in the dark. Colonies were counted four days after dilution plating.

3.2.4 Field assessments

Field trials were conducted at two field sites at the Minnesota Agricultural Experimental Station, located on the University of Minnesota's Saint Paul campus, during the 2022 growing season. One field site was not irrigated (dryland), while the other field was mist-irrigated. The mist-irrigated field was irrigated overnight for 12 minutes of every 90 min from 4:00 pm to 3:30 am. Field experiments at both locations were a completely randomized design with three replications. Treatments were wheat seed inoculated with CIX480^{Rif}, non-inoculated wheat seed, barley seed inoculated with CIX485^{Rif}, and non-inoculated barley seed. Each treatment plot consisted of four side-by-side 2-meter-long rows, spaced 30 centimeters apart. Treatments were separated by oat buffer plots, each 4-row plot also being 2 meters long and 1.2 meters wide.

Assessments were conducted on seven different tissue types during the growing season: seed at the time of planting, 3rd leaf, 4th leaf, 5th leaf, 6th leaf, flag leaf, and the spike, which was sampled at soft dough. Leaves were sampled after being fully expanded for at least one week, which meant that typically the leaf sampled was the leaf below the uppermost leaf on the plant. For each leaf tissue sample, 10 arbitrarily selected leaves within a plot were collected and bulked. At the soft dough stage of the plants, two arbitrarily selected florets from barley and

one arbitrarily selected spikelet from wheat were collected from each of 10 arbitrarily selected spikes per treatment. For each leaf collected, the percent of symptomatic leaf area was estimated visually. Bulked tissue (leaf and spike) was weighed and chopped into approximately 0.5 cm-length pieces. Tissue samples weighing under 3 g were placed in 5 ml 0.85% NaCl and tissue samples weighing over 3 g were placed in 10 ml 0.85% NaCl. Samples were shaken for 20 minutes. Tissue washes were serially diluted and 100 µl aliquots were plated on WBA-R and incubated in the dark at 28°C, and colonies were counted four days after plating. Dilutions from the dryland field were 10^{-2} to 10^{-5} and dilutions from the irrigated field were 10^{-2} to 10^{-6}.

3.2.5 Data analysis

The data collected from the tissue culture box assays and field evaluations were analyzed in the same manner. Colony forming units per gram of tissue (CFU/g) was calculated for each dilution (d) using the following calculation adapted from Duveiller et al. (1997): [(wash volume/tissue weight) $\times$ (colonies $\times$ 10^{d+1})]. For each treatment and tissue collection, the mean CFU/g was calculated from the three replications of the plated dilutions that generated countable colonies. In some cases, the colonies were so numerous on a given plate that individual colonies could not be counted; these plates were excluded from the mean calculation.

3.3 Results

3.3.1 Tissue culture box assays

Rifampicin-resistant colonies were recovered from all inoculated wheat and barley treatments at all assessed time points, whereas no colonies were recovered from the non-inoculated treatments at any time point. One day after the seed samples were inoculated, the populations of CIX480^{Rif} recovered from wheat seed in trials one and two were 1.33×10^5 and 1.17×10^5 CFU/g, respectively. Higher populations, 1.64×10^6 and 1.73×10^6 CFU/g for trials one and two, respectively, were recovered from the barley seed inoculated with CIX485^{Rif}.

The same trends in the levels of the recovery of rifampicin-resistant *X. translucens* strains were evident between each of the time points and tissues assessed in wheat and barley (Figures 3.1 and 3.2). The CFU/g recovered increased from the seed (1 dpi) to the coleoptile (4 dpi), decreased from the coleoptile to the primary leaf sampled at 10 dpi, then increased to the primary leaf sampled at 17 dpi. The tissues of the second leaf sampled at 17 dpi had a higher CFU/g than the primary leaf sampled at 10 dpi. However, the CFU/g on the second leaf at 17 dpi were lower than on the primary leaf tissue collected the same day.

The percentage of leaves that had symptoms was not consistently correlated with the population of rifampicin-resistant *X. translucens* recovered from the sample (Figures 3.1 and 3.2). In trial one, water-soaked lesions were

observed on 6.7% in both the wheat primary leaves (10 dpi) and second leaves (17 dpi), but the population of CIX480^{Rif} recovered was 1.14 $\times$ 10^6 CFU/g from the primary leaves and 2.01 $\times$ 10^8 CFU/g from the second leaves. In both trials, the barley primary leaf tissues (10 dpi) had a higher percent of symptomatic leaves (trial one: 10.0%; trial two: 13.3%) than the tissues of the second leaf (17 dpi; 3.3% in both trials), but the population of CIX485^{Rif} recovered from the primary leaves (10 dpi) was lower (trial one: 1.70 $\times$ 10^6 CFU/g; trial two: 1.08 $\times$ 10^6 CFU/g) than the amount from the second leaves (trial one: 2.63 $\times$ 10^7 CFU/g; trial two: 7.69 $\times$ 10^6 CFU/g).

For both wheat and barley, in both trials, the percentage of primary leaves that had water-soaked lesions increased between the 10 dpi and 17 dpi sampling time points, as did the CFU/g (Figures 3.1 and 3.2). In trial one, 6.7% of wheat primary leaves (sampled at 10 dpi) exhibited water-soaking symptoms and 1.14 $\times$ 10^6 CFU/g of CIX480^{Rif} were recovered, while 36.7% of the primary leaf tissues sampled at 17 dpi had symptoms and 5.92 $\times$ 10^8 CFU/g of CIX480^{Rif} were recovered. The results of the second trial were similar, with 6.7% of the wheat primary leaf tissues sampled at 10 dpi being symptomatic and 1.46 $\times$ 10^6 CFU/g were recovered, and 23.3% of the wheat primary leaf tissues sampled at 17 dpi had symptoms and 4.31 $\times$ 10^8 CFU/g of CIX480^{Rif} were recovered. For barley in trial one, 10.0% of primary leaves at 10 dpi had water-soaked symptoms and the CIX485^{Rif} population recovered was 1.70 $\times$ 10^6 CFU/g, and 16.7% of primary leaves sampled at 17 dpi had symptoms and 3.95 $\times$ 10^7 CFU/g

of CIX485^{Rif} were recovered. In trial two, 13.3% of barley primary leaves sampled at 10 dpi exhibited water-soaking symptoms and 1.08 $\times$ 10^6 CFU/g of CIX485^{Rif} were recovered, and 16.7% of the barley primary leaves sampled at 17 dpi were symptomatic and 4.09 $\times$ 10^7 CFU/g of CIX485^{Rif} were recovered.

3.3.2 Field assessments

At the time of planting (1 dpi), rifampicin-resistant *X. translucens* strains were recovered from all inoculated wheat and barley seed. No bacteria were isolated from non-inoculated seed. The populations of CIX480^{Rif} recovered from wheat seed were 3.42 $\times$ 10^5 CFU/g and 4.28 $\times$ 10^5 CFU/g in the dryland and mist-irrigated fields, respectively. The population of CIX485^{Rif} recovered from the barley seed was higher, than the wheat, being 2.94 $\times$ 10^6 CFU/g and 3.13 $\times$ 10^6 CFU/g in the dryland and mist-irrigated field, respectively.

CIX480^{Rif} was recovered in the mist-irrigated field from wheat throughout the growing season and the populations recovered ranged from 5.90 $\times$ 10^7 to 2.63 $\times$ 10^8 CFU/g (Figure 3.3). The highest population of CIX480^{Rif} recovered came from the 5th leaf samples. The populations of the rifampicin-resistant strain decreased at each sampling after the 5th leaf, with the lowest population of CIX480^{Rif} being isolated from the spike at soft dough. Incidence of disease generally increased at each subsequent assessment, as did the severity of disease observed (Figure 3.3). Symptoms were also observed in the non-inoculated wheat plots from the 4th leaf sampling onward, though the symptoms were less severe than those in the inoculated wheat plots (data not shown).

In the dryland field, CIX480^{Rif} was not recovered from wheat at the 3rd leaf or flag leaf samples. From the 4th leaf to the 6th leaf samplings the population of recovered CIX480^{Rif} increased from 9.75 $\times$ 10^{2} to 2.02 $\times$ 10^{7} CFU/g (Figure 3.3). However, it should be noted that colonies were only recovered from one of the three replications at the 4th, 5th, and 6th leaf sampling times, reducing the mean CFU/g values for these observations. In the one replication where CIX480^{Rif} was recovered the populations were 2.92 $\times$ 10^{3}, 4.04 $\times$ 10^{5}, and 6.06 $\times$ 10^{7} CFU/g, from the 4th, 5th, and 6th leaf samples, respectively. Due to bird damage to the wheat spikes, no data were collected at the soft dough stage in the dryland field. BLS symptoms were not observed on any tissue types in either the inoculated or non-inoculated wheat plots, except for the flag leaf where 3.3% of the leaves showed symptoms and the disease severity was only 0.03% (Figure 3.3). The flag leaves of the wheat in the non-inoculated treatments had similarly low levels of BLS (data not shown).

In the mist-irrigated field, CIX485^{Rif} was recovered from the barley at all growth stages sampled, with the populations recovered ranging from 1.07 $\times$ 10^{7} to 2.45 $\times$ 10^{8} CFU/g (Figure 3.4). The population of CIX485^{Rif} recovered increased with growth stage from the 3rd to the 6th leaf sampling, from which the highest population was recovered. The recovered population of CIX485^{Rif} decreased from the 6th leaf sampling to the spike sampled at soft dough. BLS symptoms were first observed on the 4th leaf samples and disease incidence and severity generally increased with crop development, although the disease

severity never exceeded 1.1% (Figure 3.4). Symptoms in the non-inoculated barley plots were first observed on the 5th leaf samples, though disease incidence and severity were lower than in inoculated plots (data not shown).

In the dryland field, CIX485^{Rif} was not isolated from the barley flag leaves or the spikes at soft dough stage. CIX485^{Rif} was recovered from all samplings from the 3rd leaf to the 6th leaf, with the population recovered ranging from 1.43 $\times$ 10^5 to 2.01 $\times$ 10^7 CFU/g (Figure 3.4). The lowest population of rifampicin-resistant *X. translucens* pv. *translucens* was recovered from the 4th leaf samples, while the 6th leaf samples had the highest population. Symptoms were only observed on the 5th, 6th, and flag leaf samples and disease incidence decreased between each of the subsequent sampling points. Disease severity was less than 1.0% on these leaf samples. Mild symptoms of BLS were observed on the flag leaf samples, though CIX485^{Rif} was not recovered from the flag leaves (Figure 3.4). Some BLS symptoms were also observed in the non-inoculated barley plots on the 5th and 6th leaf samples (data not shown).

3.4 Discussion

In this study, rifampicin-resistant strains of *X. translucens* pv. *undulosa* and *X. translucens* pv. *translucens* associated with wheat and barley seed, respectively, were shown to colonize the tissues of the plant throughout the plant's development. Tissue culture boxes placed in growth chambers presumably provided a favorable warm and humid environment for *X. translucens* to colonize and infect host tissues (Bamberg 1936; Duveiller et al.

1997). Under these conditions, the transmission rate from the seed into the young wheat and barley seedlings was 100%.

Similarly, the transmission rate from infested wheat and barley seed into maturing plants was 100% in conditions that favor the dispersal and colonization of the pathogen, such as was observed in the mist-irrigated field. Under these conditions, CIX480^{Rif} and CIX485^{Rif} were both recovered from all tissues sampled in this study from the 3rd leaf to the soft dough growth stage. In contrast, CIX480^{Rif} was only recovered from a single replication of the 4th, 5th, and 6th leaf samples of wheat in the dryland field. CIX485^{Rif} was recovered from barley at all growth stages sampled in the dryland field, except at flag leaf and soft dough. The 2022 growing season in St. Paul, MN was very dry, precipitation was 182 mm below average (NOAA 2023). The differences in strain recovery between the two field conditions suggests that moisture is particularly important for *X. translucens* pv. *undulosa* associated with wheat seed, facilitating the ability to infect seedlings, colonize developing host tissues, and maintain a population in those tissues as the plant matures. We observed the BLS symptoms on wheat to increase over time in the mist-irrigated field, though symptoms were not observed in the dryland plots. Moisture appeared to play a less important role in the ability of *X. translucens* pv. *translucens* to colonize barley. The pathogen population and disease development were lower on barley in the dryland versus the mist-irrigated plots, but the pathogen was still recovered from the 3rd leaf to the 6th leaf samplings. Moisture has been indicated to support colonization and

infection, but also in the movement of bacteria on leaves, within a plant, and from plant-to-plant (Bamberg 1936; Duveiller et al. 1997; Duveiller & Maraite 1995). This study found that moisture likely plays a role in the development of BLS from seedborne inoculum, but the significance appears to differ between the wheat and barley pathosystems.

Where *X. translucens* is localized in and/or on wheat and barley seed is unknown. In leaves, *X. translucens* pv. *undulosa* colonizes the mesophyll tissue and does not colonize the vascular tissue, whereas *X. translucens* pv. *translucens* can colonize both the vascular and mesophyll tissue (Bragard et al. 1997; Gluck-Thaler et al. 2020; Pesce et al. 2017; Sapkota et al. 2020). The different lifestyles of these two pathogens suggests that the colonization of seed also differs between *X. translucens* pv. *undulosa* and *X. translucens* pv. *translucens*. The location of the bacteria in the seed may affect how the environment influences the survival of the bacteria in association with the seed and the subsequent colonization and infection of the host plant. How long bacterial cells remain viable over time, or the efficacy of seed treatment applied with the goal of reducing primary inoculum, would also be impacted by where in the seed tissues the bacterial cells reside.

Since the seed in this study was artificially inoculated, our results may not fully represent how a natural infection may develop. It would be informative to examine the seed harvested from the plants grown from the inoculated seed in field experiments to determine if rifampicin-resistant strains could be recovered

from the subsequent generation of plants. The seed from a second generation would more closely mimic a natural infection and provide insights into the ability of *X. translucens* to survive in association with cereal crops over multiple generations. Clearly, more research is needed to understand *X. translucens* localization in and/or on wheat and barley seed and how the environmental conditions impact these bacterial pathogens.

This study demonstrated that BLS can be transmitted from both wheat and barley seed when associated with *X. translucens* pv. *undulosa* and *X. translucens* pv. *translucens*, respectively. It also shows that the pathogen can colonize new plant tissues throughout the plant's life. This work also highlights the importance of environmental conditions in the development of BLS epidemics and supports our understanding of how the pathogenesis of BLS in wheat and barley differ in ways that have significance for disease management practices.

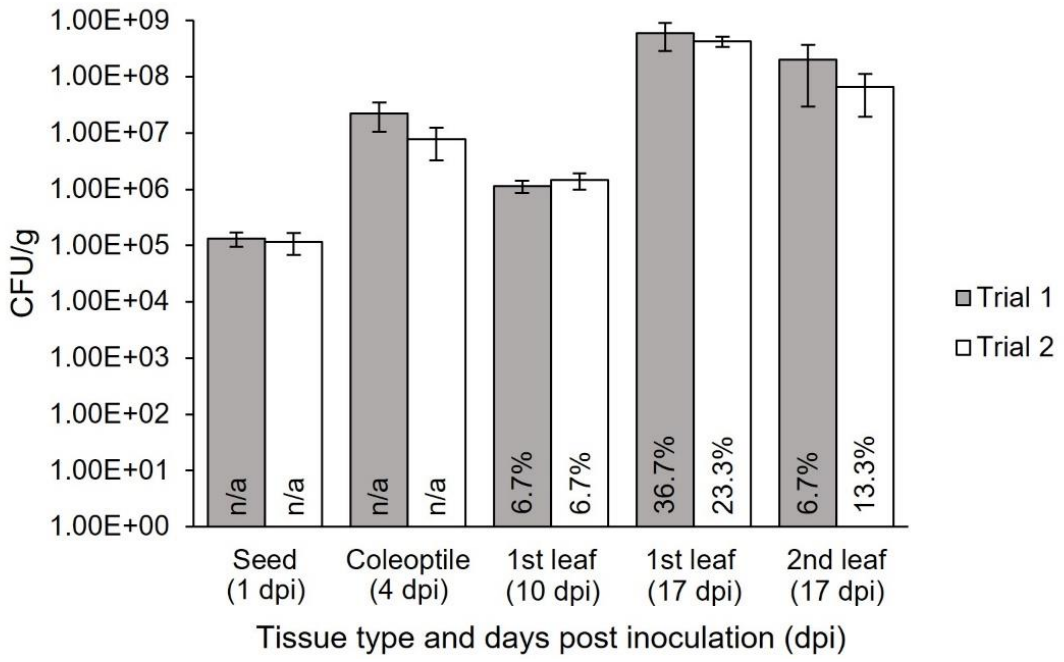


Figure 3.1 Colony forming units per gram of tissue (CFU/g) of rifampicin-resistant *X. translucens* pv. *undulosa* recovered from seed, coleoptile, primary leaf (1st leaf), and second leaf tissues sampled at one of four time points (days post inoculation (dpi)). Wheat ('Mayville') seed was inoculated with the rifampicin-resistant *X. translucens* pv. *undulosa* strain CIX480 by vacuum infiltration and placed on 1% water agar in sterile tissue culture boxes for up to 17 dpi. Two trials were conducted. The percentages within each bar represent the percent of leaves that had water-soaking symptoms at the time of sampling; n/a = not assessed. Error bars represent the standard error of CFU/g value across three replications.

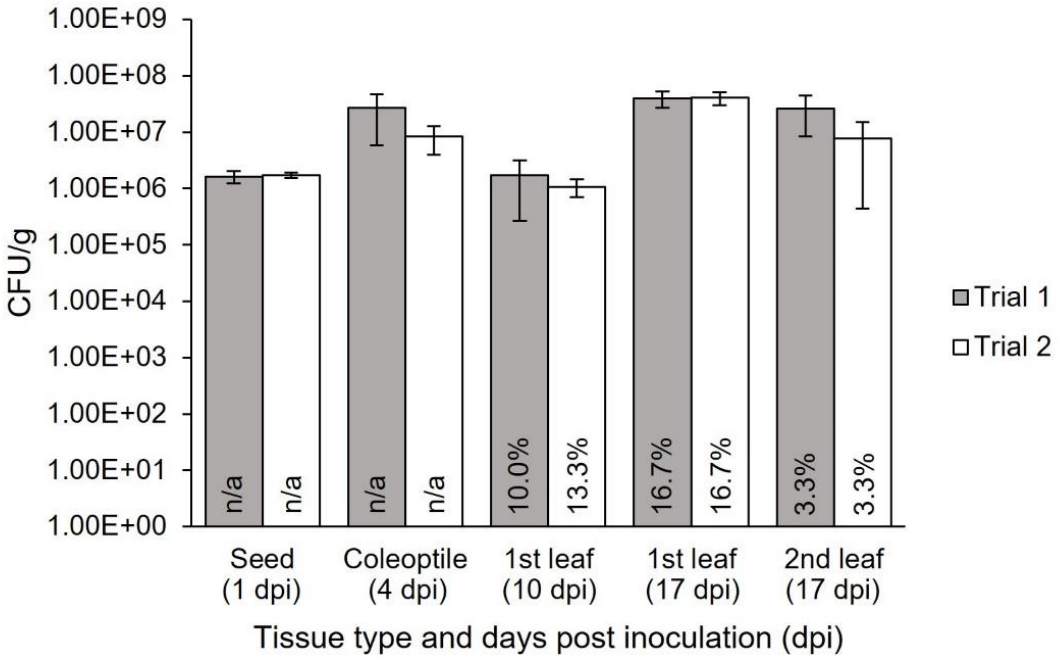
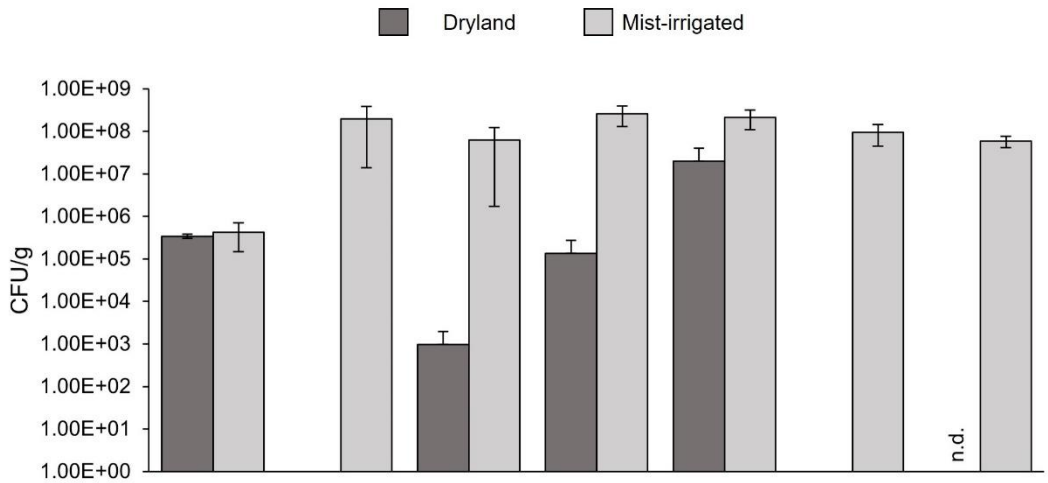
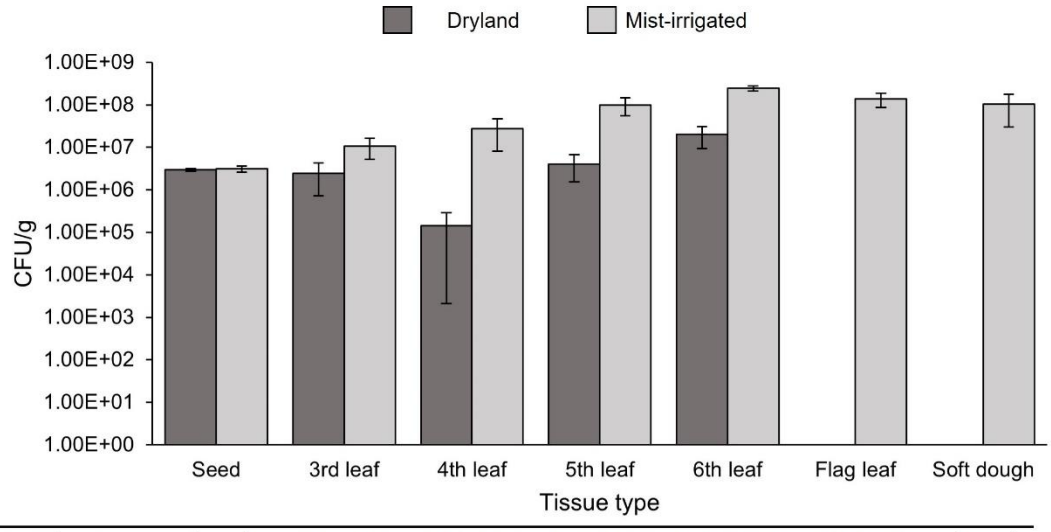


Figure 3.2 Colony forming units per gram of tissue (CFU/g) of rifampicin-resistant *X. translucens* pv. *translucens* recovered from seed, coleoptile, primary leaf (1st leaf), and second leaf tissues sampled at one of four time points (days post inoculation (dpi)). Barley ('Pinnacle') seed was inoculated with the rifampicin-resistant *X. translucens* pv. *translucens* strain CIX485 by vacuum infiltration and placed on 1% water agar in sterile tissue culture boxes for up to 17 dpi. Two trials were conducted. The percentages within each bar represent the percent of leaves that had water-soaking symptoms at the time of sampling; n/a = not assessed. Error bars represent the standard error of the CFU/g value across three replications.



Disease Incidence	n/a	n/a	0.0	3.3	0.0	3.3	0.0	63.3	0.0	96.7	3.3	93.3	n/a	n/a
Disease Severity	n/a	n/a	0.0	1.0	0.0	0.2	0.0	7.0	0.0	9.5	0.03	18.5	n/a	n/a

Figure 3.3 Colony forming units per gram of tissue (CFU/g) of rifampicin-resistant *X. translucens* pv. *undulosa* recovered from wheat seed at planting, 3rd leaf, 4th leaf, 5th leaf, 6th leaf, and flag leaf samples, and spikes at the soft dough growth stage; n.d. = no data. Wheat ('Mayville') seed was inoculated with the rifampicin-resistant *X. translucens* pv. *undulosa* strain CIX480^{Rif} by vacuum infiltration and planted in either a dryland or a mist-irrigated field. Error bars represent the standard error across three replications. In the table below the bar chart, each column is associated with the tissue type and field type identified in the bar directly above. Disease incidence is defined as the percentage of leaves that exhibited BLS symptoms and disease severity as the mean percentage of diseased leaf area from the three replications; n/a = not assessed.



Disease Incidence	n/a	n/a	0.0	0.0	0.0	3.3	20.0	30.0	13.3	36.7	3.3	36.7	n/a	n/a
Disease Severity	n/a	n/a	0.0	0.0	0.0	0.7	0.5	1.0	0.8	1.1	0.03	0.6	n/a	n/a

Figure 3.4 Colony forming units per gram of tissue (CFU/g) of rifampicin-resistant *X. translucens* pv. *translucens* recovered from barley seed at planting, 3rd leaf, 4th leaf, 5th leaf, 6th leaf, and flag leaf samples, and spikes at the soft dough growth stage. Barley ('Pinnacle') seed was inoculated with the rifampicin-resistant *X. translucens* pv. *translucens* strain CIX485^{Rif} by vacuum infiltration and planted in either a dryland or a mist-irrigated field. Error bars represent the standard error across three replications. In the table below the bar chart, each column is associated with the tissue type and field type identified in the bar directly above. Disease incidence is defined as the percentage of leaves that exhibited BLS symptoms and disease severity as the mean percentage of diseased leaf area from the three replications; n/a = not assessed.

CHAPTER 4: A rapid molecular assay to detect and quantify viable *X. translucens* pv. *undulosa* cells in wheat seed

4.1 Introduction

Xanthomonas translucens pv. *undulosa* is an economically significant pathogen causing bacterial leaf streak (BLS) in wheat (*Triticum aestivum*). Yield losses have been reported up to 60% in North Dakota with an estimated loss of $8 million U.S. dollars in 2020 (Friskop et al. 2022). BLS is generally considered to be a seed transmitted disease, however the importance of seed as a primary source of inoculum and the conditions necessary for seed transmission are still unclear.

Molecular diagnostics allow for rapid detection, and in some cases quantification, of plant pathogens. Recently, two quantitative PCR (qPCR) protocols have been developed for *X. translucens* (Clavijo et al. 2022; Hong 2021). Neither of these qPCR assays distinguish *X. translucens* pv. *undulosa* from *X. translucens* pv. *translucens*, though *X. translucens* pv. *translucens* infects barley and not wheat (Jones et al. 1917) so they can be distinguished using host range assays. Quantitative PCR can be used to estimate the pathogen population in a sample; however qPCR cannot distinguish living versus dead cells.

Viability PCR (vPCR) has been used to differentiate viable and non-viable bacterial cells by treating samples with a photoactive dye, propidium monoazide (PMA), prior to DNA extraction and qPCR. PMA binds to DNA of dead cells,

following photoactivation, after permeating those cells with compromised cytoplasmic membranes. The bound PMA inhibits DNA amplification during qPCR (Nocker et al. 2006; Sicard et al. 2019; Temple et al. 2013) and with a paired untreated sample, both the viable and non-viable bacterial cells may be quantified. Temple et al. (2013) developed a vPCR assay for the detection of *X. hortorum* pv. *carotae* to quantify living cells following the hot-water treatments of carrot seed, designed to kill the pathogen.

The objectives of this study were to develop a vPCR protocol for *X. translucens* pv. *undulosa* by utilizing a previously described qPCR method (Hong 2021) and validate the vPCR assay on seed harvested from wheat that exhibited BLS symptoms in the field.

4.2 Materials and Methods

4.2.1 Wheat seed samples from naturally and artificially infected fields

Wheat seed, naturally infected with *X. translucens*, was obtained following harvest from the LeCenter location of the 2022 Minnesota on-farm variety trials (MOFVT). The three wheat varieties used in this study all exhibited BLS foliar symptoms and black chaff symptoms when at the milk ripe growth stage. The three wheat varieties used were ‘Buns’ (Peterson Farms Seed), ‘MN Rothsay’ (University of Minnesota), and ‘AP Smith’ (Syngenta/AgriPro).

Wheat seed was also obtained from the St Paul 2022 bacterial leaf streak coordinated nursery (BLSCN) located at the Minnesota Agricultural Experimental Station of the University of Minnesota in St Paul, MN. This nursery was spray

inoculated using a gas-powered backpack sprayer with *X. translucens* pv. *undulosa* at a concentration approximately 1 $\times 10^8$ CFU/ml at tillering (Curland et al. 2020a). Seed was harvested from three plots that had low, medium, and high ratings of BLS on a scale of 0 to 9. The three plots harvested were varieties 'Driver' (low = 3, South Dakota State University), 'Select' (medium = 5, South Dakota State University), and 'LCS Ascent' (high = 7, Limagrain Cereal Seeds) (Curland, R. D., Liu, Z, Ali S., and Dill-Macky, R., *unpublished results*).

All seed was mechanically threshed, dried, and stored in plastic bottles at 4°C for approximately one year, until used for vPCR.

4.2.2 Seed washes and dilution plating

For each seed sample, 3 g of seed were placed into 6 ml of cold (4°C) 0.85% NaCl (wt/vol) and shaken for 20 minutes to wash bacterial cells from the seed. Two 500 µl aliquots of the bacterial suspension generated from each sample were dispensed into clear 1.5 ml microcentrifuge tubes and kept cold at 4°C until treated with PMA. The remaining seed washes were ten-fold serially diluted for quantification to 10^{-3} and 100 µl of each dilution (10^{-1} to 10^{-3}) was plated on to modified Tween Medium B (mTMB), which is a semi-selective medium for *Xanthomonas* spp. (McGuire et al. 1986; Jonathan M. Jacobs, *personal communication*). Dilution plates were incubated at 28°C for 6 to 7 days. The morphologies of colonies on the dilution plates were compared to a pure culture *X. translucens* pv. *undulosa* (CIX40) plated on mTMB. Colonies with a

yellow, slightly mucoid, round, and mounded morphology and surrounded by a crystalline halo were considered to be *X. translucens* pv. *undulosa* and counted.

The colony forming units per gram of seed (CFU/g) was calculated for each dilution (d) using the following calculation adapted from Duveiller et al. (1997): [(wash volume/seed weight) $\times$ (colonies $\times$ 10^{d+1})]. The mean CFU/g was calculated from the plated dilution(s) that generated countable colonies for each sample. In some cases, a plate had too many colonies to count and was excluded from the mean calculation.

4.2.3 PMA treatment

Each vPCR experiment performed included controls used to determine both live and dead cells. Cells from 3-day old bacterial lawns of *X. translucens* pv. *undulosa* strain CIX40 grown on Wilbrink's agar (WBA) were suspended in 0.85% NaCl (wt/vol) solution to obtain an approximate concentration of 1×10^7 CFU/ml (OD_{600} = 0.01). Four 500 µl aliquots were dispensed into clear 1.5 ml microcentrifuge tubes. Two tubes were designated as 'live cell controls' and of these two, one would subsequently be left untreated and the other treated with PMA (PMAxx, Biotium, Inc., Fremont, CA). The other two tubes were designated as 'dead cell controls' and both were placed in a heat block at 95°C for 5 minutes. After the cells in these two tubes were heat killed, one tube was left untreated while the other was treated with PMA.

The PMA treatment, applied with the goal of having PMA bind only to the DNA of dead cells, was adapted from the manufacture's standard protocol for

vPCR. One ‘live cell’ and one ‘dead cell’ control tube were treated with PMA. One tube from each seed wash sample was treated with PMA, while the other was left untreated. PMA treatments were conducted out of direct light, with 1 µl of 20 mM stock solution of PMA being added to the PMA-treated tubes for a final concentration of 50 µM (Temple et al. 2013). 1 µl of sterilized water was added to the non-PMA-treated control tubes. All tubes were incubated in complete darkness at room temperature on a shaker for 10 minutes. Then all tubes were exposed to light by placing them 20 cm below a 2175 lumen LED lamp for 5 minutes.

4.2.4 Standard curve, DNA extraction, and quantitative PCR

To standardize the quantification of cells in a sample, cells from 3-day old bacterial lawns of *X. translucens* pv. *undulosa* strain CIX40, grown on WBA, were suspended in 0.85% NaCl (wt/vol) solution to obtain a concentration of 1×10^9 CFU/ml (OD_{600} = 1.0). Ten-fold serial dilutions were made to provide a series of seven dilutions, down to a final concentration of 1×10^3 CFU/ml. Genomic DNA was extracted from the seven serial dilutions (1×10^9 to 1×10^3 CFU/ml) as described below. DNA from this series of dilutions were used to set the standard curve for quantifying DNA in each of the qPCR assays.

After the appropriate samples were treated with PMA, genomic DNA was extracted from all samples using the DNeasy blood and tissue kit (Qiagen, Germantown, MD) following the manufacture’s protocol for Gram-negative bacteria. Extracted DNA was stored at -20°C until used for qPCR.

A previously developed qPCR protocol, specific for the detection and quantification of *X. translucens* pv. *undulosa* and *X. translucens* pv. *translucens*, was used (Hong 2021). The primers used were CLS-F (5'-AACGAGCGAAGCCGTATG-3') and CLS-R (5'-GCATCCAACTTGGCTACAGT-3'). DNA amplifications were performed using 5 µl of SsoAdvanced Universal SYBR Green Supermix (Bio-Rad Laboratories, Inc., Hercules, CA), 0.4 µl each of 10µM forward (CLS-F) and reverse (CLS-R) primers, 3.2 µl sterile water, and 1 µl of genomic DNA template for a total reaction volume of 10 µl. Cycle parameters for qPCR amplification were as follows: 95°C for 5 minutes, then 40 cycles of 95°C for 15 seconds and 58°C for 45 seconds, followed by a melt curve from 65°C to 95°C in increments of 0.5°C. The qPCR assays were run with the CFX96 real-time detection system and data analysis was done with CFX Maestro 2.3 software (Bio-Rad Laboratories, Inc.). For each sample three biological and three technical replicates were used and each assay was repeated.

4.3 Results

4.3.1 Standard curve

Quantitative PCR performed on the DNA extracted from the standard dilution series of pure cultures of *X. translucens* pv. *undulosa* strain CIX40 had a linear relationship ($R^2 = 0.988$) and demonstrated that the qPCR was sensitive to populations of *X. translucens* greater than 1×10^3 CFU/ml (Figure 4.1).

4.3.2 Viability PCR and dilution plating on mTMB

X. translucens pv. *undulosa* was detected in the seed from all three wheat varieties collected from the 2022 MOFVT planted at LeCenter, MN. The mean CFU/g for each untreated sample was always higher than the mean CFU/g for the respective PMA-treated sample. In both assays, 'Buns' had the highest mean CFU/g for both the untreated and PMA-treated samples and 'MN Rothsay' had the lowest mean CFU/g (Table 4.1). The mean CFU/g determined by counting colonies on mTMB plates, following seed washing and dilution, generally corroborated the estimated population of viable cells determined from the paired PMA-treated samples. Variability between the vPCR estimates and dilution plating were less than a 3.5-fold difference, except for the 'MN Rothsay' sample in the second assay of the vPCR. In this instance, the vPCR underestimated CFU/g by nearly 10-fold, as compared to the population calculated from colonies growing on mTMB (Table 4.1).

X. translucens pv. *undulosa* was also detected in seed harvested from all three varieties harvested from the 2022 BLSCN nursery. The mean CFU/g for each untreated sample was always higher than the mean CFU/g for the respective PMA-treated sample. Population estimates of viable cells were similar between the mTMB dilution plates and the corresponding PMA treated samples, and the variability between the mean CFU/g was less than a 3.1-fold difference (Table 4.2).

4.4 Discussion

This study describes a vPCR protocol that can be used to detect and quantify viable *X. translucens* pv. *undulosa* cells. The vPCR assay was successfully used to quantify living *X. translucens* pv. *undulosa* cells from several of wheat seed lots, originating from fields exhibiting both BLS and black chaff symptoms, whether naturally or artificially infected with *X. translucens*. Dilution plating on a semi-selective media for *Xanthomonas* spp. was used to validate population estimates generated by the vPCR.

Seed samples obtained from the BLSCN field were selected to have low, medium, and high levels of BLS foliar symptoms. There was no correlation between the severity of foliar symptoms and the population of *X. translucens* pv. *undulosa* detected in association with the seed using vPCR. BLS severity on the flag leaf has been reported to correlate with the percentage of seed that is infested as well as bacterial populations (Tubajika et al. 1998). Plot scores may not be a sufficient measure to predict CFU/g of infested seed. An intentional research study with a greater diversity of germplasm and replications of the lines included in the study would be needed to identify correlations between foliar symptoms and the populations of viable *X. translucens* cells associated with the seed.

In this study, the seed samples were examined using the vPCR treatment approximately one year after harvest. Untreated samples provided estimates of the bacterial load at the time of harvest by quantifying total DNA in the sample,

whereas PMA-treated samples quantified only the viable cells in the samples. In all cases, the viability of *X. translucens* pv. *undulosa* was lower than the total number of bacterial cells detected after one year of storage. Both the vPCR method and the mTMB dilution plating provided an estimate of more than 1×10^3 CFU/g of living cells in the harvested seed. It has been reported that infested wheat seed with a concentration of $\geq 1 \times 10^3$ CFU/g has the potential to cause severe disease in a field (Forster & Schaad 1987). Our results suggest that the seed, which had been stored for a year, had maintained a pathogen population sufficiently high enough to cause disease had it been planted.

Viability PCR is a rapid method which can be completed in hours, as opposed to dilution plating which takes days, to quantify living cells. The development of a vPCR assay for *X. translucens* pv. *undulosa* is valuable tool that could be applied to test commercial seed lots, used to assess the efficacy of seed treatments, or employed in research studies to further our understanding about the role of *X. translucens*-infested seed plays in the epidemiology of BLS.

Table 4.1 Estimated mean colony forming units per gram (CFU/g) of *X. translucens* present in wheat seed samples from three varieties (Buns, MN Rothsay, and AP Smith) obtained from the Minnesota on-farm variety trial planted at LeCenter MN in 2022. Two assays of a viability PCR were performed for each sample, and an untreated sample was paired with a PMA-treated sample in each instance. For each assay, the mean CFU/g was also calculated by counting colonies grown on mTMB medium. Means for each variety were calculated from the three biological replicates in each assay.

	Mean CFU/g		
Wheat variety	Untreated	PMA-treated	mTMB plating
Assay 1			
Buns	7.11E+05	7.64E+03	2.67E+04
MN Rothsay	3.99E+04	1.25E+03	1.20E+03
AP Smith	5.02E+05	5.42E+03	5.63E+03
Assay 2			
Buns	6.53E+05	1.28E+04	2.00E+04
MN Rothsay	3.80E+04	8.20E+02	8.09E+03
AP Smith	3.24E+05	6.79E+03	1.11E+04

Table 4.2 Estimated mean colony forming units per gram (CFU/g) of *X. translucens* present in wheat seed sampled from three varieties (Driver, Select, and LCS Ascent) obtained from the BLSCN trial planted at the University of Minnesota, St. Paul in 2022. Two assays of a viability PCR were performed for each sample, and an untreated sample was paired with a PMA-treated sample in each instance. For each assay, the mean CFU/g was also calculated by counting colonies grown on mTMB medium. Means for each variety were calculated from the three biological replicates in each assay.

	Mean CFU/g		
Wheat variety	Untreated	PMA-treated	mTMB plating
Assay 1			
Driver	1.45E+05	2.73E+03	1.80E+03
Select	5.47E+06	1.15E+06	7.04E+05
LCS Ascent	2.31E+06	1.31E+05	1.67E+05
Assay 2			
Driver	4.58E+04	3.00E+04	1.75E+04
Select	3.41E+05	4.35E+04	1.33E+05
LCS Ascent	5.58E+04	5.13E+03	3.25E+03

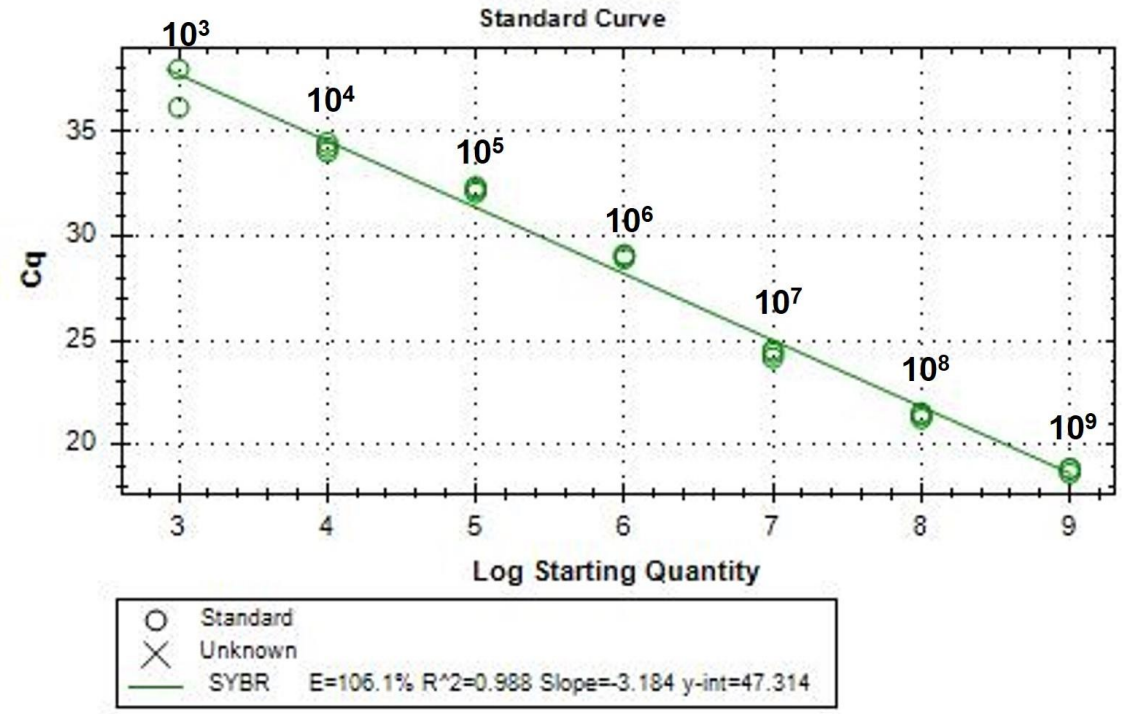


Figure 4.1 Standard curve of the quantitative PCR generated from a ten-fold serial dilution of *X. translucens* pv. *undulosa* strain CIX40, used to estimate population quantities (CFU/ml or CFU/g) in unknown samples. Data and graph were generated using CFX Maestro 2.3 software.

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