POPULATION DIVERSITY, VIRULENCE MECHANISMS OF WHEAT BACTERIAL LEAF STREAK PATHOGEN *XANTHOMONAS TRANSLUCENS* PV. *UNDULOSA* AND DEVELOPMENT OF DISEASE MANAGEMENT TOOLS FOR CANOLA CLUBROOT

A Thesis
Submitted to the Graduate Faculty
of the
North Dakota State University
of Agriculture and Applied Science

By

Ibukunoluwa Bankole

In Partial Fulfillment of the Requirements
for the Degree of
MASTER OF SCIENCE

Major Department:
Plant Pathology

June 2024

Fargo, North Dakota

North Dakota State University
Graduate School

Title

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The Supervisory Committee certifies that this ***disquisition*** complies with North Dakota State University's regulations and meets the accepted standards for the degree of

MASTER OF SCIENCE

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ABSTRACT

Bacterial Leaf streak (BLS), caused by X. *translucens* pv. *undulosa* (Xtu) is a major disease of wheat in North Dakota and surrounding areas and little is known about its local population diversity and virulence mechanisms. In my thesis research, multilocus sequence analysis and typing was used to characterize a collection of historical and contemporary worldwide Xtu strains. It was revealed that Xtu is monophyletic, and they can be grouped according to their geographic location and collection year. Additionally, targeted gene insertion mutation was used to study the role of type III effectors (Xop genes) in pathogen virulence, and two of them (*XopY* and *XopAA*) was shown to be important. In a third project, I conducted field evaluations of Hard Red Spring Wheat (HRSW) along with other commonly grown crops for their effect on the suppression of the resting spores of the canola clubroot pathogen, *Plasmodiophora brassicae*. It was shown that higher spore suppression was obtained for field pea and soybean than HRSW. My research provides important insights into wheat BLS pathosystem and management strategy for clubroot in North Dakota.

ACKNOWLEDGMENTS

I would like to express my deepest gratitude to my advisor, Dr. Zhaohui Liu, Department of Plant Pathology, North Dakota State University for his mentorship, thoughtful advice and direction during my graduate studies. I am grateful to have the opportunity to work in his lab. Under his guidance, I have learned to be a better and capable scientist.

I would like to thank my co-advisor, Dr. Venkata Chapara, Langdon Research and Extension Center, for his guidance and words of encouragement in my applied research work. I extend my sincere thanks to the members of my thesis committee, Dr. Thomas Baldwin and Dr. Jeffrey Schachterle for their valuable feedback, comments and recommendations. I would like to sincerely thank Dr. Gongjun Shi for teaching me hands-on lab techniques, for his valuable inputs and words of encouragements throughout this study. I am grateful to my current and past lab members (Fazal Manan, Md. Mukul Islam, Carly George and Grace Hong) for their friendship, support and the experiences we shared together in the lab and greenhouse.

I thank our collaborators in this study, including Dr. Frank White, University of Florida, Dr. Zhao Peng for the help with the mutagenesis study, Rebecca Curland, University of Minnesota for guiding me through the MLSA analysis, Dr. Luis Del-Rio Mendoza for helping me with the clubroot statistical analysis. Their time and efforts are greatly appreciated.

I would like to extend my heartfelt gratitude to all the faculty members, staffs and graduate students in the Department of Plant Pathology for their support, encouragement, and friendships.

Finally, my deepest appreciation goes to my family, my mother, my brother (Wale Bankole), my sister (Abiola Bankole) for their love and support. Your belief in me has been my greatest motivation especially through challenging times.

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1. LITERATURE REVIEW ON BACTERIAL LEAF STREAK

1.1. Wheat production

Wheat, including bread wheat (*Triticum aestivum* L.) and durum wheat (*T. turgidum* L.), is one of the most important staple foods consumed globally (Tack et al. 2015). Wheat is a rich source of calories, (FAO. 2019) and provides approximately 20% of all calories and protein of food consumed by humans (Ihsan et al. 2016; Obembe et al. 2021). The United States of America produces approximately 8% of the world's wheat crop, and wheat ranks third among U.S. field crops, behind corn and soybeans, in production and gross farm receipts, with a total production of 1.8 billion bushels from a harvested area of 37.3 million acres in 2023/24 (Economic Research Service, USDA, updated 10/27/2023).

North Dakota is one of the top two producers of wheat in the U.S. with a leading production of about 307.85 million bushels in 2023 (National Agricultural Statistics Service, USDA, 01/2024). North Dakota produces mainly durum and spring wheat and has historically been the nation's top producer of durum and spring wheat (Leistritz and Bangsund 1995). Spring wheat dominates North Dakota wheat production, accounting for about 86 % of all wheat productions (National Agricultural Statistics Service, USDA 2023). While durum and winter wheat typically have accounted for about 11% and 2% of all wheat produced in the state, respectively.

1.2. Bacterial leaf streak (BLS)

Bacterial leaf streak (BLS) is a common disease in almost all wheat growing regions in the world (Sapkota et al. 2020). BLS was first reported on barley in 1917 (Jones et al. 1917) and on wheat in 1919 (Smith et al. 1919), and over the years leaf streak disease had also been reported on triticale, rye, oat and brome grass (Reddy et al. 1924; Hagborg 1942; Fang et al. 1950; Cunfer and Scolari 1982). BLS on wheat and barley are caused by *Xanthomonas translucens* pv. *undulosa*

(Xtu) and *X. translucens* pv. *translucens* (Xtt), respectively (Adhikari et al. 2012; Curland et al. 2018). The occurrences of BLS have been sporadic across regions and years, however, since 2008 BLS has dramatically increased in the Upper Midwest of the United States where weather conditions are typically cool and dry (Adhikari et al. 2012; Curland et al. 2018; Kandel et al. 2012; Sapkota et al. 2020). This region is a major producer of hard red spring and durum wheat, and thus, BLS has caused great concern to the wheat production in this region.

1.2.1. Signs and symptoms

The symptoms of BLS disease can be observed on the leaves and spikes (Sapkota et al. 2020). Early symptoms appear as small water-soaked lesions primarily on the leaf tissue. These lesions expand and progress parallel with leaf veins, becoming chlorotic and having a greasy appearance (Ledman et al. 2023). As environmental conditions become favorable, the disease progresses from the infection point toward the leaf tip and down toward the leaf sheath. Streaked lesions will then coalesce to expand across the width of the leaf blade, eventually becoming translucent necrotic lesions (Jones et al. 1917; Sapkota et al. 2020; Ledman et al. 2023). The initial signs of the pathogen can appear as milky droplets and later as yellow exudates or as ooze present in and around leaf lesions under warm and humid conditions (Bamberg 1936; Jones et al. 1917). On the spike, symptoms of BLS are referred to as black chaff. This refers to the dark-purple streaks on the glumes, with a characteristic banding pattern of dark discoloration alternating with healthy tissue on the awns (Bamberg 1936; Smith et al. 1919). Although not as common as on the leaves, bacterial ooze may be present on the glumes of infected spikes (Smith et al. 1919; Osdaghi et al. 2023).

1.2.2. Distribution and economic importance

BLS diseases have a global distribution (Duveiller et al. 1997). BLS has been reported in almost all continents, including North America (United States, Canada, and Mexico), South America (Brazil, Peru, and Argentina), Asia (China, Japan, India, and Iran), Africa (Ethiopia, Kenya, and Israel), Australia, and most parts of Europe (France, Russia). Although the disease has been reported to be sporadic, epidemics of BLS diseases have occurred in several major wheat growing regions in the Upper Midwest regions of the United States, including North Dakota, South Dakota, Minnesota, and Idaho which is likely due to the favorable environmental conditions, buildup of primary inoculum and high susceptibility of current major cultivars (Jones et al. 1917; Smith et al. 1919; Adhikari et al. 2012).

Significant yield reductions occur when the bacterium colonizes flag leaves of susceptible varieties, leading to high disease severities, and a drastic reduction in photosynthetic activity (Friskop et al. 2023). Yield losses also occur from a reduction of grain test weight and/or the number of grains per spike (Duveiller et al. 1997; Waldron 1929). Early previous studies on yield loss have been reported to be 10% or less, but severe infections cause as high as a 40% yield loss from severely diseased fields. (Forster et al. 1986; Duveiller et al. 1997). Recently, in North Dakota, a study done by Friskop et al. (2023) demonstrated that BLS can cause yield loss of up to 60% in highly susceptible varieties, estimating up to $8 million U.S. dollars in economic losses.

1.2.3. Disease cycle and epidemiology

Duveiller et al. (1997) proposed a general disease cycle for wheat-BLS, however, many aspects about pathogen survival and disease initiation remain unclear. The most important source of pathogen inoculum is considered to be seed because the bacteria can be detected in and isolated from seeds harvested from the infected field (Sapkota et al. 2020). Although, there have been

several reports of low transmission from wheat seeds to seedlings (Bamberg 1936; Boosalis 1952; Braun 1920; Tubajika et al. 1998; ledman et al. 2023), the exact rate of bacterial transmission from the infected seeds to the next crops has not been clearly investigated. The bacterium can also survive on crop debris, and grassy weeds and can serve as a source of primary inoculum (Forster and Schaad 1989; Curland et al. 2018; Ledman et al. 2021, 2023). However, reports have shown that bacterial pathogens cannot survive very well in soil and crop debris of annual crops, suggesting crop residues are not a significant source of inoculum (Boosalis 1952; Milus and Mirlohi 1994; Duveiller et al. 1997). Although the exact conditions conducive to BLS development are not well known, warm and humid conditions are thought to be important for BLS development because the disease has been found more in wet seasons or in sprinkler-irrigated fields with warm temperatures (Sapkota et al. 2020).

1.2.4. Disease management

Management of BLS in cereal crops is difficult because not much research has been done to develop effective controlling methods. Crop rotation is ineffective because the pathogen cannot survive for long in crop debris or soil (Duveiller et al. 1997). Since seed is considered a primary source of inoculum, planting pathogen-free seed may be an effective way to reduce disease incidence (Duveiller et al. 1997). Seed disinfection methods including physical (heat) or chemical methods have been shown to reduce the bacterial concentrations in the infected seeds (Atanasoff and Johnson 1920; Forster et al. 1990). However, reports from these treatments have been inconsistent and cannot completely eliminate bacteria in the seeds nor stop the spread of BLS inoculum between fields (Duveiller et al. 1997). No effective means of chemical control have been identified, although these studies have been limited (Sapkota et al. 2020; Lux et al. 2020). Copper based compounds have been used to manage other bacterial pathogens, as both seed and

leaf protectants, but the use of copper on crops with large acreage poses a number of risks, including phytotoxicity, altering the soil microbiota (Lamichhane et al. 2018), and does not protect yield (Lux et al. 2023).

Planting resistant cultivars is the best approach to BLS management. However, the majority of wheat cultivars cultivated in mid-west region is moderately to highly susceptible to BLS, and a large amount of variability exists in susceptibility among commonly grown wheat varieties (McMullen and Adhikar 2011; Lux et al. 2020) with only a few having partial resistance. Thus, breeding varieties of spring wheat with improved resistance to the pathogen could reduce the BLS incidence and epidemics.

1.2.5. Identification and mapping of host resistance

Genetic resistance is the most effective and economical control strategy for BLS of wheat. Disease screenings to identify sources of resistance to BLS have been conducted under greenhouse conditions or field conditions on a diversity of wheat germplasm from different sources including, commercially grown varieties, breeding lines, landraces, and related species (Hagborg 1974; Akhtar and Aslam 1986; Duveiller et al. 1993; Alizadeh et al. 1994; Milus and Mirlohi 1994; El Attari et al. 1996; Milus et al. 1996; Tillman et al. 1996; Adhikari et al. 2011, 2012b; Kandel et al. 2012, 2015). The results from these several screenings showed that a low percentage of resistant accessions or lines are present in wheat germplasms, and the majority of the lines has only partial resistance, some of which could serve as potential to improve BLS resistance in wheat cultivar (Tillman et al. 1996; Adhikari et al. 2011; Kandel et al. 2012). For example, the wheat lines identified with partial resistance included ‘Pavon 76’ and ‘Mochis’, 88, and ‘Thornbird’ (Duveiller et al. 1993), ‘Terral 101’ (Milus and Mirlohi, 1994), Daqingshan no.3, Daqingshan No.4, MN 81319, GP 5012 (Tillman et al. 1996), and ‘SD4205’ (Kandel et al. 2012). Several triticale (×

Triticosecale) lines have also been shown to have high levels of resistance to BLS (Cunfer and Scolari 1982; Johnson et al. 1989; Sapkota et al. 2018). Four lines, including UP 7th ITSN#20, UPT 72142, M2A-Bgc, and Siskiyou were identified by Cunfer and Scolari (1982) during the evaluation of 35 triticale lines.

BLS resistance has been shown to be quantitative, 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 and Harrison 1996). Using classic genetic analysis to characterize resistance in three partially resistant varieties, Duveiller et al. (1993) identified a total of five genes: *Bls1*, *Bls2*, *Bls3*, *Bls4*, and *Bls5* with *Bls1* present in all three partially resistant wheat cultivars and having the largest effect. Over the years, Quantitative trait locus (QTL) analysis and genome-wide association mapping efforts have been done to locate BLS resistance genes in various wheat chromosomes (Adhikari et al. 2012; El Attari et al. 1996; Kandel et al. 2015; Ramakrishnan et al. 2019). The high level of resistance in the triticale line "Siskiyou" shown to be controlled by a major gene *Xct1* (Johnson et al. 1989) have been mapped to the rye chromosome 5R using recombinant bred line triticale population (Wen et al. 2018).

1.3. *Xanthomonas translucens*

X. translucens are a group of gram-negative bacteria, that causes disease on cereals and grasses. First isolated from barley, it was named *Bacterium translucens* by Jones et al. (1917) due to its translucent symptoms on the leaves. Smith et al. (1919) later isolated a similar bacterial pathogen on wheat and called the organism *B. translucens* var. *undulosum*. Over the later years, closely related bacterial pathogens to the wheat and barley pathogens were identified from other cereal crops, such as rye, triticale, oat and many Poaceae grass species which were integrated into

the genus *Xanthomonas* (Hagborg 1942). Taxonomy for this group of bacteria has undergone quite some changes in the last century (see below).

Under the transmission electron microscope, they are non-spore forming, rod-shaped, 0.5–0.8 × 1.0–2.5 μm in size and contain a single polar flagellum (Sapkota et al. 2020). They usually form pale yellow colonies on many nutrient agar media such as Wilbrink's agar (WBA). Several other selective media including KM-1, XTS and WBC have also been developed for isolating *X. translucens* from seeds and leaf tissues (Duveiller et al. 1997; Osadaghi et al. 2023).

1.3.1. Classification and nomenclature

Early taxonomy was based on pathogenicity tests and host range. Thus, due to the high levels of morphological similarities and overlapping host ranges, the classification of *X. translucens* has been confusing over the years and have undergone many revisions in the previous years. Up until the 1930s, the genus *Bacterium* was used to describe the non-sporing rod-like bacteria. The genus *Xanthomonas* was first established to include the species *X. translucens* that caused BLS disease on small grains by Dowson (1939). Hagborg (1942) later re-classified *X. translucens* into five formae speciales (f. sp.) based on their observed hosts of origin f. sp.; *hordei* (barley), *undulosa* (wheat, barley, and rye), *hordei*-avenae (barley and oat), *secalis* (rye), and *cerealis* (wheat, barley, rye, and oat). Later, Fang et al. (1950) distinguished five formae speciales including f. sp. *hordei* (barley), *undulosa* (wheat), *secalis* (rye), *cerealis* (*B. inermis*), and *phleipratensis* (timothy). The *X. translucens* f. sp. *cerealis* was given its name to strains that naturally occur on smooth bromegrass and quack grass but can infect wheat, barley, rye, and oat. The use of *forma specialis* was replaced by pathovar because *Xanthomonas* species have very similar morphological, physiological and biochemical characteristics and could only be differentiated by host range. Thus, Dye and Lelliott (1974) classified all *Xanthomonas translucens*

on cereals into the species of *X. campestris* as different pathovars, including pv. *translucens*, *undulosa*, *cerealis*, *hordei*, and *secalis.* Accordingly, closely related bacterial pathogens that cause bacterial wilt in forage grasses were placed into *X. campestris* as pvs. *graminis*, *phlei*, *poae*, and *arrhenatheri* (Egli and Schmidt 1982; Van den Mooter et al. 1987).

Technological advancements, including DNA-DNA hybridization, modern biochemical and molecular tools, such as protein electrophoresis, and gas chromatography of fatty acids, revealed a large amount of heterogeneity within the *X. campestris* group (van den Mooter 1987; Azad and Schaad 1988; Kersters et al. 1989; Vauterin et al. 1992). This led to the re-classification of the genus *Xanthomonas.* The species *X. translucens* was then re-established to include all Xanthomonades that cause disease on cereals and forage grasses. The two main groups identified within *X. translucens* were called the 'translucens' group, which causes BLS of small grains, including pathovars *undulosa*, *translucens*, *cerealis*, *hordei*, and *secalis*, and the 'graminis' group, including pv. *graminis*, *poae*, *phlei*, and *arrhenatheri*, which causes bacterial blight of forage grasses (Vauterin et al. 1992, 1995). With the aid of more sensitive genetic and biochemical methods such as such as sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of proteins, gas chromatography of fatty acid methyl esters (FAME), and amplified fragment length polymorphism (AFLP), three pathogenicity groups were identified within the cereals group: *X. translucens* pv. *cerealis*, *X. translucens* pv. *translucens*, and *X. translucens* pv. *undulosa* (Vauterin et al. 1992; Yang et al. 1993; Bragard et al. 1995; Peng et al. 2016; Langlois et al. 2017). Recently, evidence from phylogenies based on multilocus sequence analyses of housekeeping genes, and genome sequence data, have further separated *X. translucens* into three major groups; Xt-I which are the cereals pathovars including, pvs *undulosa, translucens, secalis*, and *hordei*. Xt-II include the pathovar *cerealis* pathogenic on cereals and grasses while the last group, Xt-III

include pvs *arrhenatheri, graminis*, *phlei*, *phleipratensis, and poae* which are pathogenic on only grasses (Curland et al. 2018; Ledman et al. 2021; Goettelmann et al. 2022).

1.3.2. Diversity

Understanding of the genetic diversity of bacterial plant pathogens is a key component for successful breeding and deployment of host plant resistance (Leung et al. 1993). *X. translucens* pv. *undulosa* (Xtu) and *X. translucens* pv. *translucens* (Xtt) are the major pathogens associated with recent cereals BLS outbreaks in the Upper Midwest of the United States (Adhikari et al. 2011; Curland et al. 2018, 2020; Ledman et al. 2021). Although there is an overlapping host range between these two pathovars, some key aspects of genetic diversity vary between them. In several, phylogenetic studies, Xtu forms a single lineage and is genetically more homogenous than Xtt which usually form three distinct lineages: Xtt A, B and C (Curland et al. 2018, Ledman et al. 2021). Xtt A is more closely related to the Xtu group than Xtt groups B and C. Multilocus sequence typing revealed that the two most commonly identified STs distributed in the Upper Midwest are ST 36 and ST 38 for Xtu and are ST 23 and ST 43 for Xtt (Curland et al. 2018, Ledman et al. 2021). A global-scale phylogeographic analysis using *X. translucens* strains isolated in Iran and available sequences from six different countries denoted the multilocus haplotype (MH2) as the founder for Xtu, which included many strains from North America as well as two reference pathotype strains from Canada: *X. translucens* pv. *undulosa* (LMG 892^{T}) and *X. translucens* pv. *secalis* (LMG 883^{T}) isolated from *Triticum turgidum* and rye respectively (Khojasteh et al. 2019).

Xtu has been shown to be monophyletic in a number of studies, either within a regional collection of *X. translucens* isolated from wheat and barley (Curland et al. 2018) or in a whole-genome single-nucleotide polymorphism analysis of *X. translucens* from different hosts, countries, and years (Charkhabi et al. 2017). However, Adhikari et al. (2011) investigated the

genetic diversity of Xtu strains from five locations in North Dakota using rep- PCR and insertion sequence-based (IS)-PCR techniques. The genotyping revealed a high level of molecular polymorphisms among Xtu strains, but no correlation was found between pathotypes and haplotypes (Adhikari et al. 2011).

1.3.3. Detection and diagnosis

In the previous decades, characterization of *Xanthomonas translucens* pathogens have been done using various methods, including dilution plating with the use of selective media, seedling infection and pathogenicity tests, DNA-DNA hybridization, membrane protein assay, biochemical and serological tests (Azad and Schaad 1988; Bragard et al. 1995; Elrod and Braun 1947; Vauterin et al. 1995; Rademaker et al. 2006). Colony morphology on a general or semi-selective media is not a reliable diagnostic tool for *X. translucens* pathogens because they resemble other *Xanthomonas* species or yellow- pigmented saprophytic bacteria commonly associated with plants or seeds. DNA-based methods such as polymerase chain reaction (PCR) protocols have been developed to detect *X. translucens* from other Xanthomonads from infected field seeds but could not differentiate the *X. translucens* pathovars (Maes et al. 1996). Diagnostic Loop- Mediated Isothermal Amplification (LAMP) primers that amplify targeted nucleotide sequences at a constant temperature, have been developed to distinguish *X. translucens* pathovars in the translucens group (causing disease on cereals) from those in the graminis group (causing disease on forage grasses) (Langlois et al. 2017), however they could not differentiate between the highly related pathovars: *X. translucens* pv. *translucens* (Xtt) and *X. translucens* pv. *undulosa* (Xtu). Recently, DNA markers that specifically identify Xtt from Xtu isolated from plant materials have been developed (Alvandi et al. 2023; Hong et al. 2023). Román-Reyna et al. (2023) developed a multiplex PCR with a set of six primers to detect and differentiate pathovars in *X. translucens.*

1.3.4. Genomic sequences

Genome sequencing and comparative genomics have aided in the classification of *X. translucens* strains, and also help to identify genes that are critically involved in pathogenicity and virulence. The first genome sequence of *X. translucens* was obtained from *X. translucens* pv. *graminis* strain Xtg29 (Wichmann et al. 2013). Many of the sequences of *X. translucens* previously deposited in the NCBI genome GenBank have been sequenced with short-reads platforms and have assembled into contigs (Sapkota et al. 2020). However, in the last decade, using the long reads sequencing techniques such as Oxford Nanopore PromethION or Pacific Biosciences (PacBio), complete genome sequence for various *X. translucens* strains have been obtained and are publicly available. The strains having complete genome sequences include *X. translucens* pv. *undulosa* strains Xtu 4699, P3 and LW16 (Peng et al. 2016, 2019), ICMP 11055 (Charkhabi et al. 2017); *X. translucens* pv. *translucens* strains DSM 18974^{T} (Jaenicke et al. 2016), UPB886 (Roman-Reyna et al. 2020), and DSM 18974^{PT} (Goettelmann et al. 2022), *X. translucens* pv. *cerealis* strains CFBP 2541 and NXtc01 (Pesce et al. 2017; Shah et al. 2019). Seven *X. translucens* strains representing an entire set of genetic diversity of two pathovars Xtu and Xtt in Iran were reported by Shah et al. 2021. These genomic sequence resources are important for us to understand the taxonomy, biology and virulence of *X. translucens*.

1.3.5. Pathogenicity and virulence mechanisms

Plant pathogenic bacteria enter through natural openings of plants, such as stomata and hydathodes, or wounds (Kay and Bonas 2009) followed by the colonization of plant tissues by using sophisticated pathogenicity and virulence systems. In turn, plants utilize the immune system to defend themselves from bacterial pathogens. Hosts and pathogens are continuously intertwined in a co-evolutionary arms race (Abrams 1986; Dawkins and Krebs 1979). The PAMP-triggered

Immunity (PTI) is the first layer of plant immunity that a pathogen encounters, where extracellular receptors known as pattern recognition receptors (PRRs) recognizes conserved microbial-associated molecular patterns (MAMPs), or pathogen-associated molecular patterns (PAMPs), such as flagellin from the pathogen. This leads to a series of biochemical and structural changes in the host cell, such as cell wall strengthening and oxidative burst (Agrios 2005; Jones and Dangl 2006). However, the pathogen can suppress this first line of defense response by injecting secreted proteins called effectors into a host cell. To counteract that, plant cells have a second line of defense known as effector-triggered immunity (ETI) where intercellular receptors called resistance (R) genes recognize these effectors to induce stronger defense responses often leading to programmed cell death known as hypersensitive response (HR). To overcome ETI, pathogen can mutate their effector being recognized or evolve new effector to cause disease, known as effector-triggered susceptibility (ETS) (Francis et al. 2002; Stavrinides et al. 2008; Dillon et al. 2019; Lindeberg et al. 2012). Plant could evolve with new resistance gene to recognize new pathogen effector leading to ETI again. The cycle of ETS and ETI continues as time goes on.

Type III secretion system (T3SS): Like other Gram-negative bacteria, *X. translucens* utilizes the type III secretion system (T3SS) for pathogenicity and virulence. The T3SS is a syringe-like structure that spans the bacterial cell membranes and plant cell membranes to inject type III effectors (T3Es) which are secreted proteins into host cells to promote infection (Büttner and Bonas 2002, 2010), and mediates the processes of pathogen adaptation to specific host tissue, species, and genotypes (White et al. 2009). T3SS is encoded by the chromosomal hrp (hypersensitive response [HR] and pathogenicity) gene cluster and is highly conserved (Büttner and Bonas 2010; White et al. 2009). In the *X. translucens* species, the core *hrp* cluster consists of 23 genes, with 8 *hrp* genes, 11 *hrc* genes, and 4 *hpa* genes (Wichmann et al., 2013; Pesce et al.

2017; Goettelmann et al. 2022). Mutations of the *hrp* cluster in both *X. translucens* pv. *translucens* and *X. translucens* pv. *undulosa* strains eliminate pathogenicity demonstrating that the T3SS is essential in disease development in barley and wheat (Peng et al. 2016; Pesce et al. 2017).

Transcription activator-like (TAL) effectors : TALES are a unique class of T3Es found only in *Xanthomonas* and some *Ralstonia* strains (Bogdanove et al. 2010). Once delivered into the plant cytoplasm, they are transported into the nucleus, bind to specific DNA sequence(s) called effector-binding elements (EBEs) in the promoter of a particular host gene functioning like a transcription activator (Bogdanove et al. 2010, Timilsina et al. 2020). The transcription of a particular host gene or genes can lead to either susceptible or resistant reactions. A typical TALE consists of T3SS secretion signal in N terminus, the nuclear localization signal (NLS), an acidic activation domain (AAD) in C terminus, and most importantly, the central repeat region (Bogdanove et al. 2010). All TALEs are nearly identical in N and C-terminus sequences but differ in the central repeat region which contains amino acids for DNA binding (Mahfouz et al. 2010, Mak et al. 2012). The TALE central region usually has 17-18 repeats with each repeat typically containing 33-34 amino acid (aa). The amino acids for each repeat are almost identical except the 12th and 13th aa, known as repeat variable di-residue (RVD) which dictate the DNA binding specificity of each TALE (Bogdanove et al. 2010, Mak et al. 2012).

The number of TALE genes is variable within *X. translucens*, which have been identified using high-quality genome sequences. In Xtu, usually 7-8 TALES are identified (Peng et al. 2016, Charkhabi et al. 2017, Goettelmann et al. 2022), while in Xtt, usually 4-8 TALES are identified (Roman-Reyna et al. 2020, Shah et al. 2021, Schachterle et al. 2022, Heiden et al. 2023), except Xtkm33 which has 12 TALES (Khojasteh et al. 2020). So far, only four TALEs from different Xtu strains have been shown to play a significant role in virulence. Tal2 and Tal4b from the Xtu strain

ICMP11055 were shown to individually contribute to virulence on wheat (Charkhabi et al. 2017). Xtu 4699_Tal8 was functionally characterized to induce the expression of the wheat gene TaNCED located on the short arm of chromosome 5B to promote disease susceptibility (Peng et al. 2019). Interestingly, the Xtc NXtc01_ Tal1, but not Tal 2 was shown to significantly contribute to virulence (Shah et al. 2019).

Non-transcription activator-like (Non-TAL) effectors: *Xanthomonas* outer proteins (Xops) are the non-TALEs T3Es delivered to the host by the T3SS and grouped into 53 classes from XopA to XopBA (Büttner and Bonas 2010; White et al. 2009). A few effectors were named as avirulence proteins, for example AvrBS1 to AvrBS2 because they can be recognized by plant resistance genes. With the advancements in genome sequencing, many Xop effectors have been revealed in *X. translucens,* and 21 to 36 classes of Xop effectors were found in a given strain (Goettelmann et al. 2022; Heiden et al. 2023; Peng et al. 2017; Shah et al. 2021). Using comparative genomics, highly conserved Xops have been identified between strains within each pathovar and across pathovars, but a few Xops are specific to individual pathovars. In *X. translucens*, the core sets of Xops are XopZ, XopX, XopR, XopQ, XopP, XopN, XopL, XopK, XopF, and AvrBS2. Specific Xops to Xtt are XopAJ, XopAL1, XopE3, and XopM, while XopE1 and XopE5 are specific to Xtu (Peng et al. 2017; Shah et al. 2021; Goettelmann et al. 2022).

These effectors are key virulence factors because when translocated into the host cell, they can target the different pathways of the host, allowing the pathogen to acquire nutrients or evade or suppress host defenses. A few of these Xops have been demonstrated to have major contributions to virulence in *Xanthomonas* pathosystems. XopR, XopN and XopZ, have been demonstrated to be individually required for full virulence in *X. oryzae* pv. *oryzae* (Akimoto-Tomiyama et al. 2012; Sinha et al. 2013; Song and Yang 2010). AvrBS2 and XopN contribute to

full virulence in *Xanthomonas campestris* pv. *campestris* (Jiang et al. 2008) and *Xanthomonas oryzae* pv. *oryzicola* (Xoc) strain GX01 (Liao et al. 2020), while only AvrBS2 contribute to full virulence in another Xoc Chinese strain RS105 (Li et al. 2014). Interestingly, *xopAA* and *xopQ* deletion mutant was shown to have an enhanced virulence of Xoc to the rice cultivars, Jingang 30 (Li et al. 2014) and IR28 (Pei et al. 2010) respectively. Similarly, XopX contributes to *X. campestris* pv. *vesicatoria* virulence in host pepper and tomato plants (Metz et al. 2005).

In addition, several host targets of the Xops have been identified in various plant-*Xanthomonas* pathosystems. For example, XopAA and XopY in *X. oryzae pv. oryzae* interact with the host gene OsSERK1/2 and OsRLCK185 to inhibit rice immunity, respectively (Yamaguchi et al. 2013a and b). While the *X. campestris* pv. *vesicatoria* XopD functions as a SUMO protease that interacts with the transcription factor SlERF4, to inhibit ethylene production and to promote pathogen growth in tomato (Kim et al. 2008). Also, XopQ in *X. campestris* pv. *vesicatoria* suppresses ETI by targeting the tomato 14-3-3 isoform SlTFT4, an important component of ETI (Teper et al. 2014). However, no functional study has been conducted to test the role of these Xops in virulence of *X. translucens* (Goettelmann et al. 2022; Ledman et al. 2023; Liu et al. 2023). Characterization of every individual T3E for its molecular functions will allow us to better understand not only the genetic determinants of host specificity in *X. translucens* but also the host-pathogen interactions for breeding durable resistance against BLS in wheat cultivars.

1.4. Objectives of this study on wheat BLS

Bacterial leaf streak (BLS) disease on wheat has increased drastically over the last decade especially in wheat-growing regions of the United States. The impact of this disease on wheat production has majorly been due to the lack of effective management tools. Host resistance is the most desirable method for the management of BLS disease, as it is sustainable and environmentally

friendly. However, the development of genetic resistance relies on a better understanding of the diversity and biology of the pathogen in a local scale and the host-pathogen interactions. Therefore, this research sought first to increase our understanding on the phylogenetic relationships within different Xtu/Xtt populations and then functionally characterize Xtu Xop effectors for their contribution to BLS development. The overall goal of my research is to provide advanced understanding of disease systems in wheat and barley BLS diseases, which will be useful for breeding durable resistance to these diseases.

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2. LITERATURE REVIEW ON CLUBROOT ON CANOLA

2.1. Canola production

Canola, the trade name for Oilseed rape (*Brassica napus* L.) in the Brassicaceae family (Stefansson and Kondra 1975), is the second largest oil seed crop in the world, providing 13% of the world's oil supply (Raymer 2002). Canola seeds are important sources of oil and is ranking the third most edible oil produced and used worldwide (Economic Research Service, USDA 2023). Canola oil is widely used for cooking, salad, and in making margarine because it has the lowest saturated fat content of all major edible vegetable oils. The United States is one of the top 10 leading producers of canola globally, cultivated in around 2 million acres in 2023 (Economic Research Service, USDA 2023).

North Dakota leads the United States in canola production (Rahmen and Mendosa 2019) accounting for 96% of the nation's production, which is 2% of the world production in 2023. Thus, ND serves as a key contributor to the country's overall output followed by Washington and Montana (Economic Research Service, USDA 2023). The distribution of canola production across these states has been attributed to favorable climatic conditions, including adequate rainfall and temperature patterns conducive to crop growth. In North Dakota, canola cultivation provides farmers with a profitable alternative to other crops, contributing to the overall farm income. The *Brassica napus* varieties are mainly grown in North Dakota, of which the spring type is best adapted to North Dakota conditions (NDSU extension 2022). These varieties have erucic acid content of less than 2% and less than 30 micromoles of glucosinolates per gram of seed and are grown for edible purposes, while the high-erucic acid oil rapeseed (mainly produced in Europe and few places in Canada and the U.S) is grown and used for industrial lubricants.

2.2. Clubroot disease of canola

Clubroot, caused by *Plasmodiophora brassicae* (Woronin), is one of the most destructive diseases of canola and other cruciferous vegetables worldwide (Dixon 2009). Club root on canola was first reported in the Canadian prairies in 2003, and ever since the pathogen has been spreading rapidly in several canola fields (Dokken-Bouchard et al. 2010; Strelkov and Hwang 2014). In the United States, Clubroot on rapeseed (*B. napus*) was first identified in Cavalier County, North Dakota (Chittem et al. 2014). Since then, it has been reported every year especially in the upper northeastern part of North Dakota (NDSU extension 2022) and has become a threat to the oilseed rapeseed production (Chapara et al. 2019a, 2021).

2.2.1. Signs and symptoms of clubroot

The symptoms of club root disease on canola can include wilting, stunting, reduction of seed production, a thin stand and ultimately premature plant death (NDSU extension 2022). However, these above ground symptoms are not the diagnostic features of this disease, as they mimic symptoms from abiotic stresses or infection with other diseases. Clubroot is a soil borne disease that causes swellings, or galls on the plant roots of Brassicae family (Dixon 2009). Examination of roots for galls by digging up from the affected areas is the fastest way to identify the signs of clubroot pathogen (Khangura and Wright 2012). The galls are usually small as they begin to develop but may be up to 1 inch in diameter by the time of maturity. These galls allow for the colonization of the plant's root systems by the clubroot pathogen which obtains moisture and nutrient from the plant root tissues instead (Javed et al. 2023).

2.2.2. Geographical distribution and economic significance

Clubroot has been widely distributed around the world since the 18th century (Olsson 1939; Wallenhammer et al. 2014). Currently, clubroot is reported to be distributed in over 88 countries

around the world (Chapara and Meena 2021). However, historically, the impact of clubroot has been greatest on cruciferous vegetables in Canada, Australia and South America (Howard et al. 2010; Chapara et al. 2019a). Within the last couple of decades, clubroot has emerged as a devastating disease of canola throughout Canada. Recent reports in the international clubroot work groups have also indicated an increase in clubroot of canola in infected fields in the countries such as the United States, Canada, Germany, Poland, France, Japan, South Korea, China, Columbia, India, Nepal, and Czech Republic (Chapara and Meena 2021).

While clubroot is a cause for concern worldwide, experimental studies to determine the yield or economic losses associated with infection of canola have been very limited. Most yield loss estimates are based on anecdotal reports or approximations of grower-expected yields vs. harvested amounts (Botero-Ramírez et al. 2022). In Australia, yield losses in canola were estimated to be around 50% (Donald and Porter 2003), while in western Canada, 30% yield loss were estimated in heavily infested soils (Tewari et al. 2005). In at least one canola field in central Alberta, the clubroot severity was so severe that the crop was not harvested, so the yield loss was effectively 100% (Strelkov and Hwang 2014).

2.2.3. Disease cycle and epidemiology

Plasmodiophora brassicae is a soil borne obligate parasite that requires the plant host to complete its life cycle but thrives in the soil as resting spores. These resting spores are long-lived, with a half-life of about four years, but can survive in the soil up to 20 years (Wallenhammar 1996). Thus, resting spores are the primary source of inoculum on the field. Each resting spore germinates to release one primary zoospore that swims in water films of the soil to infect root hairs by penetration of the cell wall, and cause malformations of the root hairs. However, this primary infection or root hair infection stage do not cause macroscopic symptoms and are not responsible

for significant yield and quality losses (Howard et al. 2010). Within the root hairs, primary plasmodia develop and cleave into zoosporangia, each containing 4–16 secondary zoospores, which then cause secondary infection by infecting the root cortex. Here, the pathogen induces local hypertrophy, resulting in the gall formation (Kageyama and Asano, 2009; Chapara and Meena 2021).

After this secondary infection, the secondary plasmodia develop within the root cortex, while the infected roots become friable, decompose and release the resting spores in large quantities into the soil. Every individual gall of clubroot has the capability of releasing approximately 8 x 10^8 resting spores back to the soil, indicating that the field will never be free of clubroot resting spores if brassica hosts such as canola are exposed to the soil quite often (Hwang et al. 2012). Disease infection and severity is mostly favored by high soil moisture, acidic soil (low pH) and temperatures around 68 to 77F. In addition, poorly drained areas or parts of fields commonly flooded are more prone to infection (NDSU extension 2022). Clubroot spread over significant distances occurs mainly via movement of resting spores in soil or in infected plant material. However, strong wind gusts and flooding also have been identified as capable of transferring spores.

2.3. *Plasmodiophora brassicae*

The club root pathogen *P. brassicae* belongs to the plasmodiophorids, a group of plant pathogenic protists (Neuhauser et al. 2014) which were formally considered as a group of myxomycetes (Fungi and fungi-like). However, the genus *Plasmodiophora* was established for *P. brassicae* and other plasmodiophorids (Woronin 1877) to different them from Fungi. *P. brassicae* and other plasmodiophorids have common features such as chitinous cell wall of the resting spores with Fungi (Muirhead and Pérez-López 2022; Thornton et al. 1991), but have distinctive features

including a multinucleate plasmodium, biflagellate secondary zoospores, uninucleate resting spores, cruciform nuclear division, and lack of filamentous growth which make them clearly divergent from fungi and oomycetes (Bulman et al. 2011; Neuhauser et al. 2014). In the recent years, they have been classified as Phytomyxea in the eukaryotic Rhizaria group (Bi et al. 2019; Neuhauser et al. 2014; Sierra et al. 2016) that includes obligate biotrophic parasites and have a diverse range of host plant hosts. *P. brassicae* infects more than 300 members from the brassica family (Dixon, 2009; Hwang et al. 2012). However, a few non-cruciferous plants such as nasturtium (*Tropaeolum majus* L.), papaya (*Carica papaya* L.), corn poppy (*Papaver rhoeas* L.), and clover (*Trifolium repens* L.) have been listed as hosts of *P. brassicae* (Ludwig-Müller et al. 1999). In the United States several brassica crops grown have been reported as potential hosts of the clubroot pathogen (Tewari et al. 2005; Strelkov et al. 2006).

2.3.1. Genomics and genetics

Being an obligate biotrophic organism, *P. brassicae* is still impossible to grow in axenic culture due to its intracellular growth in the host cells. The first genome of the pathogen was published in 2015 (Schwelm et al. 2015), which was obtained for the European single SSI e3 isolated from a stubble turnip. The genome draft was generated using Illumina short sequencing technologies and assembled into a total size of 24 Mb (Schwelm et al. 2015). Later, using PacBio long sequencing technology and building on the first e3 assembly, a nearly complete assembled genome version of the e3 genome have been achieved (Stjelja et al. 2019), with a small genome size of 25.1 Mb that includes 19 nuclear contigs. Since then, draft genomes of several canola isolates from Canada (Rolfe et al. 2016), Europe (Daval et al. 2019), and China (Bi et al. 2019), which derived from the same club as the e3 isolate have been generated.

Currently, there are now 50 draft genomes deposited publicly in the NCBI database (Javed et al. 2022). Comparative genomics of available genome data reveal features in *P. brassicae* different from other biotrophic plant pathogens such as presence of an incomplete metabolic pathways hence the dependency of the pathogen on the host, and the inability to synthesize fatty acids de novo (Schwelm et al. 2015), hence the high amounts of lipid structures in the resting spores (Bi et al. 2016) that serve as lipid reservoir for the clubroot pathogen until it can refuel after a host infection. About half of the predicted *P. brassicae* proteins show no similarities to proteins from other species. Also, these proteins lack known functional domains, or have only a predicted unknown or general function (Rolfe et al. 2016; Schwelm et al. 2015; Stjelja et al. 2019).

2.3.2. Pathotypes

The clubroot pathogen have been shown to have differential physiological response on brassicas, of which have been used to differentiate *P. brassicae* isolates as races (Ayers 1957; Williams 1966; Buczacki et al. 1975). In recent times, the term pathotypes have been used instead of races to distinguish between *P. brassicae* isolates with differential virulence profiles in brassica species (Somé et al. 1996; Strelkov et al. 2018). Three pathotyping systems have been widely used for many years to pathotype *P. brassicae* isolates; Williams clubroot differential (Williams 1966), European clubroot differential (ECD) (Buczacki et al. 1975), and Somé clubroot differential (Somé et al. 1996). In the early 2000s, there was an appearance of clubroot-resistant (CR) cultivars in the market (Strelkov et al. 2011). Given the effectiveness of this resistance, the planting of CR canola cultivars soon became the most effective clubroot management tool. Since then, many CR varieties have been generated, by introgression of CR genes from other brassicas like rutabaga or Chinese cabbage (Liu et al. 2018). However, due to the high selection pressure, the resistance in most commercial cultivars became overcome by the emergence of new virulent pathotypes, leading to

the introduction of a new clubroot differential system known as the Canadian Clubroot Differential (CCD) (Strelkov et al. 2018). Currently, based on CCD system, more than 35 different clubroot pathotypes have been identified in Canada, using 13 hosts, one of them being CR *Brassica napus* (canola).

2.3.3. Detection and quantification

A reliable and sensitive diagnostic method is needed for an efficient disease management. The resting spores of *P. brassicae* are small, usually 3 to 5 mm and lack a characteristic ornamentation that aids in quick and accurate identification. Thus, extracting and quantifying resting spores by microscopy can give highly variable results and is time consuming (Ward et al. 2004). Several PCR-based methods have been developed in the last decade, targeting diverse genes in *P. brassicae* to detect the pathogen in infected fields, quantify the resting spores, or differentiate between the pathotypes with different virulence profiles (Czubatka-Bieńkowska et al. 2020; Gossen et al. 2019; Tso et al. 2021, 2022). Using, the nuclear ribosomal DNA (rDNA), standardized assays such as the conventional polymerase chain reaction (PCR) (Li et al. 2013; Zhou et al. 2018), quantitative (q)PCR (Cao et al. 2014; Deora et al. 2015), digital droplet PCR (ddPCR) (Gossen et al. 2019; Wen et al. 2020), loop-mediated isothermal DNA amplification (LAMP) (Yang et al. 2022) have been utilized for quantification of the resting spores in soil.

However, present in the soil are other components such as clay particles, pheonolic compounds, and heavy metals that can inhibit PCR and quantification reliability (Bilodeau 2011). Thus, multiplex TaqMan assays have been developed to quantify inhibition in each sample, together with the target sample using two primers (van Gent-Pelzer et al. 2010). Furthermore, to reduce primer interference, a competitive internal positive control (CIPC) approach have been introduced to the above assay to ensure amplification of nontarget amplicon of exogenous DNA,

with the primer-binding regions identical to those of the target sequence, an internal sequence similar to the target sequence in length and base composition, and a unique region where the probe binds to the amplicon that differentiates the CIPC from the target locus (Deora et al. 2015). This ensures amplification of both the CIPC and the target locus with one primer pair only. This have the advantage of saving time and resources.

2.4. Clubroot management

Disease management of club root is difficult because resting spores of *P. brassicae* can remain viable in the soil for many years (Wallenhammar 1996), and soil inoculum levels increase quickly in the presence of susceptible hosts (Hwang et al. 2012). Canola growers are thus faced with the near impossibility of the removal of *P. brassicae* inoculum from an infested field. Also, this persistence of the resting spores leads to another difficulty for canola growers on how to prevent the pathogen from spreading to other fields (Rampel et al. 2014). A number of individual strategies have been proven useful for clubroot control in vegetable brassicas, however most are not practical or cost-effective in canola, which is typically grown on a much larger scale (Howard et al. 2010; Hwang et al. 2014). An integrated strategy of genetic resistance, cultural practices, biological and chemical control are often proposed for management of clubroot disease (Dixon 2003; Donald and Porter 2009).

2.4.1. Chemical control

Currently, there is almost no or limited data available on effectiveness of pesticides that are available to control clubroot on canola (Chapara and Meena 2021). The use of fungicides has been part of clubroot management strategies since the 1980s (Doyle and Clancy 1987). Several synthetic fungicides, such as fluazinam, pentachloronitrobenzene, metalaxyl-mancozeb, azoxystrobin, difenoconazole, and carbendazim have been tested against the clubroot pathogen.

However, results on effectiveness to control the pathogen have been highly variable depending on the crop, geographical location, and application strategies (Hwang et al. 2012; Liao et al. 2022; Peng et al. 2014). The modes of action for most of these fungicides against infection and symptom development caused by *P. brassicae* remain largely unknown (Peng et al. 2014). In addition, on canola, a soil-drench application of fungicide is not feasible because of the large volumes of water required. Fungicide seed treatments have also been examined but none of the treatments was effective in field trials (Hwang et al. 2011a). Seed treatments may have a role in reducing the risk of seed borne dissemination of *P. brassicae* by seed produced in low disease pressure infested fields but are ineffective in heavily infested fields (Rennie et al. 2011).

2.4.2. Biological control

Among the several micro-organisms with known bio-control properties, *Trichoderma* sp and *Bacillus* sp have been shown to control clubroot disease in Asia, North America, and Latin America (Botero-Ramírez et al. 2015; Peng et al. 2014; Zhao et al. 2022; Zhu et al. 2020). Comparative genomics have shown that the genome of two *Bacillus* strains are effective in controlling the clubroot disease as they harbor genes responsible for antibiotic biosynthesis and antimicrobial peptide production (Zhu et al. 2020). In addition, Extensive research have been conducted in Canada to investigate the potential of biocontrol for management of clubroot on vegetables using indigenous microorganisms or commercial bio fungicides (Peng et al. 2014). These studies have shown that Serenade (Bayer CropScience, Germany), and Prestop (Verdera Oy, Finland) were generally more effective than the indigenous bio-control agents and are as effective as the synthetic fungicides, especially under low *P. brassicae* inoculum pressure (Peng et al. 2011a). However, the efficacy of these biofungicides varied among several trials, crops soil types and test sites (Kasinathan 2012). In canola, a soil-drench application with aqueous

formulations of biofungicides is needed for an effective control clubroot disease, however this is not practical and economical for canola growers (Hynes and Boyetchko 2011).

2.4.3. Genetic resistance

The use of clubroot-resistant (CR) varieties has been widely used for the effective management of club root disease of canola. The first clubroot-resistant (CR) canola cultivars were registered in 2009 and 2010, showing strong resistance to the most common pathotypes (2, 3, 5, 6 and 8) of *P. brassicae* found in Canada (Rahman et al. 2014; Peng et al. 2014; Strelkov and Hwang, 2014). Also, several genetic analysis and fine mapping studies have identified major quantitative trait loci (QTLs) and genes from different *Brassica* species involved in *P. brassicae* resistance (Hasan et al. 2021; Lv et al. 2020). In *B. napus* (canola, genome AACC) germplasm, up to 19 R-genes/QTL have been mapped for clubroot resistance (Werner et al. 2007; Chapara and Meena 2021), whereas most CR genes have been identified from the canola progenitor *B. rapa* (Chinese cabbage, genome AA). For example, *CRa* and *Crr1a* have been successfully isolated and characterized from Chinese cabbage lines of *B. rapa*, and both confer resistance to clubroot (Hatakeyama et al. 2013; Ueno et al. 2012). Yang et al. (2022) also identified 62 candidate CR genes and functionally validated two of them, *CRA3.7.1* and *CRA8.2.4*. to encode club root resistance by transforming them in susceptible *B. napus.*

While CR canola is now the most important clubroot management tool available (Peng et al. 2014), the repeated cultivation of these resistant cultivars exerted selection pressure on *P. brassicae* populations (LeBoldus et al. 2012). This led to the emergence of new strains of *P. brassicae* that overcame the genetic resistance in many of the canola cultivars and has been confirmed recently in commercial fields in Alberta (Hwang et al. 2014; Strelkov et al. 2016a). Thus, since the pathogen causing clubroot is highly variable, the same resistant hybrid should not

be used in consecutive years of planting canola. Rotation of resistance genes and hybrids is critical for protecting the effectiveness of the resistance genes and limiting yield loss, while putting in place other strategies.

2.4.4. Cultural control

While chemical, biological control and genetic resistance are some strategies that are based on the genomics of *P. brassicae* and make take longer time to develop. Cultural practices are measures that can be easily applied to minimize spread of clubroot disease but should be adapted to each country or carefully tailored by each grower in different regions (Javed et al. 2022).

Field sanitation and weed control: Several reports have shown that a few spores can turn into billions of spores over the course of one season, thus overall sanitation of weeds, field equipment and machinery should be mandated for all farmers in areas where clubroot has been detected, and to farmers who feel they are at risk of getting clubroot pathogen in their fields (Chapara and Meena 2021). Field sanitation limits the movement of soil particles which may help prevent the spread of the pathogen within a club root infested field or prevent introduction of the pathogen into new fields (Hwang et al. 2014). Moreover, weeds and volunteer cruciferous crops can provide alternative hosts for the clubroot pathogen, thus they should be adequately managed in the field (Hennig et al. 2022).

Field survey: Disease surveys have played a crucial role in management of clubroot disease worldwide (Chapara and Meena 2021). It has helped in early detection, assessment of disease spread, monitoring of the disease trend and an evaluation of the control measures practiced in each region. In central Sweden, 78% of 190 canola fields surveyed were reported to be infested with clubroot (Wallenhammer 1996). In Manitoba, the yearly survey report indicated the significant and fast spread of clubroot in canola fields, as within the span of two years from 2012

to 2014, more than 600 fields were infected (Strelkov et al. 2018; Chapara and Meena 2021). Likewise, in Alberta Canada, survey report confirmed over 2000 fields that were infected with clubroot (Cao et al. 2009). In the state of North Dakota, U.S. an annual survey conducted indicated clubroot presence and a rapid spread from one to 33 new fields by 2018 in a single County (Chapara et al. 2019). Hence, continuous surveying is important as it will help farmers take timely control measures.

Soil amendments: Research studies have shown that clubroot disease is often favored by acidic soil conditions (Karling 1968; Myers and Campbell 1985; Rastas et al. 2012). Clubroot incidences diminishes rapidly when the soil pH is at 7.0 or above and is correlated to the inhibition of spore germination (Chapara et al. 2019). Thus, maintaining or raising the soil pH by addition of calcium cyanamide, boron, wood ash and especially different forms of lime has been used as a strategy to reduce clubroot in high-value horticultural crops (Murakami et al. 2002), and oil seed rape (Graham et al. 2016). The impact of these products on clubroot severity and yield has been assessed in field trials on canola in different regions, over several growing seasons. Several studies showed that there was reduced severity and increased yield compared with the non-treated control, but the disease reduction and yield increase were too small to justify the expense of lime application (Donald et al. 2001; Hwang et al. 2011b; Chapara and Meena 2021).

Moreover, the results were inconsistent across years and soil types. Other researchers have also concluded that this option is not practical or cost effective for use in canola production, because several tons of lime per hectare are required to increase the pH of an acidic soil to a level at which club root severity is significantly reduced (Webster and Dixon 1991; Murakami et al. 2002). Likewise, soil fumigants may have the potential to reduced club root severity on canola, as they have been effective in vegetables (White and Buczacki 1977). However, this option is also

not practical for canola production, as fumigation is very costly and can destroy the natural soil microbial community.

Crop rotation: A longer break from brassica hosts is the most effective and practical management option for clubroot (Rampel et al. 2014; Chapara and Meena 2021). Proximity to the roots of both host and non-host plants can stimulates the germination of the resting spores of P. brassicae (Friberg et al. 2006). A wide range of plant species, including common velvet-grass, perennial ryegrass, and Indian cress can allow for germination of resting spores and root hair infection, (Friberg et al. 2006). However, in the absence of host plants, germinated spores are likely to survive only for short periods (Suzuki et al. 1992; Takahashi 1994). Thus, since the resting spores can survive in the soil for more than a decade, a long-term management option to manage clubroot on canola would be longer years of rotations with other nonhost crops (Hwang et al. 2019; Peng et al. 2014) alongside limiting soil movement, controlling clubroot host weeds and volunteers of canola (Chapara and Meena 2021).

In the study done by Peng et al. (2014), it was shown that a two-year canola breaks reduced *P. brassicae* resting spore concentrations by >90% relative to growing continuous oilseed rape (OSR) or a one-year break in heavily infested field plots. In addition, after each two-year canola break, recommendations are to grow resistant canola variety and different variety each time (NDSU Ext. 2022). Lengthening crop rotations and using different varieties will slow the development of new pathotypes of *P. brassicae* that can overcome genetic resistance (Hwang et al. 2019). Soybean, barley, flax, field pea and lentil have been reported as non-host crops of *P. brassicae* that could be planted successfully in a rotation with canola (Hwang et al. 2019; Yang et al. 2020). Moreover, since canola is likely to shatter seeds, volunteer plants are a probability during

the next canola season, thus cereals are best to follow canola to allow the use of certain broadleaf phenoxy herbicides for volunteer canola control (NDSU Ext. 2022).

2.5. Objectives of this study on clubroot of canola

Plasmodiophora brassicae, the causal agent of clubroot of canola, is endemic to northeastern North Dakota, particularly in Cavalier County. Clubroot is now a regular yield-robbing disease in North Dakota since its identification in 2013. A limited number of clubroot resistance genes are available for deployment, however, single gene resistance to clubroot has broken down quickly in four years in canola in Alberta, Canada. Therefore, genetic resistance should be considered as just one component in managing clubroot in canola in North Dakota. In other places like Canada, recommended management practices are the use of resistant cultivars and long crop rotation with non-host crops such as cereals and pulses. Also, based on previous research, a two to three-year rotation with a non-host crop is a commonly recommended practice. However, the recommended length of rotation practice is rarely seen in the clubroot prevalent areas in North Dakota. In addition, since wheat is one of the most cultivated and highly beneficial crops in North Dakota, we wanted to determine the suitability of wheat as the suppressant rotation crop of *Plasmodiophora brassicae* population in the soil in North Dakota. Thus, the objectives of this study were aimed at addressing this need to help canola growers find a suitable non-host crop to rotate with CR canola cultivars to delay or prevent genetic resistance in North Dakota, while also maximizing profit even during fallow or non-host rotation periods.

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3. MULTILOCUS SEQUENCE ANALYSIS AND TYPING TO DIFFERENTIATE AND CHARACTERIZE CEREAL-SPECIFIC *XANTHOMONAS TRANSLUCENS* STRAINS[1]

3.1. Abstract

Bacterial Leaf Streak (BLS) has been a major concern for wheat and barley production in the Upper Midwest region of the US and elsewhere in the world. *Xanthomonas translucens* pathovars *X. translucens pv. undulosa (Xtu)* causing BLS on wheat and *X. translucens pv. translucens (Xtt)* causing BLS on barley are closely related. While pathovar identifications are by definition based on host range and symptom profiles, these assays are time-consuming and sometimes give inconsistent results. In this study, multilocus sequence analysis (MLSA) of four house-keeping genes (*rpoD, dnaK, fyuA, and gyrB*) was used to characterize and examine the phylogenetic relationship of a diverse collection of *X. translucens* strains including strains from historical collections and contemporary strains collected in North Dakota . MLSA of the four housekeeping genes validated the pathovar assignation of those strains and revealed distinct subclades within both Xtu and Xtt groups. Furthermore, multilocus sequence typing (MLST) of these strains identified seven and three sequence types for Xtu and Xtt strains, respectively. The establishment of efficient Xtt/Xtu differentiation methods and characterization of these strains will

[1] The material in this chapter contains content previously published in Hong, E., Bankole, I. A., Zhao, B., Shi, G., Buck, J. W., Feng, J., Curland, R. D., Baldwin, T., Chapara, V. and Liu, Z. 2023. DNA markers, pathogenicity test, and multilocus sequence analysis to differentiate and characterize cereal-specific *Xanthomonas translucens* strains. Phytopathology 113:2062-2072. Ibukunoluwa Bankole is a co-first author of the paper and had the primary responsibility of performing DNA extraction, sequencing, multilocus sequence analysis and typing of the strains used in this chapter. Ibukunoluwa Bankole drafted and revised all versions of this chapter. Zhaohui Liu served as the principal investigator and corresponding author and checked the analysis conducted by Ibukunoluwa Bankole.

be useful in studying disease epidemiology and host pathogen interactions for breeding programs when screening for sources of resistance for these two important bacterial pathogens.

3.2. Introduction

Bacterial leaf streak (BLS) is a common disease in almost all wheat- and barley-growing regions in the world (Sapkota et al. 2020). In recent years, BLS has dramatically increased in the Upper Midwest of the United States, where weather conditions are typically cool and dry (Adhikari et al. 2012; Curland et al. 2018; Kandel et al. 2012; Sapkota et al. 2020). This region is a major producer of hard red spring wheat, durum, and barley; thus, BLS has caused great concern to the wheat and barley productions in this region. Yield losses due to BLS have been reported to be 10% or less, but severe infections can cause up to a 40% yield loss, especially on highly susceptible varieties (Duveiller et al. 1997; Forster and Schaad 1988; Waldron 1929). In addition, BLS affects protein levels, leading to quality reduction in barley (Shane et al. 1987). Despite the fact that BLS has been one of the major factors impacting wheat and barley production in many areas, controlling BLS in the field is nearly impossible due to a lack of effective management tools (Sapkota et al. 2020). This is mainly because there is currently not a complete understanding of the disease systems, including disease epidemiology, pathogen pathogenicity/virulence mechanisms, and host resistance/susceptibility mechanisms.

The two pathovars causing disease on wheat and barley are *X. translucens* pv. *undulosa* (Xtu) and *X. translucens* pv. *translucens* (Xtt), respectively (Bragard et al. 1997; Vauterin et al. 1995). Although Xtu and Xtt are not distinguishable morphologically, they differ in many aspects. First, the two pathovars have a distinctive difference in host range. Xtu has a broad host range, capable of causing disease on wheat, triticale, barley, rye, oat, intermediate wheatgrass, and cultivated wild rice by artificial inoculations, whereas Xtt has a narrow host range, causing disease

only on barley (Adhikari et al. 2012; Curland et al. 2019, 2021; Duveiller et al. 1997; Hagborg 1942; Jones et al. 1917; Smith et al. 1919; Vauterin et al. 1995). Second, modern phylogenetic studies using multilocus sequences and whole genome sequences have revealed that Xtu and Xtt are genetically distinct, belonging to two distinct clades (Curland et al. 2018, 2020; Goettelmann et al. 2022; Langlois et al. 2017; Peng et al. 2016). Third, the two pathovars likely have specific tissue niches, with Xtu mainly colonizing mesophyll tissues and Xtt being specialized in vascular tissue (Gluck-Thaler et al. 2020). Recently, Gluck-Thaler et al. (2020) identified a hydrolase gene, *CbsA*, conferring vascular tissue specificity in Xtt. Fourth, genome sequence comparison studies have shown that Xtu and Xtt have different sets of type III effector genes, including transcriptional activator-like effector (TALE) genes (Charkhabi et al. 2017; Jaenicke et al. 2016; Peng et al. 2016). The difference in effector repertoires suggests distinctive pathogenicity and virulence mechanisms for the two pathogens.

All the differences mentioned above suggest that the two pathovars have unique mechanisms to cause disease; therefore, different methods should be applied when designing plant assays to study these two pathosystems. Multilocus sequence analyses (MLSA) of housekeeping genes can be used to differentiate closely related taxa as well as to provide fine resolution for population structures within taxa (Fayette et al. 2016; Pe´rez-Losada et al. 2013). Previous studies have shown that MLSA can differentiate species and sometimes pathovars of *Xanthomonas* (Bragard et al. 1995; Fayette et al. 2016; Young et al. 2008, 2010). Langlois et al. 2017 conducted phylogenetic analyses within the *X. translucens* group using genome sequence data, and clearly separated *X. translucens* pathovars affecting cereals from other noncereal pathovars but did not separate Xtu from Xtt. In contrast, a MLSA study based on four housekeeping genes separated Xtu from Xtt, clearly divided the pathovars into different clades (Peng et al. 2016). Furthermore,

based on majorly Minnesota strains, Xtt was shown to divide into three subclades XttA, XttB and XttC, while Xtu strains were either monophyletic or grouped into two clades (Curland et al. 2018, 2020, and Ledman et al. 2021). Given the fact that the two pathovars can be isolated from the same hosts but differ in many aspects, thus a simple diagnostic method to separate the two pathovars is needed. In our lab, DNA markers that can separate Xtu and Xtt has been developed and used to characterize a diverse collection of *X. translucens* strains isolated from different hosts of origin (cereals and non-cereals). Thus, the aim of this study is to use MLSA and MLST to validate their identities and also provide more insights into the genetic diversity and relatedness of cereal pathovars associated with BLS epidemics in North Dakota and other places.

3.3. Materials and methods

3.3.1. Bacterial strains and culturing

A total of 84 bacterial strains were used in this study. This includes, a historical collection of 51 *X. translucens* strains maintained at the University of Georgia, Griffin Campus (originally curated by Barry M. Cunfer, retired professor), hereafter referred to as the Cunfer collection, which were collected from various hosts across different years and locations in and outside of the United States. A few strains in this collection originated from the National Collection of Plant Pathogenic Bacteria, Harpenden, United Kingdom, and the Culture Collection of Plant Diseases Division Auckland, New Zealand (Cunfer and Scolari 1982). This collection and 33 North Dakota strains were used for MLSA and typing (Table 3.1). In addition, five *X. translucens* strains representing two Xtu MLSA subclades (CIX40 and W1-9) and three Xtt MLSA subclades Xtt A (CIX43), Xtt B (CIX84), and Xtt C (CIX41) as reported by Curland et al. (2018) were included in the analysis, with the sequences downloaded from NCBI GenBank (Table 3.2). Furthermore, reference sequences of *X. translucens* strains representing different pathovars were included in MLSA/

multilocus sequence typing (MLST): Xtt (LMG 876^{T}), Xtu (LMG 892^{PT}), *X. translucens* pv. *secalis* (LMG 883^{PT}), *X. translucens* pv. *cerealis* (B50 = NCPPB 1943, LMG 679^{PT}), *X. translucens* pv. *arrhenatheri* (LMG 727^{PT}), *X. translucens* pv. *poae* (B86 = LMG 728^{PT}), *X. translucens* pv. *graminis* (LMG 726^{PT}), *X. translucens* pv. *phlei* (LMG 730^{PT}), *X. translucens* pv. *phleipratensis* (LMG 843^{PT}), and *X. translucens* pv. *pistachiae* (CFBP 8304^{PT}) (Table 3.2). Additionally, five bacterial strains of other Xanthomonads, *X. hyacinthi* (LMG 739^{T}), *X. sacchari* (LMG 471^{T}), *X. theicola* (LMG 8684^{T}), *X. oryzae* pv. *oryzae* (ICMP 3125^{PT}), and *X. oryzae* pv. *oryzicola* (ICMP 5743^{PT}), were used as outgroups in the MLSA, with the sequences downloaded from GenBank (Table 3.2). The bacterial strains were re-streaked from the single-colony- derived stock cultures stored at −80°C and were cultured at 28°C for 2 days on Wilbrink's agar (WBA) media (0.5% Bacto Peptone, 1% sucrose, 0.05% K_2HPO_4 , 0.025% $MgSO_4 \cdot 7H_2O$, 0.005% Na_2SO_3, and 1.5% agar; Sands et al. 1986). Fully formed colonies were seen on plates after the incubation period, which were used for DNA extraction.

3.3.2. Bacterial genomic DNA extraction

Genomic DNA was isolated from the Cunfer collection and the North Dakota bacterial strains. A small amount of pure culture was transferred into 3 ml of nutrient broth and cultured overnight. Bacterial cells were collected for genomic DNA extraction following the protocol described by Leach et al. (1990). The extracted DNA was quantified using a Nanodrop One spectrophotometer (Thermo Fisher Scientific, Wilmington, DE), and the concentrations were adjusted to 5 to 10 ng/µl for use in PCR.

3.3.3. Multilocus sequence analysis (MLSA)

MLSA was performed using four housekeeping genes (*rpoD*, *dnaK*, *fyuA*, and *gyrB*) as described in Curland et al. (2018). A total of 96 strains were subjected to MLSA, including 33

strains from North Dakota, 51 strains from the Cunfer collection (Table 3.1), and 23 reference strains (Table 3.2), with the sequences obtained from the NCBI GenBank. The four genes (*rpoD*, *dnaK*, *fyuA*, and *gyrB*) were amplified from each strain using previously published primers and conditions (Curland et al. 2018). For each PCR, a portion of the product (10 µl) was first checked on 1% agarose gel. The remaining product of confirmed amplifications was cleaned with ExoSAP-IT (Thermo Fisher Scientific) following the provided user manual. The purified PCR products were sent for Sanger sequencing at Eurofins Genomics (Louisville, KY).

All sequences were assembled, aligned, and trimmed using Qiagen CLC Genomics Main Workbench 8 (Qiagen, Germantown, MD). In line with the previous MLSA studies (Curland et al. 2018, 2020; Ledman et al. 2021; Young et al. 2008; Zacaroni et al. 2012), the sequences for each gene and strain were trimmed to the following sizes, *rpoD* (674 bp), *dnaK* (762 bp), *fyuA* (522 bp), and *gyrB* (687 bp), to a final concatenated length of 2,645 base pairs (bp) for the four-gene loci concatenation. For strains that were neither Xtu nor Xtt, the sequences from two loci (*rpoD* and *gyrB*) were used with a concatenated length of 1,366 bp. The sequences for those genes have been submitted to NCBI through the BankIt portal under the accession number OR575931- OR576202. Model testing of individual loci and concatenated loci using CLC Genomics Main Workbench 8 identified the general time-reversible (GTR+G+T) model as the best fit for all phylogenetic tree constructions. The Bayesian Evolutionary Analysis Sampling Trees (BEAST.v1.10.4) program was used to perform Bayesian Markov chain Monte Carlo analysis assuming a strict clock and 10 million generations. The output was analyzed with Tracer v1.7.2 (Rambaut et al. 2014) and visualized in FigTree v.1.4.4 (http://tree.bio.ed.ac.uk/software/figtree). Meta-data layers and final tree edits were completed in CLC Genomics Main Workbench 8.

3.3.4. Multilocus sequence typing (MLST)

DnaSP v.6 was used to generate haplotype data and analyze variation among sequences (Rozas et al. 2003). Allele numbers were given unique sequences for each of the four loci, and sequence type (ST) numbers were assigned to each unique concatenated sequence, consistent with previous studies (Curland et al. 2018, 2020; Ledman et al. 2021) for comparison of ST data across studies. Any sequence type not previously described was assigned the next available consecutive number. Clonal complexes, along with their predicted founding ST, were analyzed and identified using the goeBURST algorithm to construct a minimum spanning tree in PHYLOVIZ 2.0 (Nascimento et al. 2017). Metadata layers and final tree edits were done in Python v3.10.5.

Table 3.1. List of bacterial strains used in this study.

Strain ID	Host	Geographic origin	Collection/ Year [a]	MLSA group [b]	Sequence type [c]
LW16	*Triticum aestivum*	North Dakota	2009	Xtu	36
P3	*Triticum aestivum*	North Dakota	2009	Xtu	29
LB10	*Triticum aestivum*	North Dakota	2009	Xtu	36
Xt4699	*Triticum aestivum*	Kansas	1999	Xtu	38
LG 1	*Triticum aestivum*	North Dakota	2008	Xtu	36
LG 10	*Triticum aestivum*	North Dakota	2008	Xtu	59
LG 15	*Triticum aestivum*	North Dakota	2008	Xtu	36
LG 25	*Triticum aestivum*	North Dakota	2008	Xtu	36
LG 3	*Triticum aestivum*	North Dakota	2008	Xtu	38
LG 48	*Triticum aestivum*	North Dakota	2009	Xtu	38
LG 54	*Triticum aestivum*	North Dakota	2009	Xtu	36
LB 11	*Triticum aestivum*	North Dakota	2008	Xtu	36
LB 3	*Triticum aestivum*	North Dakota	2008	Xtu	30
LB 41	*Triticum aestivum*	North Dakota	2008	Xtu	36
LB 5	*Triticum aestivum*	North Dakota	2009	Xtu	36
LB 58	*Triticum aestivum*	North Dakota	2008	Xtu	38
LB 8	*Triticum aestivum*	North Dakota	2008	Xtu	38
CS 2	*Triticum aestivum*	North Dakota	2008	Xtu	36
CS 22	*Triticum aestivum*	North Dakota	2009	Xtu	36
CS 27	*Triticum aestivum*	North Dakota	2008	Xtu	36
CS 28	*Triticum aestivum*	North Dakota	2008	Xtu	36
CS 32	*Triticum aestivum*	North Dakota	2008	Xtu	38
CS 35	*Triticum aestivum*	North Dakota	2008	Xtu	36
CS 4	*Triticum aestivum*	North Dakota	2009	Xtu	54
CR 10	*Triticum aestivum*	North Dakota	2008	Xtu	59
CR 12	*Triticum aestivum*	North Dakota	2008	Xtu	29
CR 25	*Triticum aestivum*	North Dakota	2008	Xtu	36
CR 3	*Triticum aestivum*	North Dakota	2008	Xtu	36
CR 31	*Triticum aestivum*	North Dakota	2009	Xtu	36
CR 41	*Triticum aestivum*	North Dakota	2008	Xtu	36
B1	*Hordeum vulgare*	North Dakota	2013	Xtt	15
B2	*Hordeum vulgare*	North Dakota	2013	Xtt	43
B1FA	*Hordeum vulgare*	North Dakota	2017	Xtt	43

Table 3.1. List of bacterial strains used in this study (Continued).

Strain ID	Host	Geographic origin	Collection/ Year [a]	MLSA group [b]	Sequence type [c]
B8GF	*Hordeum vulgare*	North Dakota	2017	Xtt	43
Xt-1	*Hordeum vulgare*	Other places in US	NCPPB #973	Xtt	15
Xt-2	*Secale cereale*	Other places in US	NCPPB #1836	Xtu	36
Xt-3	*Hordeum vulgare*	Other places in US	NCPPB #1943	Xtc	NA
Xt-5	*Phleum pratense*	Brazil	NCPPB #1839	Xtg	NA
Xt-6	*Triticum aestivum*	Canada	NCPPB #1945	Xtu	36
Xt-7	*Hordeum vulgare*	Other places in US	PDDCC #5752	Xtt	15
Xt-8	*Dactylis glomerata*	Other places in US	PDDCC #1409	Xtc	NA
Xt-9	*Hordeum vulgare*	India	PDDCC #5735	Xtt	64
Xt-11	*Phleum pratense*	Other places in US	PDDCC#5744	Xtg	NA
Xt-12	*Secale cereale*	Canada	PDDCC #5749	Xtu	33
Xt-13	*Triticum aestivum*	Canada	PDDCC #5755	Xtu	36
Xt-102	*Secale cereale*	Georgia	1977	Xtu	33
Xt-103	*Secale cereale*	Georgia	1977	Xtu	36
Xt-104	*Secale cereale*	Georgia	1977	Xtu	33
Xt-105	*Secale cereale*	Georgia	1977	Xtu	36
Xt-108	*Secale cereale*	Georgia	1976	Xtu	33
Xt-109	*Secale cereale*	Georgia	1976	Xtu	36
Xt-110	*xTriticosecale*	Georgia	1976	Xtu	36
Xt-111	*xTriticosecale*	Georgia	1978	Xtu	33
Xt-112	*Secale cereale*	Georgia	1979	Xtu	36
Xt-113	*xTriticosecale*	Alabama	1978	Xtu	38
Xt-114	*xTriticosecale*	Alabama	1978	Xtu	38
Xt-115	*Secale cereale*	Georgia	1978	Xtu	36
Xt-116	*Triticum aestivum*	South Dakota	1979	Xtu	33
Xt-118	*Triticum aestivum*	South Dakota	1979	Xtu	36
Xt-121	*Hordeum vulgare*	Montana	1981	Xtu	38
Xt-122	*Triticum aestivum*	Georgia	1982	Xtu	38

Table 3.1. List of bacterial strains used in this study (Continued).

Strain ID	Host	Geographic origin	Collection/ Year [a]	MLSA group [b]	Sequence type [c]
Xt-125	*Secale cereale*	Georgia	1980	Xtu	33
Xt-126	*Secale cereale*	Georgia	1980	Xtu	33
Xt-127	*Triticum aestivum*	Montana	1983	Xtu	43
Xt-128	*xTriticosecale*	Florida	1984	Xtu	29
Xt-129	*xTriticosecale*	Florida	1984	Xtu	29
Xt-130	*Secale cereale*	Florida	1984	Xtu	33
Xt-131	*Triticum aestivum*	Georgia	1984	Xtu	29
Xt-132	*Hordeum vulgare*	Florida	1984	Xtu	36
Xt-133	*Triticum aestivum*	Georgia	1991	Xtu	36
Xt-134	*Triticum aestivum*	Georgia	1991	Xtu	36
Xt-202	*xTriticosecale*	Mexico	1978	Xtu	29
Xt-203	*Secale cereale*	Mexico	1978	Xtu	38
Xt-205	*Secale cereale*	Mexico	1978	Xtu	38
Xt-206	*xTriticosecale*	Mexico	1978	Xtu	38
Xt-207	*xTriticosecale*	Mexico	1978	Xtu	38
Xt-209	*Secale cereale*	Mexico	1978	Xtu	38
Xt-210	*xTriticosecale*	Mexico	1978	Xtu	38
Xt-211	*xTriticosecale*	Mexico	1978	Xtu	38
Xt-214	*Triticum aestivum*	Mexico	1978	Xtu	36
Xt-215	*xTriticosecale*	Mexico	1978	Xtu	38
Xt-216	*xTriticosecale*	Mexico	1977	Xtu	38
Xt-217	*xTriticosecale*	Mexico	1977	Xtu	38
Xt-218	*Triticum aestivum*	Brazil	1982	n.d.	NA
Xt-226	*xTriticosecale*	Ethiopia	1978	Xtu	38

[a] Strains from Xt-1 to Xt-226 were from the Cunfer collection.

[b] n.d. = not determined by multilocus sequence analysis. Xtu = *Xanthomonas translucens* pv. *undulosa*, Xtt = *X. translucens* pv. *translucens*, Xtc = *X. translucens* pv. *cerealis*, and Xtg = *X. translucens* pv. *graminis*. [c] NA = not analyzed.

Table 3.2. List of previously described *Xanthomonas translucens* species and pathovars used as reference for comparison of strains used in this study.

Strain designation [a]	Species	Pathovar	Host of Origin	Geographic origin	Source [b]
LMG 892^{PT}	*X. translucens*	*undulosa*	*Triticum turgidum*	Canada	1
LMG 876^{T}	*X. translucens*	*translucens*	*Hordeum vulgare*	Minnesota, US	1
LMG 883^{PT}	*X. translucens*	*secalis*	*Secale cereale*	Canada	1
LMG 727^{PT}	*X. translucens*	*arrhenatheri*	*Arrhenatherum elatius*	Switzerland	1
B50 = NCPPB 1943	*X. translucens*	*cerealis*	*Hordeum vulgare*	United States	1
LMG 679^{PT}	*X. translucens*	*cerealis*	*Bromus inermis*	United States	1
LMG 726 ^{PT}	*X. translucens*	*graminis*	*Dactylis glomerata*	Switzerland	1
LMG 730^{PT}	*X. translucens*	*phlei*	*Phleum pratense*	Norway	1
LMG 843^{PT}	*X. translucens*	*phleipratensis*	*Phleum pratense*	United States	1
B86 = LMG 728^{PT}	*X. translucens*	*poae*	*Poa trivialis*	Switzerland	1
LMG 728^{PT}	*X. translucens*	*poae*	*Poa trivialis*	Switzerland	1
CFBP 8304^{PT}	*X. translucens*	*pistachia*	*Pistacia vera*	Australia	3
LMG 739^{T}	*X. hyacinthi*		*Hyacinthus orientalis*	Netherlands	1
LMG 471^{T}	*X. sacchari*		*Saccharum officinale*	Guadeloupe	1
LMG 8684^{T}	*X. theicola*		*Camelia sinensis*	Japan	1
ICMP 3125^{PT}	*X. oryzae*	*oryzae*	*Oryza sativa*	unknown	4
ICMP 5743 ^{PT}	*X. oryzae*	*oryzicola*	*Oryza sativa*	unknown	4
CIX 40	*X. translucens*	*undulosa*	*Triticum aestivum*	Minnesota, US	2
W1-9	*X. translucens*	*undulosa*	*Triticum aestivum*	Minnesota, US	2
CIX 43	*X. translucens*	*translucens*	*Hordeum vulgare*	Minnesota, US	2
CIX 41	*X. translucens*	*translucens*	*Hordeum vulgare*	Minnesota, US	2

Table 3.2. List of previously described *Xanthomonas translucens* species and pathovars used as reference for comparison of strains used in this study (Continued).

Strain designation [a]	Species	Pathovar	Host of Origin	Geographic origin	Source [b]
CIX 84	*X. translucens*	*translucens*	*Hordeum vulgare*	Minnesota, US	2

[a] Type strains (^{T}); Pathotype strains (^{PT}); [b] 1= Belgian Co-ordinated Collections of Microorganisms, LMG Bacteria Collection; 2= University of Minnesota, Curland et al. (2018, 2020); 3= NCBI Reference Sequence: NZ_CP074365.1; 4= Young et al. 2008.

3.4. Results

3.4.1. Characterization of the *X. translucens* collection by MLSA

Four housekeeping genes were unable to be amplified from Xt-218; thus, it was not included in the MLSA. Only two genes (*rpoD* and *gyrB*) were successfully amplified for Xt-3, Xt-5, Xt-8, and Xt-11. All four genes were successfully amplified from all Xtu (42) and Xtt strains (4). A phylogenetic tree was constructed for the four strains (neither Xtu nor Xtt) and the reference strains from different species and pathovars based on Bayesian analysis with the two gene concatenations. Xt-5 and Xt-11 clustered as a subclade with *X. translucens* pv. *graminis* (LMG 726^{PT}). Xt-3 and Xt-8 clustered with *X. translucens* pv. *cerealis* (LMG 679^{PT} and NCPPB 1943) (Figure 3.1). In the constructed phylogenetic tree, strains identified as Xtu and Xtt formed two close clusters together with their reference strains but were clearly separated from the other *X. translucens* pathovars. Within the Xtt and Xtu groups, three and two distinct subclades were observed, respectively (Figure 3.2). Within Xtt, five strains (B1FA, B2, B8GF, Xt-9, and Xt-127) clustered with the reference strains CIX84 in Xtt A, whereas three strains (Xt-7, B1, and Xt-1) clustered with the reference strain CIX43 in Xtt C. The Xtu clade contained a variety of strains from different hosts of origin (wheat, triticale, rye, and barley). The largest of the Xtu subclade grouped with LMG 883^{PT} (*X. translucens* pv. *secalis*), LMG 892^{PT} (*X. translucens* pv. *undulosa*),

and reference strain CIX40 from Minnesota (Curland et al. 2018). The second subclade included reference strain W1-9 from Minnesota (Curland et al. 2020) (Figure 3.2).

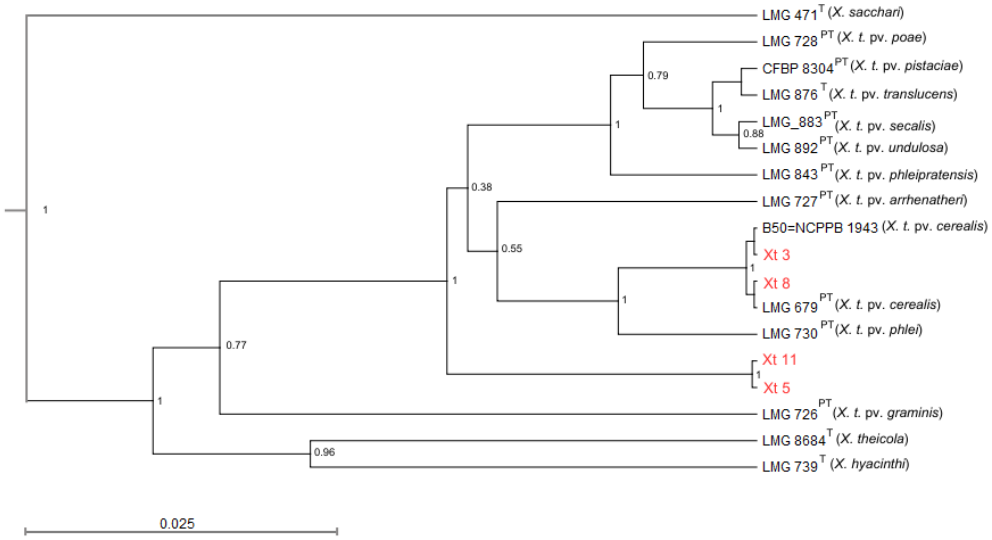

Figure 3.1. Phylogenetic tree constructed for the bacterial strains that were not characterized by marker analysis.
Note: Four strains in red were assigned to individual *Xanthomonas* species or *X. translucens* pathovars using reference strains and concatenated sequences from two housekeeping genes (*rpoD* and *gyrB*). ^{PT} = reference pathotype strains, ^{T} = reference type strains; numbers at nodes represent posterior probabilities of clade division.

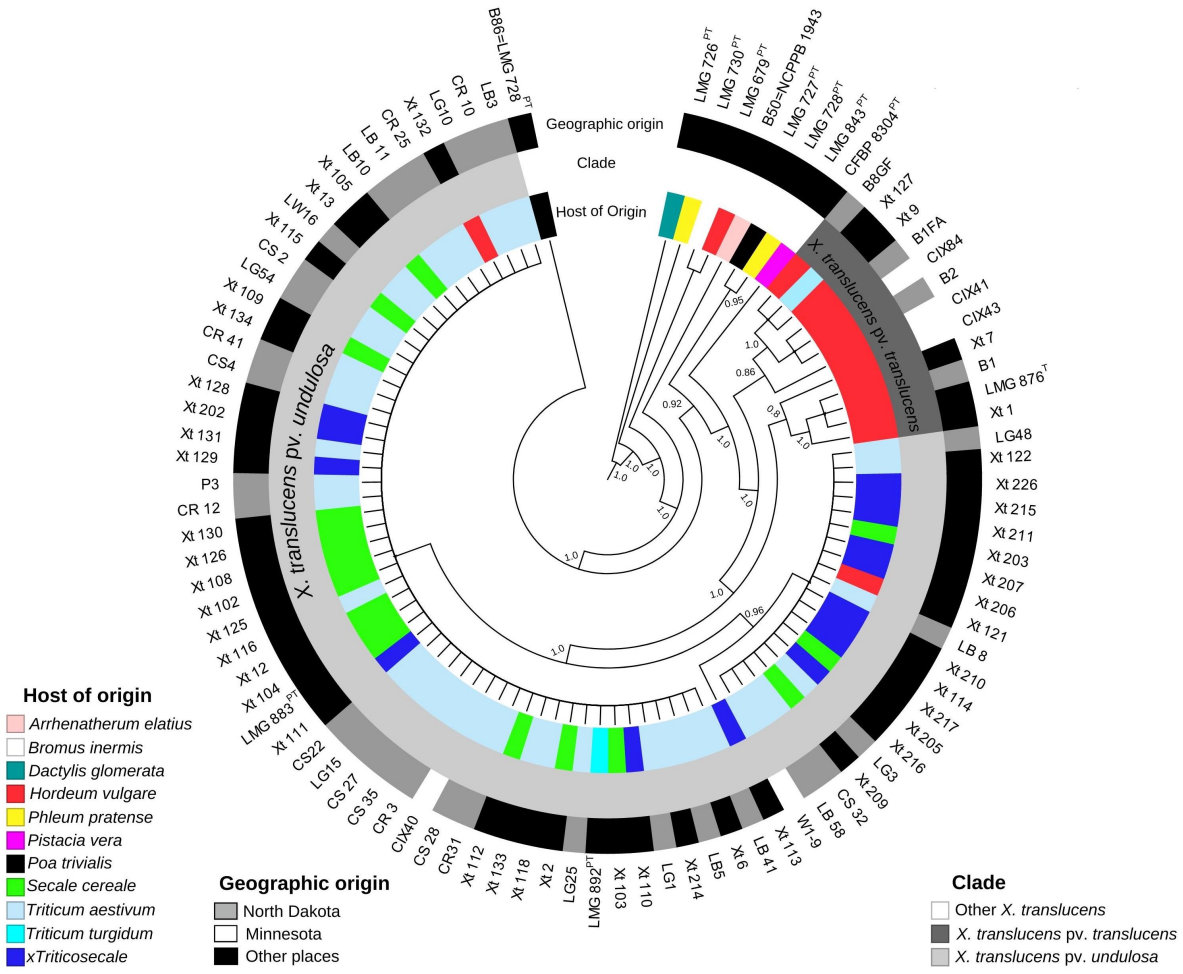

Figure 3.2. Circular cladogram of *Xanthomonas translucens* strains based on Bayesian analysis. Note: The analysis was performed on concatenated sequences (2,645 bp) from four housekeeping genes (*rpoD*, *dnaK*, *fyuA*, and *gyrB*) for a total of 79 *X. translucens* strains, including strains from North Dakota and the Cunfer collection, as well as reference type strain LMG 876^{T} *X. translucens* pv. *translucens* and pathotype strains LMG 892^{PT} *X. translucens* pv. *undulosa*, LMG 883^{PT} *X. translucens* pv. *secalis*, LMG 679^{PT} and B50 = NCPPB 1943 *X. translucens* pv. *cerealis*, LMG 727^{PT} *X. translucens* pv. *arrhenatheri*, LMG 728^{PT} and B86 = LMG 728^{PT} *X. translucens* pv. *poae*, LMG 726^{PT} *X. translucens* pv. *graminis,* LMG 730^{PT} *X. translucens* pv. *phlei*, LMG 843^{PT} *X. translucens* pv. *phleipratensis*, and CFBP 8304^{PT} *X. translucens* pv. *pistachiae*.

3.4.2. MLST on Xtu and Xtt strains

We further performed MLST to investigate the relatedness and genetic diversity among Xtu and Xtt strains in reference to all STs previously described (Curland et al. 2018, 2020; Ledman et al. 2021). Ten STs were identified, with seven for Xtu and three for Xtt (Figure 3.3). In the Xtu complex, founder ST 36 contained the most abundant strains collected across all locations and from different hosts(*n*=32), followed by ST38(*n*=20), ST33(*n*=8), and ST 29 (*n* = 7). The majority of North Dakota and Georgia strains were assigned to ST 36, whereas strains from Mexico were mainly distributed in ST 38. ST 30 and ST 54 contained only one strain each, whereas ST 59 contained two, all of which were from North Dakota. Four sequence types (ST 29, ST 30, ST 33, and ST 38) in the Xtu complex were single-locus variants of founder ST 36, whereas ST 54 and ST 59 were identified as double-locus variants from ST 36. In the Xtt complex, three STs were identified, including previously described ST 43 (*n* = 4) and ST 15 (*n* = 3) and newly assigned ST 64 (*n* = 1) (Figure 3.3).

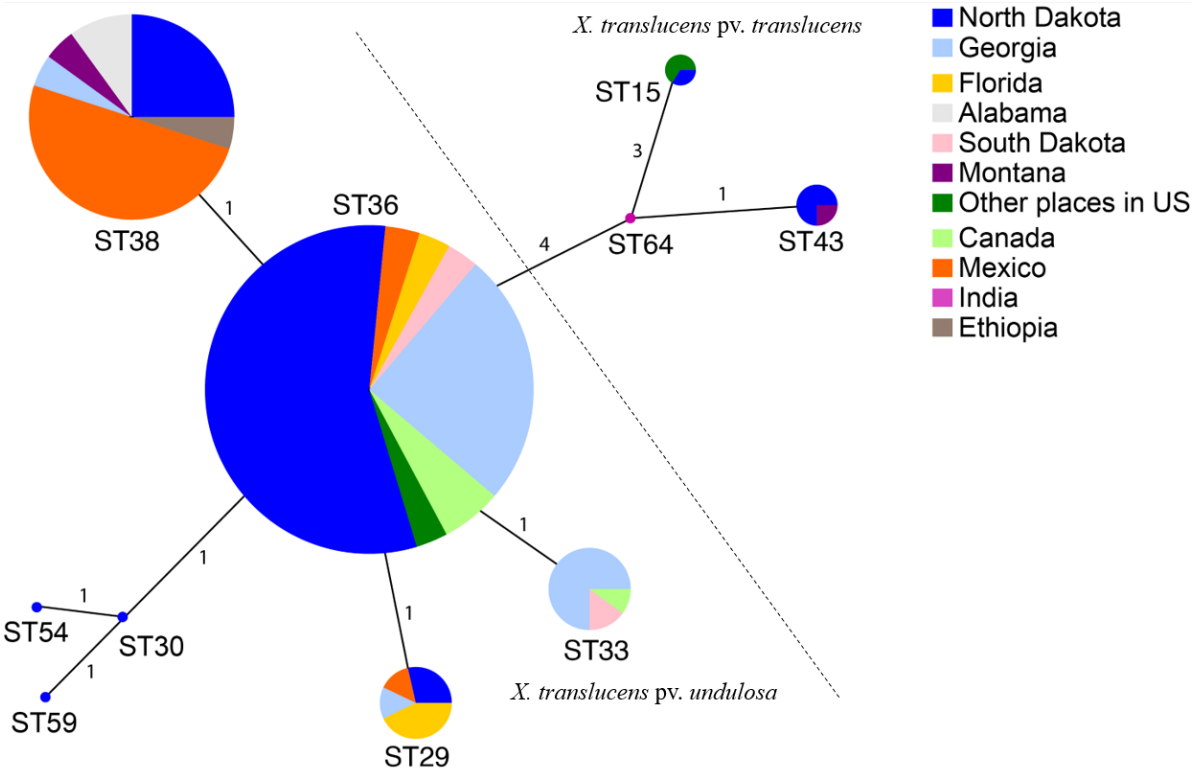

Figure 3.3. Minimum spanning tree of 79 *Xanthomonas translucens* pv. *undulosa* and *X. translucens* pv. *translucens* strains from diverse geographic locations based on sequence types (STs).
Note: Each node represents an ST based on concatenated STs from four housekeeping genes (*rpoD*, *dnaK*, *fyuA*, and *gyrB*) and aligns with the STs assigned in previous studies (Curland et al. 2018, 2020; Ledman et al. 2021). The sizes of nodes are relative to the number of individuals sharing the same ST. The numbers on the branch indicate variants at four loci relative to each other. A dashed line separates the groups of *X. translucens* pv. *undulosa* and pv. *translucens*.

3.5. Discussion

Wheat and barley production in North Dakota and surrounding areas has been greatly impacted by BLS. The two pathovars causing BLS on wheat and barley differ in their host range and tissue specificity as well as pathogenicity or virulence mechanisms (Adhikari et al. 2012; Bragard et al. 1995; Duveiller et al. 1997; Gluck-Thaler et al. 2020; Hagborg 1942; Jones et al. 1917; Sapkota et al. 2020; Smith et al. 1919; Vauterin et al. 1995), suggesting that the two pathosystems should be studied separately. However, the two pathovars are morphologically indistinguishable and can be isolated from the same hosts. Therefore, there is a great need to

develop a simple method to separate the two pathovars. As part of the study done by Hong et al. (2023), DNA markers that can be used to separate the two pathovars were developed and successfully applied them to differentiate and characterize a collection of diverse *X. translucens* strains. In this study, MLSA was used to further characterize and validate the robustness of the developed markers, and MLST was used to determine the genetic diversity and relatedness of Xtu strains in North Dakota and other places. This study will aid the in characterization of Xtu/Xtt strains from wheat and barley which is useful in disease diagnosis and epidemiology studies.

Four strains in the Cunfer collection (Xt-3, Xt-5, Xt-8, and Xt- 11) could not be assigned to a pathovar by the developed Xtt and Xtu DNA markers. However, MLSA from the two loci concatenations clearly showed that they are neither Xtu nor Xtt because these strains clustered away from the pathotype reference strains LMG 892^{PT} (Xtu) and LMG 876^{T} (Xtt) (Figure 3.1). In the phylogenetic tree, Xt-5 and Xt-11 cluster together and are located closer to *X. translucens* pv. *graminis*, and Xt-3 and Xt-8 group together and are closer to *X. translucens* pv. *cerealis*. Furthermore, the pathogenicity tests done by Hong et al. (2023) supported the groupings where Xt-5 and Xt-11 caused more water-soaking on both wheat and barley but caused weak chlorosis on barley and not many symptoms on wheat, suggesting that they are away from the cereal BLS group. Cunfer and Scolari (1982) previously designated Xt-5 and Xt-11 as *X. translucens* pv. *phleipratensis*. However, Xt-5 and Xt-11 did not group with *X. translucens* pv. *phleipratensis* reference strain LMG 843^{PT} but were closer to pv. *graminis* LMG 726^{PT} in our phylogenetic analysis. Further host range assays including the host of origin for each non-Xtt/Xtu strain in this study are needed to validate the pathovar identity with corresponding reference strains.

Results from the MLSA of four housekeeping genes (*rpoD*, *dnaK*, *fyuA*, and *gyrB*) correlated with the Xtu or Xtt designations by the previous molecular marker analysis and

pathogenicity tests. Strains designated as Xtu by the molecular markers grouped with the pathotype reference strain LMG 892^{PT} (Xtu), and strains designated as Xtt grouped with the type reference strain LMG 876^{T} (Xtt) (Figure 3.2). Congruent with previous studies (Curland et al. 2018, 2020), the Xtu clade could be divided into two subclades, represented by the reference strains W1-9 and CIX40, respectively (Figure 3.2). Although the W1-9 subclade had a smaller number of strains compared with the CIX40 subclade, both subclades contain strains from different hosts and geographic locations. The North Dakota strains did not form a unique group, suggesting a co-divergence of the Xtu populations from North Dakota and other geographic locations. Using Xtu strains from wheat and wild grasses, Ledman et al. (2021) identified more variations within the Xtu population. Although a limited number of Xtt strains were used in this study, these strains grouped within the Xtt clade and showed consistency with previous studies, where three subgroups, Xtt A, Xtt B, and Xtt C, were identified (Figure 3.2). This suggests a greater genetic diversity within the Xtt population than within the Xtu population (Curland et al. 2018, 2020; Ledman et al. 2021). More Xtt strains from barley production areas in the United States, such as North Dakota, Idaho, and Montana, are needed to confirm this observation.

Through MLST, we identified ST 36 as the most dominant sequence type for Xtu, as well as the founder ST, which is consistent with previous findings (Curland et al. 2018, 2020; Ledman et al. 2021). The majority of North Dakota strains were identified as ST 36, which further supports the hypothesis that ST 36 is adapted to the Upper Great Plains. However, this ST was also detected in the Xtu strains from the U.S. states of Georgia, Florida, and Montana, as well as other countries (Table 3.1), indicating that this ST is not just limited to the Upper Great Plains. Strains with the same genotype as ST 36 have been described in Uruguay and Iran, suggesting that this ST is globally distributed (Clavijo et al. 2022; Khojasteh et al. 2019). After ST 36, ST 38 was the second

most common sequence type for the North Dakota strains. ST 38 was also present in the Minnesota Xtu strains from wheat and wild grasses at a frequency second to ST 36 (Curland et al. 2018, 2020; Ledman et al. 2021). Interestingly, the majority of Mexico strains belonged to ST 38, not ST 36. This may suggest that ST 38 is better adapted to the Mexican environment. However, more Xtu strains from that region are needed to make a solid conclusion. One strain each was identified for ST 30, ST 54, and ST 59, indicating the low frequency of those STs present in North Dakota. We only analyzed four North Dakota Xtt strains with three in ST 43 and one in ST 15, both of which were reported in Minnesota. More Xtt strains are needed to obtain the complete population structure in North Dakota. One Xtt strain from India was identified as ST 64, which has not been previously reported. Although the biological relevance of STs has not been determined, identifying the conserved or predominant STs in the population provides wheat and barley improvement programs with information to select appropriate strains for the development of varieties with broad resistance to BLS (Curland et al. 2018, 2020; Khojasteh et al. 2019; Ledman et al. 2021).

In conclusion, MLSA and typing of four housekeeping genes were successfully used to characterize a set of diverse *X. translucens* strains and provide an insight into the phylogenetic diversity and relatedness amongst the cereals pathovars. Information obtained is useful for the development and deployment of disease management strategies to BLS in wheat and barley.

3.6. References

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4. FUNCTIONAL CHARACTERIZATION OF TYPE III EFFECTORS OF WHEAT BACTERIAL LEAF STREAK PATHOGEN *XANTHOMONAS TRANSLUCENS* PV. *UNDULOSA*

4.1. Abstract

Xanthomonas translucens pv. *undulosa* (Xtu) is the causal agent of bacterial Leaf Streak (BLS) of wheat which has been increasingly important in the upper Midwest of the United States and elsewhere. Host resistance is scarce, and not much is known about pathogen virulence mechanisms, making it difficult to develop resistant cultivars. Xtu depends on a type III secretion system (T3SS) to translocate effectors into host cells for its ability to cause disease. A few transcription activator-like effectors (TALEs) have been shown to contribute to bacterial virulence. However, little is known about non-TALE Xop effectors. This study aimed to determine the roles of conserved Xops in virulence of *X. translucens* pv. *undulosa*. By using a suicide vector, target insertion mutations were successfully created for 13 Xop genes, including *XopP1*, *AvrBs2*, *XopAF*, *XopY, XopL, XopN, XopQ, XopK, XopAA, XopR, XopF1, XopAP,* and *XopZ* in a local strain. Mutant and wild-type strains were tested for virulence on a set of seven lines including wheat, durum, and barley. Compared to the wildtype strain, mutants of *XopY* and *XopAA* had a significant reduced virulence on some susceptible lines while the others did not show obvious virulence changes. This suggests that both XopY and XopAA in *Xanthomonas translucens* pv. *undulosa* play an important role in the disease development of wheat BLS. Identification of the key virulent factors in Xtu which will be useful to provide insight into the host pathogen interactions of the wheat-BLS pathosystem for breeding durable resistance in wheat.

4.2. Introduction

Xanthomonas translucens pv. *undulosa* (Xtu) is the causal pathogen for bacterial leaf streak (BLS) of wheat (Adhikari et al. 2012; Sapkota et al. 2020). Because the disease incidence and severity has dramatically increased in the upper Midwest of the United States in the last two decades, BLS has been a major concern for hard red spring and durum wheat producing states including North Dakota (Adhikari et al. 2012; Kandel et al. 2012; Curland et al. 2018; Sapkota et al. 2020). Xtu enters the plant tissue through the stomata and colonizes the mesophyll tissues, thus the early symptoms are found on the leaf tissues as water-soaked lesions, that expands and progress parallel with the leaf veins, becoming chlorotic with a greasy appearance but eventually forms translucent necrotic lesions (Jones et al. 1917; Sapkota et al. 2020; Ledman et al. 2023). BLS of wheat has a worldwide distribution and its economic impacts due to yield loss are up to 40% or less depending on disease pressure and reduction in grain quality and protein level (Forster et al. 1986; Duveiller et al. 1997). However, recently in North Dakota, an estimated yield loss of up to 60% and equivalent to $8 million loss in monetary terms was recorded on a highly susceptible hard red spring wheat cultivar (Friskop et al. 2023).

Management of BLS diseases on wheat have been limited to use of clean seed either by seed treatment with heat or chemicals. However, reports from these seed cleaning treatments have been inconsistent which cannot completely eliminate bacteria in the seeds nor stop the spread of BLS inoculum between fields (Atanasoff and Johnson 1920; Forster et al. 1989; Duveiller et al. 1997). Currently, no effective and practical means of chemical control have been developed (Sapkota et al. 2020; Lux et al. 2020). Although several greenhouse and field disease screening studies have been done to identify and map potential sources of resistance against BLS through QTL mapping and GWAS analysis, wheat lines with a good level of resistance is scarce (El Attari

et al. 1996; Adhikari et al. 2011, 2012b; Kandel et al. 2012, 2015; Ramakrishnan et al. 2019). In addition, not much is known about the pathogen virulence and host- pathogen interactions, which make breeding for resistance difficult.

The gram-negative bacteria, *X. translucens* pv. *undulosa* utilizes the needle-like structure known as the type III secretion system (T3SS) as the primary secretion system responsible for pathogenicity and virulence. This structure formed by the highly conserved chromosomal hrp (hypersensitive response [HR] and pathogenicity) gene cluster, spans the bacterial cell membranes and plant cell membranes to inject type III effectors (T3Es) into host cells to promote infection (Büttner and Bonas 2002, 2010), and mediates the processes of pathogen adaptation to specific host tissue and genotypes (White et al. 2009). T3Es are majorly of two types, transcription activator-like effectors (TALEs) and non-transcription activator-like effectors (non-TALEs), also known as Xops.

TALES are unique T3Es that localize to the nucleus, after being delivered into the host cell. They have specific central region containing amino acids known as repeat variable di-residue (RVD), which dictate each TALE binding to specific host DNA sequences called effector-binding elements (EBEs) to act like a transcription activator for pathogen benefits (Bogdanove et al. 2010, Mak et al. 2012; Timilsina et al. 2020). In Xtu, usually 7-8 TALES are identified (Peng et al. 2016, Charkhabi et al. 2017, Goettelmann et al. 2022), and only a few of the TALES identified play a significant role in Xtu virulence. For example, the Xtu 4699_Tal8 was functionally characterized to induce the expression of the wheat gene TaNCED located on the short arm of chromosome 5B to promote disease susceptibility (Peng et al. 2019).

In contrast, the non-TALES also called Xanthomonas outer proteins (Xops) tend to target different pathways of the host, allowing the pathogen to acquire nutrients or evade or suppress host

defenses pathways including PTI (PAMP -triggered immunity) and ETI (effector triggered immunity) (Büttner 2016). Genome sequencing has revealed 21 to 36 classes of Xop effectors in a single Xtu strain (Goettelmann et al. 2022; Heiden et al. 2023; Peng et al. 2016; Shah et al. 2021). The core sets of Xops in *X. translucens* are XopZ, XopX, XopR, XopQ, XopP, XopN, XopL, XopK, XopF, and AvrBS2, while specific to Xtu are XopE1 and XopE5 (Peng et al. 2017; Shah et al. 2021; Goettelmann et al. 2022). Functional studies of these Xops in virulence have been done on other *Xanthomonas* species, including *X. oryzae pv. oryzae* (Xoo), *X. oryzae* pv. *oryzicola* (Xoc), *X. campestris* pvs. *campestris* (Xcc), and *vesicatoria* (Xcv). For example, in Xoo, the causative pathogen of bacterial blight of rice, XopR, XopN, and XopZ individually contributes to full virulence of the pathogen (Akimoto-Tomiyama et al. 2012; Sinha et al. 2013; Song and Yang 2010). Similarly, Jiang et al. (2008) also showed that AvrBs2 and XopN contribute individually to Xcc virulence. XopL significantly contributes to Xcv virulence on tomato (Leong et al. 2022), Xcc virulence on Chinese cabbage (Yan et al. 2019) and Chinese radish cultivar (Jiang et al. 2009).

Identification of the key virulent factors in a pathogen is an important step to understanding the host-pathogen interaction in any pathosystem. Only a few TALEs have been shown to contribute to bacterial virulence of Xtu, however, no functional study has been done to investigate the role of these Xops in virulence of wheat BLS pathogen *X. translucens* pv. *undulosa*. The objective of this study was thus to determine the role of several Non-TAL effectors in Xtu virulence using target gene disruption. The HrcC gene encoding a conserved type III secretion system (T3SS) component was also performed in the beginning to establish the gene disruption protocol.

4.3. Materials and methods

4.3.1. Bacterial strains and culture conditions

Bacteria strains and plasmids used in this study are listed in (Table 4.1). *X. translucens* pv. *undulosa* LW16 was grown at 28ºC in nutrient broth yeast extract (NBY) liquid medium (Difco nutrient broth, 8g; yeast extract, 2g; K_2HPO_4, 2g; KH_2PO_4, 0.5g per liter) and Wilbrink's agar (WBA) plates (0.5% Bacto Peptone, 1% sucrose, 0.05% K_2HPO_4, 0.025% $MgSO_4.7H_2O$, 0.005% Na_2SO_3, and 1.5% agar (Sands et al. 1986). *Escherichia coli* strains DH5α™-T1R and PIR1 used for recombinant DNA manipulations were grown in LB (Luria-Bertani) (tryptone, 10g; yeast extract 5g; sodium chloride, 10g bacto agar, 15g per liter) medium and plates (Miller 1972) at 37ºC. Antibiotics used in this study are at the following concentrations: ampicillin, 50 μg/ml; kanamycin, 50 μg/ml; chloramphenicol,12.5 μg/ml.

4.3.2. Identification of T3SS HrcC gene and non-TAL T3Es in Xtu

The North Dakota Xtu strain LW16 was chosen to generate a collection of mutants because it is one of the well characterized *Xanthomonas translucens* pv. *undulosa* isolates that can be easily manipulated. Sequences of the conserved T3SS component HrcC and 13 non-TALEs in LW16 were obtained from NCBI database using known and previously annotated T3SS components and Xops in Xtu LW16 (Peng et al. 2019) (Table 4.1). Validation was done by subjecting the sequences to BLASTn using a database of known *Xanthomonas* effectors (http://www.Xanthomonas.org) and known T3SS components from other *Xanthomonas*. The presence of HrcC gene and each Xop used in this study was also confirmed in LW16 genome through PCR.

4.3.3. Plasmid construction

The partial fragment of approximately 500 bp length of each gene, for example HrcC was amplified by PCR from the genomic DNA with specific primers that contain the *Xba*I restriction

site and *Kpn*I restriction site on the forward and reverse primer respectively. The amplified fragment was cloned into the Topo vector through TA cloning (Table 4.1), then transformed into the *E.coli* strain DH5α^{TM}-T^{R} . The resulting recombinant plasmid, TopoTAHrcC was extracted using Qiaprep spin miniprep kit according to the manufacturer's instructions, then confirmed using the gene specific primers and Sanger sequencing with the M13F and M13R primers (Eurofins Genomics (Louisville, KY). Following Peng et al. (2016) with some modification, TopoTAHrcC containing cloned fragment was digested with XbaI and KpnI (New England Biolabs) and then ligated with XbaI/ KpnI- double digested suicide vector pKNOCK-Km for subcloning, and then transformed into *E. Coli* PIR1 strain (Table 4.1). The resulting recombinant plasmid named pKNOCK-HrcC was confirmed by PCR and Sanger sequencing using vector primers. After confirmation, resulting plasmid DNA was concentrated to 450 -500ng/ µl for electroporation.

4.3.4. Competent cells preparation and electroporation

A single colony of the Xtu wildtype strain LW16 was grown overnight in 3 ml of NBY liquid medium, which was then inoculated into 60 ml of NBY at 28°C until OD_{600} reached 0.7-1.0. Then bacterial cells were harvested and centrifuged at 4,000 rpm for 10 minutes at room temperature. The pellets were washed in 5 ml of cold TMN buffer (200 mM Tris, 1mM EDTA and 1M NaCl), followed by centrifugation for 5 minutes. The pellet was re-suspended in 0.5 ml of cold TMN buffer (50 mM Tris, 50 mM $MgSO_4$ and 80 mM NaCl), and incubated on ice for 2 hours. After incubation, cells were centrifuged at 8, 000 rpm for 2 minutes. The pellet was washed three times with 1ml cold and deionized water, with each wash followed by centrifugation at 8, 000 rpm for 2 minutes. Final resuspension of competent cells was done in 0.5 ml of cold deionized water. Plasmid DNAs (pKNOCK-HrcC) in 10 µl of 400-500 ng/µl was mixed with 100 µl of *X. translucens* competent cells and then electroporated using a gene pulser (Bio-rad gene Pulser

Xcell, USA), with a voltage of 2.5kV, capacity of 25F, resistance of 200Ω, 0.2cM cuvette to create a transient shock of 4.7-5.0 ms. The electroporated cells were added to 900ml of NBY recovery medium and regenerated for 1h at 28°C. The cells were then plated on WBA plates containing kanamycin and grown at 28 °C for 3 days for selection of kanamycin-resistant insertion mutant.

4.3.5. PCR screening of mutants

The mutants of the highly conserved type III secretion gene HrcC and 13 Xop genes (Table 4.1) were generated using targeted gene insertion method by incorporating a partial gene as well as the whole vector sequence into the wildtype strain as described above. Genomic DNA from a single colony of each kanamycin-resistant mutant was obtained using Qiagen DNeasy UltraClean microbial kit according to the manufacturer's instructions. Insertion in each gene was then confirmed and validated by PCR using gene specific primers located outside the cloned fragment, designated as XtuHrcCkoF2 and XtuHrcCkoR2. Each mutant was also re-streaked on non-selective media plates (WBA without Kanamycin) for an effect in overall growth in comparison to the wildtype strain LW16.

4.3.6. Virulence assays and statistical analysis

Each Xop mutant were tested along with the wildtype strain, LW16 for virulence on seven plant genotypes of wheat, triticale and barley (Table 4.2). However, ΔhrcC mutants were tested only on RB07 wheat genotype. The pathogenicity test and virulence assay were done in a greenhouse room set at 28°C with a 12-h photoperiod and 75% humidity at NDSU.

For ΔhrcC pathogenicity test, two days old bacterial cells of mutants and wildtype were scraped off the WBA plates and resuspended in 1x PBS buffer. Bacterial suspensions were adjusted to OD600 = 0.2 using a BioPhotomer (Eppendorf AG, Germany) for the needleless syringe spot infiltration and to an OD600 = 0.5 was used for the spray inoculation. For the

needleless syringe infiltration assay, bacteria were infiltrated into secondary leaf of 14-day-old plants as spots. Disease development was assessed 7DAI (days after inoculation). For the spray inoculation method, plants at three leaf stage were sprayed using a spray gun. Inoculated plants were placed in the misting chamber for 2 days and then incubated in a plastic tent for 3 days at 75% humidity. Disease development was then assessed 7DAI.

For virulence test of other mutants, the tip inoculation method was used, and the mutant strains and wildtype were applied to a same leaf side-by-side using 200mL tips. The tip was first dipped in bacterial cell suspension which was adjusted to an OD600 = 0.2 and then forcibly touched onto leaf surface to allow a creation of wounds. Both wildtype and mutant strain were applied four times in the up-middle side of the leaf. Disease scoring based on the intensity of the water-soaked lesions was done at 3DAI as +++ (high water-soaking), ++(medium water-soaking), + (low water-soaking), 0 (no water-soaking) and ch (chlorosis development). To quantify virulence of XopY and XopAA mutants, lesion length measurement was done at 6DAI for 16 infiltrated spots on a toatal of 3 plants for wildtype and each mutant. The tip inoculation virulence assay was repeated once for ΔxopY and ΔxopAA mutants, while for the rest of the mutants, experiment was done once. One-way analysis of variance (ANOVA) statistical analyses was performed on all lesion length measurements using JMP® PRO, 17 (SAS Institute Inc., Cary, NC). Mean separation was conducted using the Student t-test and significant difference test was used set at $\alpha = 0.05$.

Table 4.1. Bacterial strains and plasmids used in this study.

Strains / Plasmids	**Relevant characteristics** [a]	**Source**
Plasmids		
PCR2.1-Topo	3.9kb, Amp^r/ Kn^r	Invitrogen Corp, San Diego, CA
pKNOCK-km	2.0 kb, suicide vector, Neo^r/Kn^r	Alexeyev 1999
Escherichia coli		
DH5α^{TM}-T^R	*hsdR, mcrA, lacZΔM15, recA1,* Str^R	Invitrogen Corp, San Diego, CA
PIR1	*hsdR, F–Δlac169, recA1*	Invitrogen Corp, San Diego, CA
X. translucens* pv. *undulosa		
LW16	Wildtype, isolated 2009 in North Dakota	Peng et al. 2019
LW16ΔhrcC	LW16 with the mutation of HrcC, Kn^r	Peng et al. 2016; This study
LW16ΔxopY	LW16 with mutation of XopY, Kn^r	This study
LW16ΔxopAA	LW16 with mutation of XopAA, Kn^r	This study
LW16ΔxopP	LW16 with mutation of XopP, Kn^r	This study
LW16ΔavrBS2	LW16 with mutation of AvrBS2, Kn^r	This study
LW16ΔxopAF	LW16 with mutation of XopAF, Kn^r	This study
LW16ΔxopAA	LW16 with mutation of XopAA, Kn^r	This study
LW16ΔxopAP	LW16 with mutation of XopAP, Kn^r	This study
LW16ΔxopN	LW16 with mutation of XopN, Kn^r	This study
LW16ΔxopR	LW16 with mutation of XopR, Kn^r	This study
LW16ΔxopL	LW16 with mutation of XopL, Kn^r	This study
LW16ΔxopQ	LW16 with mutation of XopQ, Kn^r	This study
LW16ΔxopK	LW16 with mutation of XopK, Kn^r	This study
LW16ΔxopF1	LW16 with mutation of XopF1, Kn^r	This study

[a] Amp^r = Ampicillin resistance; Kn^r = Kanamycin resistance; Neo^r = Neomycin resistance; $Str^{R=}$ Streptomycin resistance.

Table 4.2. List of plant genotypes used for virulence assay in this study.

Name	Relevant Characteristics	Source /Reference
RB07	Hard red spring wheat (*Triticum aestivum L.*); Hexaploid (6n)	University of Minnesota Agricultural Experiment Station; Anderson et al. 2009
Chinese spring	Hard red spring wheat (*Triticum aestivum L.*); Hexaploid (6n)	Sears and Miller, 1985
Boost	Hard red spring wheat (*Triticum aestivum L.*); Hexaploid (6n)	PI 678681; SDSU-HRSW breeding program (2015); Glover et al. 2021
PI41025	Durum wheat (*Triticum turgidum ssp. Dicoccum*); Tetraploid(4n)	USDA Agricultural Research Service; Faris et al. 2014
Siskiyou	Triticale (*xTriticale*); Hexaploid (6n), spring type	International Maize and Wheat Improvement Center, Mexico, and the University of California, Davis, USA; Qualset 1985
UC38	Triticale (*xTriticale*); Hexaploid (6n), spring type	University of California, Davis; Gustafson 1974
Bowman	Barley (*Hordeum vulgare*); two-rowed feed barley	North Dakota Agricultural Experiment Station and USDA-ARS (1984)

4.4. Results

4.4.1. Pathogenicity test of type III secretion HrcC mutants in LW16

No obvious effect on the overall growth of bacteria was observed in LW16ΔhrcC mutants as compared to the wildtype strain on non-selective media plates while the mutants, but not wild type can grow on the media with kanamycin (Figure 4.1). These mutants were tested for their ability to cause disease using the infiltration method on the highly susceptible spring wheat cultivar RB07. Infiltration of wild type produced strong water-soaking symptoms while infiltration with the mutant strain LW16ΔhrcC did not show any reaction indicating a complete loss of pathogenicity (Figure 4.2). Similarly, using spray inoculation assay, no disease symptom was observed for the LW16ΔhrcC while strong water soaking symptoms as well as necrosis were observed for the wildtype at 7 DAI (Figure 4.2).

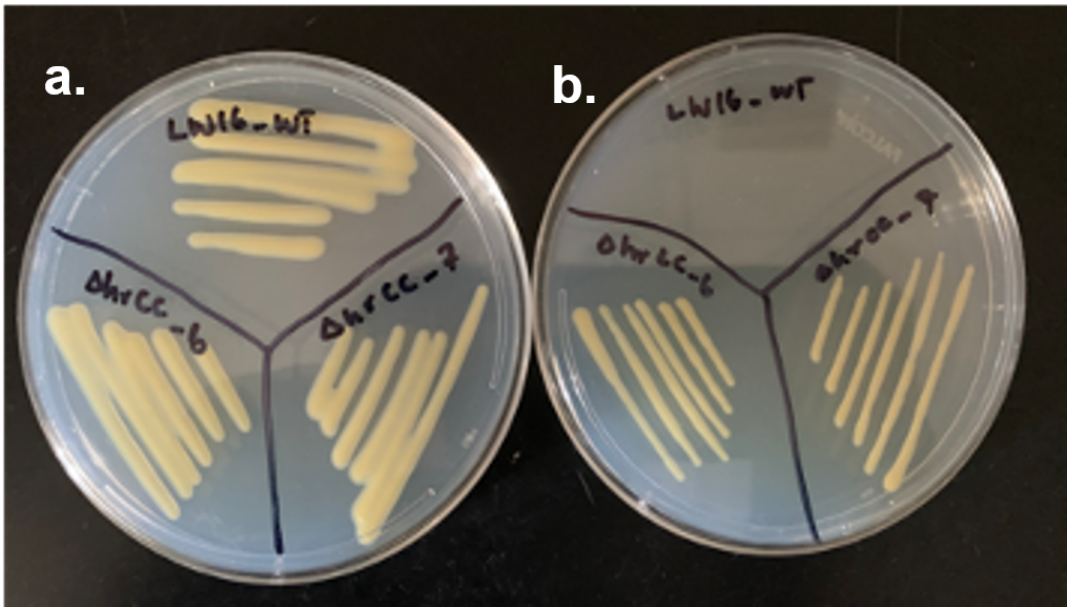

Figure 4.1. Growth comparison of T3SS-deficient ΔhrcC mutant with wildtype strain LW16 on media plates.
Note: (a) Non-selective media plate of WBA containing LWI6 wild type (on top), and ΔhrcC mutants' bacteria (below). (b) Selective media plates of WBA with kanamycin containing LWI6 wild type (on top), and two ΔhrcC mutants' bacteria (below).

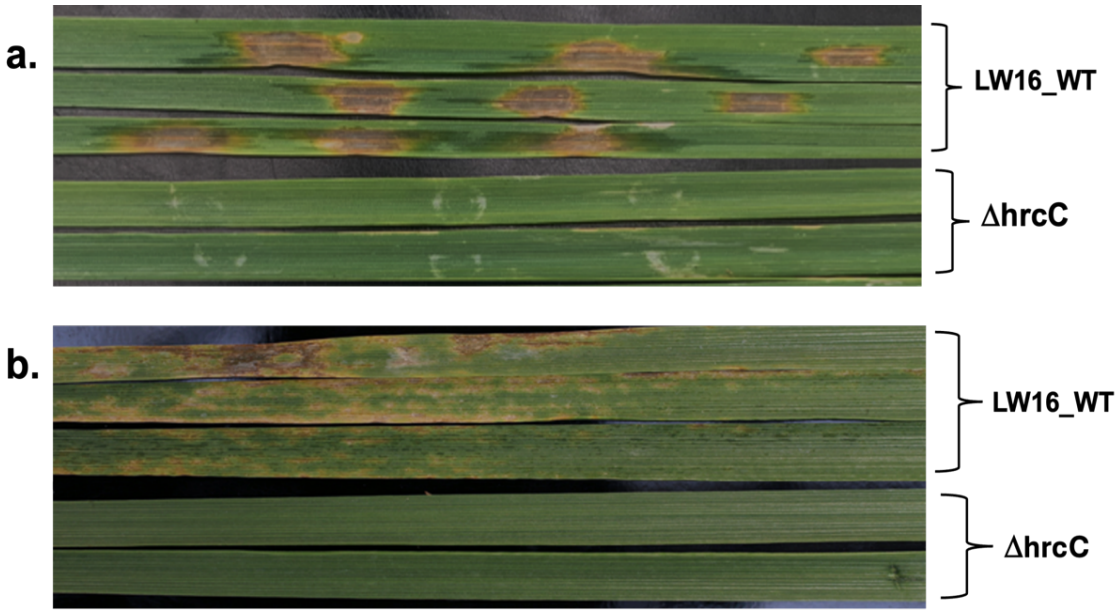

Figure 4.2. Pathogenicity test of T3SS-deficient ΔhrcC mutant of Xtu on highly susceptible wheat line RB07.
Note:(a) Results from needleless syringe infiltration of wildtype LW16 caused strong water-soaking while ΔhrcC mutants showed no symptoms on the plants 7DAI. (b) Results using spray inoculation method. ΔhrcC mutants had no disease symptom while LW16 caused strong water-soaking and necrosis 7DAI.

4.4.2. PCR confirmation of Xop mutants in LW16

All mutants grew identically to the wild type strain LW16 in WBA medium without selective antibiotics (data not shown). The results of PCR amplification with the gene specific primer for mutants and wildtype were shown in Figure 4.3. All mutants had a larger size of band of about 3kb compared to the wild type because of the insertion of the entire pKNOCK-km vector.

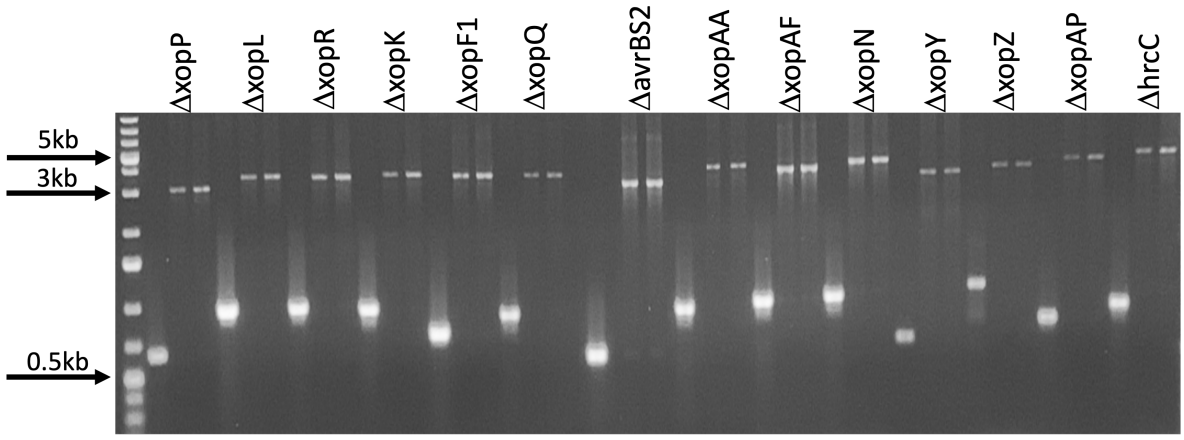

Figure 4.3. Validation of gene mutation by PCR.
Note: Genomic DNA of the mutants for individual genes and wildtype strain LW16 was subjected to polymerase chain reaction (PCR) using designated flanking primers, F2 and R2. The DNA marker was loaded in the first lanes, and the size of four bands was denoted at the left sides of the gels. Wildtype bands are represented for each gene having size between 0.5kb- 1kb. Mutant bands for each gene are represented with size of 3kb - 4kb.

4.4.3. Virulence assay of individual mutants on plant genotypes

Based on intensity of water soaking symptoms, reduced virulence was seen for ΔxopY and ΔxopAA mutants as compared to the wildtype strain LW16 3DAI (Table 4.3). For ΔxopY mutants, reduced water soaking symptoms was observed only on highly susceptible plant genotype of RB07, UC38, Chinese spring and PI41025, and the moderately resistant line, Siskiyou. For ΔxopAA mutants reduced water soaking symptoms was observed only on highly susceptible RB07, UC38, PI41025, and moderately resistant line Siskiyou. For the rest of the 11 Xop mutants tested, no obvious virulence change was observed as compared to the wildtype (Table 4.3).

Virulence quantification done at 6DAI further showed a shorter lesion length for both ΔxopY and ΔxopAA mutants as compared to the wildtype strain (Figure 4.4 and 4.5). For ΔxopY mutants, on RB07, UC38, Chinese spring, PI41025, and Siskiyou, the mean lesion length were 0.58, 0.33 , 0.31, 0.36, and 0.21cm respectively as compared to the wildtype with lesion lengths of 0.8, 0.54 , 0.52 and 0.55 cm respectively (Figure 4.6). Statistical analysis based on student's t-

test showed that the mean length of ΔxopY on these lines are significantly different ($P < 0.0001$) from those of the wildtype at $\alpha = 0.05$ (Figure 4.6). However, no significant differences were seen on the moderately spring wheat line, Boost, and Bowman.

A significant difference (P-value < 0.0001) in the lesion length was also observed 6DAI between the ΔxopAA mutants and the wildtype strain at $\alpha = 0.05$. On RB07, UC38, PI41025, and Siskiyou, the mean lesion length are 0.58, 0.38, 0.43, and 0.3 cm respectively, as compared to the wildtype lesion length of 0.86, 0.63, 0.65, and 0.47 cm respectively (Figure 4.6). No significant differences in Chinese spring , Boost, and Bowman was observed.

Table 4.3. Virulence assay of individual mutants on plant genotypes at 3DAI.

S/N	T3Es		RB07	Chinese spring	Boost	UC38	PI41025	Siskiyou	Bowman
1	XopQ	WT	+++	++	++	++	+++	++	+/ch
		KO1	+++	++	++	++	+++	++	+/ch
		KO2	+++	++	++	++	+++	++	+/ch
2	XopK	WT	+++	++	++	++	+++	++	+/ch
		KO1	+++	++	++	++	+++	++	+/ch
		KO2	+++	++	++	++	+++	++	+/ch
3	XopL	WT	+++	++	++	++	+++	++	+/ch
		KO1	+++	++	++	++	+++	++	+/ch
		KO2	+++	++	++	++	+++	++	+/ch
4	XopN	WT	+++	++	++	++	+++	++	+/ch
		KO1	+++	++	++	++	+++	++	+/ch
		KO2	+++	++	++	++	+++	++	+/ch
5	XopAA	WT	+++	++	++	++	+++	++	+/ch
		KO1	++	++	++	+	++	+	+/ch
		KO2	++	++	++	+	++	+	+/ch
6	XopY	WT	+++	++	++	++	+++	++	+/ch
		KO1	++	+	++	+	++	+	+/ch
		KO2	++	+	++	+	++	+	+/ch
7	XopZ	WT	+++	++	++	++	+++	++	+/ch
		KO1	+++	++	++	++	+++	++	+/ch
		KO2	+++	++	++	++	+++	++	+/ch
8	XopP1	WT	+++	++	++	++	+++	++	+/ch
		KO1	+++	++	++	++	+++	++	+/ch
		KO2	+++	++	++	++	+++	++	+/ch
9	AvrBS2	WT	+++	++	++	++	+++	++	+/ch
		KO1	+++	++	++	++	+++	++	+/ch
		KO2	+++	++	++	++	+++	++	+/ch
10	XopR	WT	+++	++	++	++	+++	++	+/ch
		KO1	+++	++	++	++	+++	++	+/ch
		KO2	+++	++	++	++	+++	++	+/ch
11	XopF1	WT	+++	++	++	++	+++	++	+/ch
		KO1	+++	++	++	++	+++	++	+/ch
		KO2	+++	++	++	++	+++	++	+/ch
12	XopAP	WT	+++	++	++	++	+++	++	+/ch
		KO1	+++	++	++	++	+++	++	+/ch
		KO2	+++	++	++	++	+++	++	+/ch
13	XopAF	WT	+++	++	++	++	+++	++	+/ch
		KO1	+++	++	++	++	+++	++	+/ch
		KO2	+++	++	++	++	+++	++	+/ch

+++ = (high water-soaking); ++ = (medium water-soaking); + (low water-soaking); ch = (chlorosis). Symptoms in red color shows mutant's phenotype with virulence difference from the wildtype.

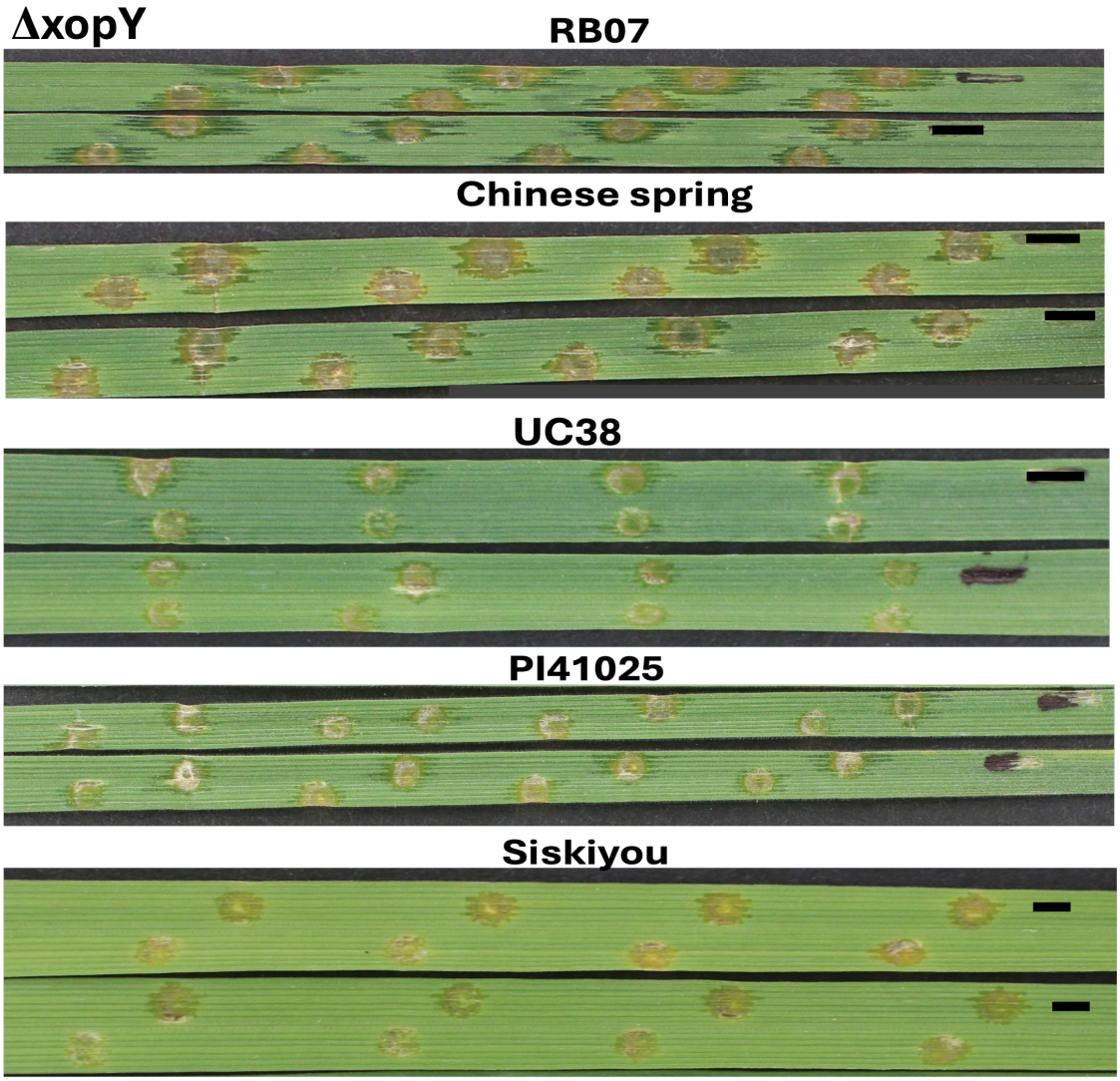

Figure 4.5. Virulence assay of *X. translucens* pv. *undulosa* LW16ΔxopY mutants.
Note: Pictorial representative of lesion length comparison of wildtype strain (with a black line in front) and mutants showed a reduced lesion length for ΔxopY mutants at 6 DAI on susceptible wheat lines: RB07, Chinese spring and PI41025; and on susceptible and moderately resistant triticale line UC38 and Siskiyou respectively.

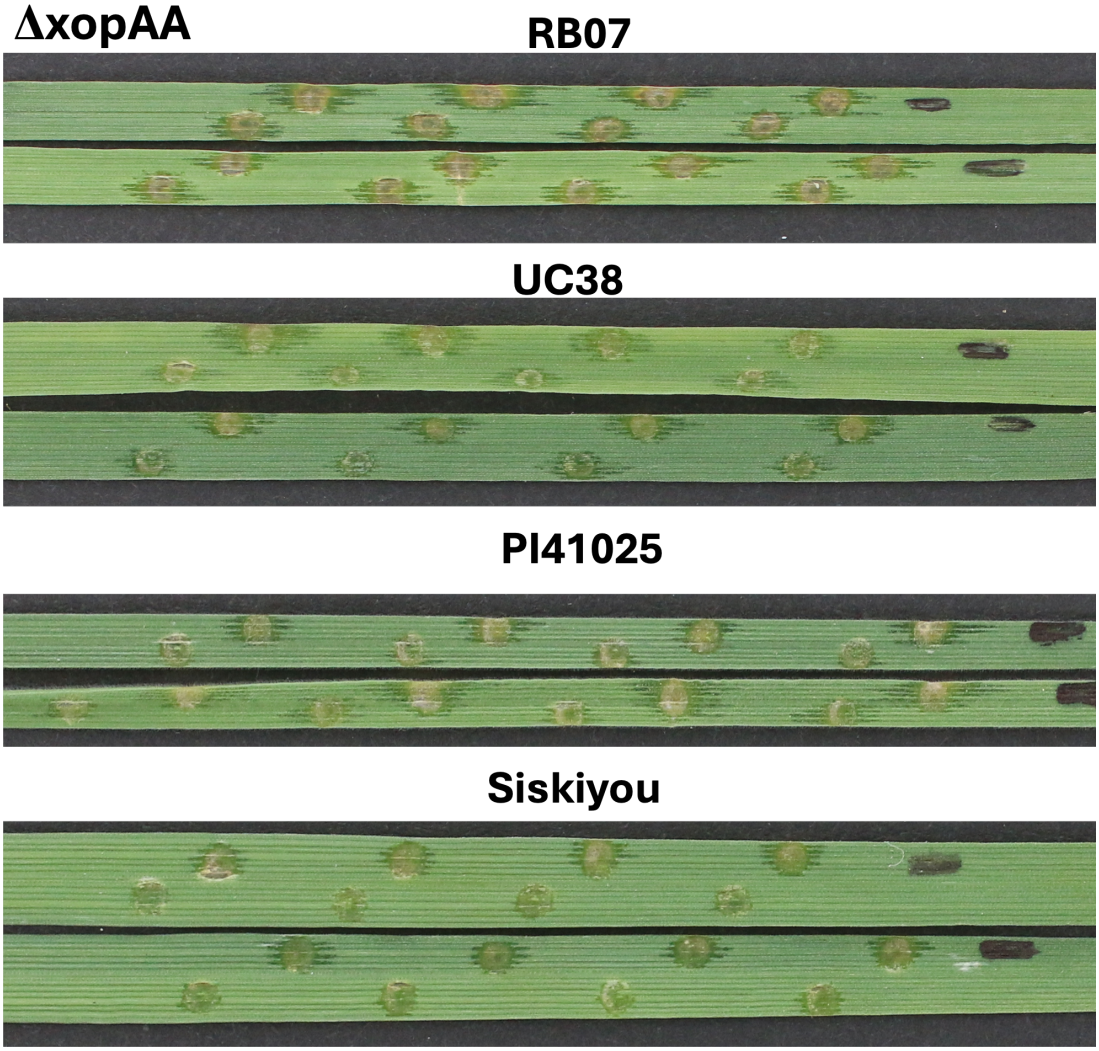

Figure 4.6. Virulence assay of *X. translucens* pv. *undulosa* LW16ΔxopAA mutants.
Note: Pictorial representative of lesion length comparison at of wildtype strain (with a black line in front) and mutants showed a reduced lesion length for ΔxopAA mutants at 6 DAI on susceptible wheat lines: RBO7 and PI41025; and on susceptible and moderately resistant triticale lines UC38 and Siskiyou respectively.

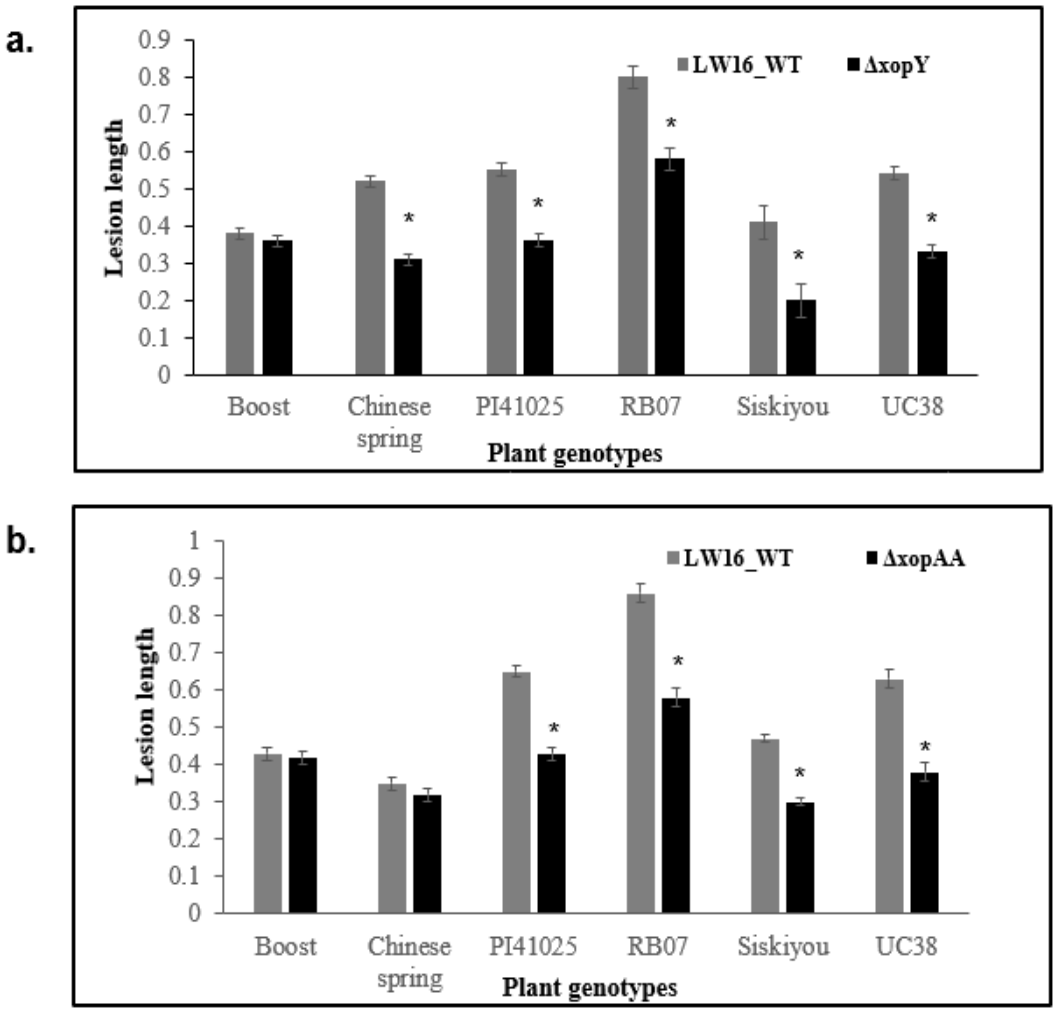

Figure 4.7. Virulence assays of *X. translucens* pv. *undulosa* strain LW16 type III non-TAL effectors mutants.
Note: (a) Mean lesion length of ΔxopY mutants at 6DAI. (b) Mean lesion length of ΔxopAA mutants at 6DAI. The Xop mutants have shorter lesion length on some plant genotypes compared to the wildtype. Values are the mean lesion length ± standard deviation (SD) from three replications, with 16 lesions. "*" represents marked values are significantly different from wild type in a Student's t-test ($\alpha = 0.05$).

4.5. Discussion

Bacterial Leaf Streak (BLS) disease is a major concern to hard red spring and durum wheat producing states including North Dakota (Adhikari et al. 2012; Kandel et al. 2012; Curland et al. 2018; Sapkota et al. 2020). Lack of understanding of the pathogen virulence mechanisms and host-

pathogen interactions as well as the scarcity of host resistance make breeding for BLS resistance difficult. In this study, I investigated the contribution of 13 Xanthomonas outer proteins (Xops) to virulence of the Xtu strain, LW16 by using individual target gene disruption. I successfully obtained disrupted mutant for HrcC and all 13 Xop genes and showed that ΔHrcC mutants completely lost pathogenicity and only two Xops, XopY and XopAA had a significant role in virulence of LW16 strain. This is the first functional study of the Non-TAL type III effectors in Xtu virulence and will help to provide a framework to understand the disease determinants and molecular mechanisms of host pathogen interaction in wheat BLS pathosystem.

Consistent with Peng et al. (2016) that performed mutagenesis of HrcC in another Xtu strain, Xtu4699, I also confirmed that pathogenicity of the Xtu LW16 strain is dependent on the T3SS, which likely is required for the successful delivery of the Type III effector into plant cells for virulence. The successful mutagenesis of HrcC gene also demonstrated the effectiveness of the target disruption protocol employed in this study. This provides an efficient tool for conducting gene function in Xtu in our laboratory.

XopY is a 263 amino acid protein conserved in *X. translucens* strains (Peng et al. 2019), however, studies have shown that homologues are only found in a small number of *Xanthomonas* isolates (Furutani et al., 2009; White et al. 2009). XopY contains motifs similar to subtilases (serine proteases) and interacts with a lysine motif-containing plant pattern recognition receptor (Yamaguchi et al. 2013b). Although, ΔxopY mutant strain did not exhibit any defect in virulence of Xoo on rice plants, however, using transgenic rice plants expressing XopY, it was functionally shown to interact with the receptor-like kinase gene, OsRLCK185 to inhibit downstream signaling of phosphorylation and thus preventing defense response within the plant (Yamaguchi et al. 2013b). BlastP search indicated XopY genes of Xtu and Xoo had 47% amino acid similarity.

Because this similarity is relatively lower, it is very possible that XopY plays a significant role in disease by Xtu in wheat but not in Xoo in rice. It remains to be determined if XopY interacts with receptor-like kinase gene in wheat plant to induce BLS disease.

XopAA is a 714 amino acid protein in Xtu containing motifs similar to DEAH-ATP-dependent RNA helicases, and is conserved among *X. translucens* strains (Peng et al. 2019). XopAA in Xoo has been shown to be associated with phenotypic response such as early chlorosis factor in rice plants (White et al. 2009); however, Yamaguchi et al. (2013a) showed that ΔxopAA did not have any virulence change in knockout mutants but was found to interact with the gene OsBAK1 to inhibit plant immunity. BlastP search showed that XopAA in Xtu has 88% amino acid similarity with XopAA from Xoo, which is relatively high. It is interesting to see that XopAA significantly contributes to Xtu virulence but not for Xoo. However, its molecular mechanisms to affect virulence need to be investigated.

Plant assay results showed that ΔxopY mutants showed a significant reduced lesion length at $p \leq 0.05$ on five of the plant genotypes used in this study, and not on the moderately resistant spring wheat line Boost, or the susceptible barley line, Bowman (Figure 4.6a). While ΔxopAA mutants showed a significant reduced virulence change on four of the plant genotypes, and not on other plant genotypes (Figure 4.6b). This may suggest that non-TALES may have different contributions to virulence in different plant genetic background. This has also been observed for other plant-Xanthomonas interactions. For instance, AvrBs2 and XopN contribute to the virulence in Xoc strain GX01 individually on the rice line Jiangang 30 (Liao et al. 2020). However, Li et al. (2015) found that only AvrBs2 but not XopN was required for full virulence in Xoc RS105 on rice line Nipponbare.

Other 11 non-TAL effector genes, including XopL, XopN, AvrBS2, XopZ, XopK, XopQ, XopR, XopAP, XopAF, XopP and XopF1, did not show any virulence change when individually mutated in the Xtu strain LW16. This has some difference in previous studies involving other plant-Xanthomonas systems. For example, some of the above Xop were shown to be important for various *Xanthomonas* species, including XopZ in Xoo (Song and Bang 2010), AvrBS2 in Xoc (Li et al. 2015), XopN in Xcv (Roden et al. 2004) and Xoo (Liao et al. 2020), XopL in Xcv (Leong et al. 2022) and Xcc (Yan et al. 2019). Since different *Xanthomonas* species attack different plants and cause different disease symptoms, thus, the molecular function of each Xop in virulence may differ in different pathosystems. In addition, functional redundancy or overlap of multiple effectors within a strain can prevent the detection of obvious phenotypic change of individual effector mutations in the host. It is also possible that these Xops are not important in virulence of the Xtu strain used in this study. On the other hand, the effect of individual gene mutations in virulence of Xtu may not have also been detected under the inoculation method used in this study. Some effectors can suppress preinvasive immunity, such as stomatal closure (Lozano-Durán et al. 2014). As such, the infiltration method used here to assess the virulence assay of these Xop mutants may have bypassed the first layer of plant immunity, which may be triggered before or during the natural entry of the pathogen into host extracellular spaces (Zeng et al. 2012).

In Summary, among the 13 Xop mutants generated and tested in this study, two Xops, XopY and XopAA contribute individually to the virulence of Xtu LW16 based on a reduced phenotypic change, as compared to the wildtype. Further mutation and virulence studies of these two Xops in other Xtu strains and gene complementation will validate these findings, which will be useful to provide insight into the host-pathogen interactions of the wheat-BLS pathosystem for breeding durable resistance in wheat.

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5. CHARACTERIZATION OF HRSW AS THE SUPPRESSANT ROTATION CROP OF *PLASMODIOPHORA BRASSICAE* POPULATION IN THE SOIL

5.1. Abstract

Clubroot, caused by *Plasmodiophora brassicae* is a major economically important disease of canola worldwide and is endemic to the acidic soils of northeastern North Dakota. Lack of recommended rotation practice with non-host crops can result in resistance breakdown. Thus, this study aims to assess the potential of Hard Red Spring wheat (HRSW) and other crops to suppress the soil's resting spore population of *P. brassicae*, influence the soil pH and determine the recommended length of rotation practice. The two-year rotation sequences of canola-canola, canola-wheat, canola-soybean, canola-flax, and canola-field pea were studied in a field with a clubroot history and acidic PH. Soil samples were collected at two main time points, “at planting” and “60 days after planting” and the spore concentration was quantified using Quantitative PCR (Q-PCR). Statistical analysis to compare spore populations between the two time points revealed no significant differences in canola-field pea, canola- soybean and canola- flax treatments. A 9% decrease of spore populations in canola-field pea, and 32% increase in canola-soybean treatments revealed a higher potential of field pea and soybean to suppress the resting spores as compared to a 63%, 68%, and 51% increase in canola, wheat and flax rotation respectively. No influence in the soil pH by any of the treatments was observed in both years. A significant increase in spore populations with canola, wheat and flax as previous crops and not in field pea and soybean treatments was observed in the host-interval study. This work revealed that wheat is not an optimal rotation crop to manage clubroot on canola like field pea and soybean and a longer period of years is needed for effective disease suppression when these rotation crops are used.

5.2. Introduction

Plasmodiophora brassicae is an obligate, soil borne parasite that causes clubroot disease of canola (*Brassica napus*) and many other cruciferous plants worldwide, especially in areas where cool, cloudy, and humid weather persists (Chapara and Meena 2021). The pathogen survives in the soil as microscopic well-protected resting spores and are viable in the soil for up to 20 years (Wallenhammar 1996; Kageyama and Asano 2009).

Clubroot causes local hypertrophy, characterized by swelling and abnormal growth (clubbing) of infected roots, which disrupts the uptake and transport of water and nutrients from the soil into host plants (Dixon 2009). The disruption leads to above ground symptoms such as wilting, stunting, premature ripening, and losses in both seed yield and quality (Wallenhammar et al. 1996; Strelkov et al. 2006). An estimated annual yield loss of up to 10-15% due to clubroot disease have been reported on global cruciferous crop production (Dixon 2009; Li et al. 2013) and have thus become a major threat to canola production industry. Coupled with inadequate alternative management practices, the longevity of the resting spores makes *P. brassicae* difficult to eradicate once it becomes established in a field, making the management of clubroot disease challenging for canola growers (Chapara and Meena 2021).

Several management strategies for clubroot have been used in the production of cruciferous vegetables, including an integrated approach of field sanitation, early disease detection, soil amendment with lime to increase soil pH or maintain alkalinity, and soil drench application of fungicides (Myers and Campbell 1985; Webster and Dixon 1991; Donald and Porter 2014). While these strategies may effectively reduce clubroot severity in cruciferous vegetable crops, many of the strategies are not economical or cost effective for the production of canola because they require highly mechanized operations over very large areas (Hynes and Boyetchko 2011). Host resistance

is an important tool in the management of many diseases in different plant pathosystems. Currently, only a few genes expressing strong resistance to common pathotypes of *P. brassicae* are known and have been developed and deployed as clubroot resistant (CR) varieties (Peng et al., 2014; Strelkov & Hwang, 2014). However, the repeated cultivation of these CR cultivars has exerted selection pressure on *P. brassicae* populations (LeBoldus et al. 2012), leading to the emergence of new virulent strains of *P. brassicae* that overcame those resistance genes (Hwang et al. 2014; Strelkov et al. 2016a). As such, incorporating crop rotations, using disease resistant cultivars, soil amendments and sanitation has proved more effective than any of the individual disease management components in large-scale and on-farm studies conducted in Canada and in the USA (Chapara and Meena 2021).

A longer break from brassica hosts is the most effective and practical management option for clubroot (Rempel et al. 2014; Chapara and Meena 2021). Clubroot severity increases with spore concentrations (Hwang et al. 2011a and b) and each gall on an infected canola plant can produce millions of spores (Hwang et al. 2012), leading to accumulation of inoculum on a field. Although, the roots of both host and non-host plants can stimulates the germination of the resting spores of *P. brassicae* (Friberg et al. 2006). However, in the absence of host plants, germinated spores are likely to survive only for short periods (Suzuki et al. 1992; Takahashi 1994). In previous years, the main effective strategy for crop rotation in infested field with *P. brassicae*, was rotation out of susceptible brassica crop for 4 or more years (Strelkov and Hwang 2014). For example, a 5-year fallow period with the cultivation of a CR brassicae crop, Japanese radish (*Raphanus sativus* var. *longipinnatus*) resulted in a significant decrease in the resting spore populations (Ikegami 1975). However, this was not a popular option for canola farmers given the higher returns associated with canola as compared to other cropping systems (Strelkov et al. 2011). In recent times, studies have

shown that a 3-year rotation of canola with cereals and pulses is sustainable (Cathcart et al. 2006). Furthermore, Peng et al. (2014) showed that a two-year canola breaks reduced *P. brassicae* resting spore concentrations by >90% relative to growing continuous oilseed rape (OSR) or a one-year break in heavily infested field plots.

Since its identification in 2013 in the Cavalier county, North Dakota (Chittem et al. 2014), the clubroot pathogen, *P. brassicae* has been spreading rapidly in various fields, especially in the upper northeastern part of North Dakota (Chapara et al. 2019) and is fast becoming a threat to canola production in North Dakota. The primary management practices in North Dakota are the use of resistant cultivars and a short crop rotation with non-host crops (especially wheat and soybean). However, the recommended length of rotation practice is rarely seen in these clubroot prevalent areas, and no study has been done to determine the suitable rotation sequence with canola in North Dakota. Spring wheat is one of the most widely grown crop in North Dakota and one of the major staples crops of the United States, grown within a moderately short growing season (National Agricultural Statistics Service, USDA 2023), making it an easily accessible non-host crop to rotate with canola against *P. brassicae* resting spores.

The aim of this study was thus to determine the suitability of hard red spring wheat (HRSW), soybean, flax and field pea to suppress *P. brassicae* spore populations in the soil, (2) to influence a change in soil pH, and (3) to determine the recommended year of crop rotation with non-host crop in clubroot prevalent areas in North Dakota.

5.3. Materials and methods

5.3.1. Experiment site and local climate

A 2-year rotation study from 2021- 2023, was conducted on a canola grower's field with a history of clubroot (48° 45' 36" N / 98° 22' 5" W). The local climate is humid continental

categorized as Dfb (according to Koppen's climate classification), with an average annual high temperature of 47 ºFahrenheit (ºF), average annual low temperature of 26 ºF, and mean annual precipitation of 19.42 inch. The soil type at experiment site is Svea-Barnes loam, characterized by a mix of sand, silt, and clay, providing good water retention and drainage.

5.3.2. Experimental design and soil sampling

The size of the experimental site is 50x6x4 cubic ft and was divided into 20 plots, each plot being 5x10 ft. Prior to the establishment of the experiment, the preceding crop was a susceptible canola (cv. InVigor L233P). The selected research ground was sprayed with a pre-emergent herbicide (Treflon®) and was tilled thoroughly with a rototiller. For the first year, five different treatments (non-host crops including wheat, soybean, flax and field pea and susceptible canola check) were used in the trial, and each planted four times using the Randomized Complete Block Design (RCBD) design (Table 5.1). In the second year, the same treatments were used in a different part of the same field. Using the hand planted method, with a seeding rate of 3.5 gm/ 50 sq ft or 5 lbs/ acre, the cultivars of the crops were planted as following; soybean- P009T18E; canola- cv L233P; wheat- Faller; flax- Hammond and field pea- Salamanca. For the third objective, in the following year, a clubroot susceptible canola (cv L233P) crop was seeded in all 20 plots which were previously planted with different rotation crops; soybean, wheat, field pea, flax, and susceptible canola (Table 5.1). Soil sample collection was done twice (at planting and 60 days after planting) during the research trials. Cores of soil were collected at a depth of 2-6 inches from each treatment replicate for spore quantification and pH determination at both time points.

5.3.3. Soil sample preparation, DNA extraction and pH determination

Soil samples collected from each treatment plot were first air dried at room temperature in a greenhouse room. Each soil sample was ground to fine powder using mortar and sieved thoroughly for homogenization and uniformity. Two biological replicates, each of 200mg soil from each treatment plot were then prepared for DNA extraction. Genomic DNA was extracted from these replicates using Qiagen DNAasy Power Soil kit according to the manufacturer's instructions. DNA samples were placed at -20ºC for long-term storage. Soil samples that were collected from each treatment plots at planting and 60 days after planting were also subjected to pH determination at the NDSU soil testing laboratory.

5.3.4. Quantification of *P. brassicae* spore populations

Molecular assays using real-time quantitative PCR (Q-PCR) were used to quantify the resting spores of P. brassicae from the soil collected from each plot. All Q-PCR analyses were performed using a multiplexed TaqMan assay that included a competitive internal positive control (CIPC) (Deora et al. 2015) for the detection and quantification of P. brassicae in the soil. A primer pair of DC1F (5'-CCTAGCGCTGCATCCCATAT-3') and DC1R (5'-CGGCTAGGATGGTTCGAAA-3') was used to amplify a 90-bp fragment of internal transcribed spacer (ITS) 1 region of P. brassicae (Cao et al. 2007), along with two CIPC probe sets; PB1 (5'-6-FAMCCATGTGAACCGGTGAC-NFQ-MGB-3') and GFP1 (5'- VICACCATTACCTGTCGACACAATCTGCCCT-NFQ-MGB-3') (Deora et al. 2015). The assays were conducted in a 96-well Bio Rad real-time system according to the manufacturer's instructions.

The reaction mixture of a total volume of 20µl included 10µl of 2x Sso Advanced Universal Probes Supermix (Bio-Rad), 0.8µl of 22.5µm the *P. brassicae* primers, 1.0µl of 5Mm CIPC probes (Applied Biosystems, Thermo Fisher scientific), 2µl of target DNA and 4.4 µl of distilled water as

negative control. The thermal cycling conditions were run at 95 °C for 3 mins, followed by 39 cycles at 95 °C for 15 s and 62 °C for 1min. There were two technical replicates for each of the two-biological replicate of each treatment plot in each assay. Water was also included as a negative control. For spore estimation, a 10-fold dilution series of *P. brassicae* gDNA resting spores prepared from clubbed root galls collected from field ranging from 10^2 to 10^8 was used to generate a standard curve as previously described by (Deora et al. 2015). Estimates of spore concentration were generated as the output for Cq values and starting quantity (SQ) using CFX Maestro software (version 2.3, Bio-Rad). The cq value of the CIPC was adjusted to 20 for a correction of inhibition (Bilodeau 2011, Deora et al. 2015).

The lowest Cq value on the standard curve for 10^2 spores was selected as the threshold point for Cq values for all the samples. Thus, Cq values of samples greater than the threshold Cq was considered absent of the pathogen and denoted as zero spores. For Cq values of samples less than the cut off Cq but within the standard curve concentrations, an average of the starting quantity (SQ) values obtained from the four technical replicates was used to estimate the spore population for each sample and multiplied by 5 to give the number of spores/1g soil.

5.3.5. Statistical analysis

All statistical analysis was performed using the JMP®, Version 17. (SAS Institute Inc., Cary, NC, 2024). The spore population obtained from the soil samples obtained from each treatment, “at planting’ and at “60 days after planting” were used to statistically analyze and identify suitable non-host crop after clubroot susceptible canola was planted. The homogeneity of variance was checked with Levene’s Test prior to pooling the resting spore data from the two years for analysis of variance (ANOVA). Quantification data of resting spores were Log10 transformed prior to analysis, and the transformed data showed a normal distribution based on the Shapiro–

Wilk Test. Change in resting spore concentrations at the two sampling time points were assessed using a paired two-sample t-test. Fisher's Protected LSD was used to separate the means when ANOVA was significant at ($P \leq 0.05$).

The pH value obtained from the soil samples obtained from each treatment, "at planting' and at "60 days after planting" were also used to statistically analyze to determine crop rotation on soil pH. The homogeneity of variance was checked with Levene's Test. Change in soil pH at the two sampling time points in each treatment were assessed using a paired two-sample t-test. Fisher's Protected LSD was used to separate the means when ANOVA was significant at ($P \leq 0.05$).

5.3.6. Disease assessment

For the host interval study where canola was planted after the first year of non-host rotation, the disease severity of clubroot on canola were visually assessed 60 days after planting. Canola stubbles (N= 25 plants) of each plot were uprooted from the soil, excess soil from the roots was removed by a gentle shake. The galls on the roots were rated by using the 0-3 scale developed by (Kuginuki et al. 1999; Strelkov et al. 2006), where 0=no galls, 1=a few small galls (small galls on less than one-third of the roots), 2 = moderate galling (medium to large-sized galls on one-third to two-thirds of the roots), and 3 = severe galling (medium to large-sized galls on greater than two-thirds of the roots). A disease index (DI) was calculated using the formula of Horiuchi and Hori (1980) later modified by Strelkov et al. (2006):

$$\mathrm{DI}(\%) = \frac{\sum(n\times 0+n\times 1+n\times 2+n\times 3)\times 100}{N\times 3} \quad (1)$$

where Σ is the sum total, "n" is the number of plants in each severity level; "N" is the total number of plants evaluated; and 0,1,2 and 3 are the symptom severity classes.

Table 5.1. Crop rotation sequences used in this study from 2021-2023.

	Non-Host interval (First & second year)		**Host interval (After the first year)**	
Entry	Experiment ID	Rotation sequence	Experiment ID	Rotation sequence
1	CRROTATION_R1	canola-soybean	CRTCLA_R1	canola-soybean-canola
2	CRROTATION_ R2	canola-canola	CRTCLA_R2	canola-canola-canola
3	CRROTATION_ R3	canola-wheat	CRTCLA_R3	canola-wheat-canola
4	CRROTATION_ R4	canola-flax	CRTCLA_R4	canola-flax-canola
5	CRROTATION_ R5	canola-field pea	CRTCLA_R5	canola-field pea-canola

5.4. Results

5.4.1. Effect of non-host rotation sequences on *P. brassicae* spore populations in the soil

The effect of different non-host rotation sequence on *P. brassicae* spore populations were investigated by comparing soil spore populations "at planting" and "60 days after planting". Leven's Homogeneity test of variance for spore quantification data obtained for two years showed an insignificant p-value of 0.1955, indicating a homogenous variance among the groups. Thus, quantification data from the two years were pooled together.

A significant increase in spore concentrations by 63% was observed in canola-canola treatment with an actual spore number of 1.29×10^5 g/soil at planting to 3.51×10^5 g/soil 60 days after planting (Figure 5.1). Similarly, a significant increase was observed for canola-wheat treatment by 68% with an actual spore number of 9.54×10^4 g/soil at planting to 3.1×10^5 g/soil 60 days after planting. An insignificant increase was seen in canola-flax and canola-soybean, with a percent change of 51% and 32% respectively. Canola-field pea had a reduction of spores by 9% from 1.7×10^5 g/soil to 1.62×10^5 g/soil, however, analysis of variance (ANOVA) performed at $P \leq 0.05$ showed that actual spores obtained from the two time points are insignificant (Figure 5.1).

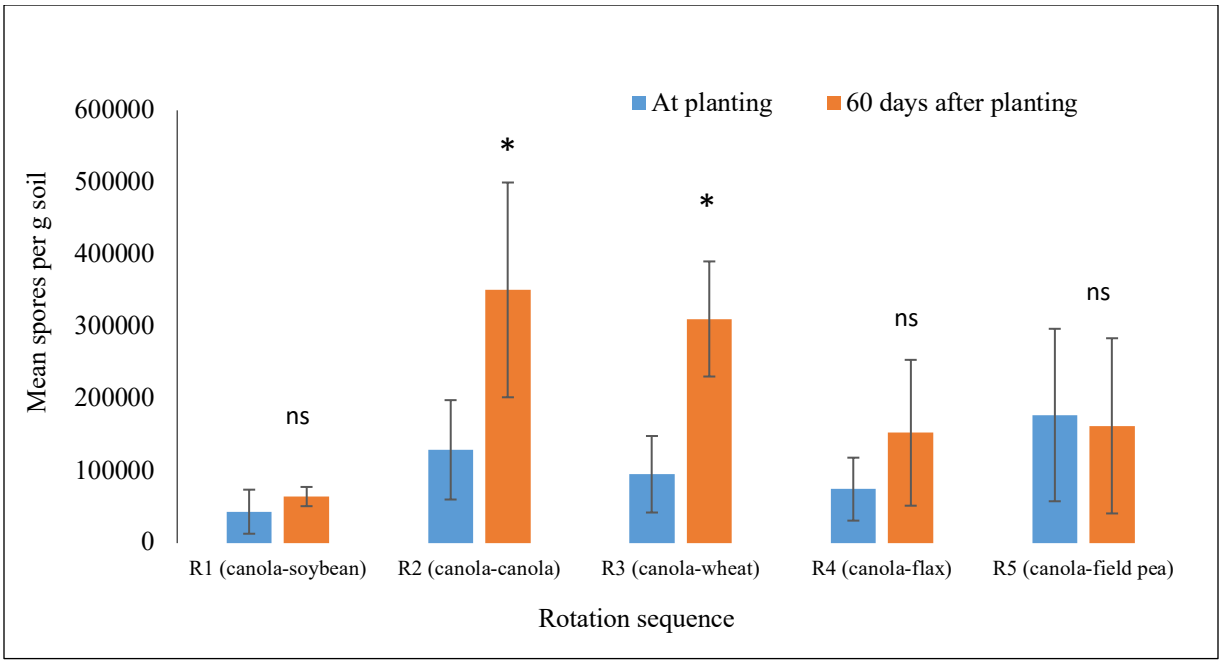

Figure 5.1. Comparison of mean resting spores that were obtained at planting and 60 days after planting in the two years with canola (cv L233P) as the previous crop.
Note: n.s= non-significant; * = significance obtained based on the Tukey-Kramer's test at $P \leq 0.05$.

5.4.2. Effect of non-host crop rotation on the soil pH value

The effect of the rotating non-host crops after planting susceptible canola on the spore populations of *P. brassicae* was also assessed by comparing soil pH values "at planting" and "60 days after planting" for the two years individually. Leven's Homogeneity test of variance for soil pH obtained for two years showed a significant p-value of 0.006.

In the first year, ANOVA showed that there was no significance in the average soil pH at the two time points in each of the treatments. An average soil pH between 6.1- 6.6 was observed across treatments (Table 5.2). Likewise, ANOVA analysis performed for the second-year rotation study, with an average pH between 6.0-6.4 showed no significance in each treatment between both time points.

Table 5.2. Comparison of mean soil pH that were obtained at planting and 60 days after planting (canola (cv L233P) as the previous crop) from 2021-2023.

	YEAR 1		YEAR 2	
Rotation patterns (treatments)	**pH (at planting)**	**pH (60 DAP)**	**pH (at planting)**	**pH (60 DAP)**
R1 (canola - soybean)	6.1$^{n.s}$	6.2$^{n.s}$	6.1$^{n.s}$	6.4$^{n.s}$
R2 (canola - canola)	6.1$^{n.s}$	6.3$^{n.s}$	6.1$^{n.s}$	6.4$^{n.s}$
R3 (canola - wheat)	6.1$^{n.s}$	6.3$^{n.s}$	6.0$^{n.s}$	6.4$^{n.s}$
R4 (canola - flax)	6.2$^{n.s}$	6.4$^{n.s}$	6.1$^{n.s}$	6.3$^{n.s}$
R5 (canola - field pea)	6.4$^{n.s}$	6.6$^{n.s}$	6.0$^{n.s}$	6.3$^{n.s}$

DAP = days after planting; Data represent the means of four replications. The F values were not significant at $P \leq 0.05$ by analysis of variance. n.s= non-significant; ***** = significance obtained based on the Tukey-Kramer's test at $P \leq 0.05$.

5.4.3. Effect of host interval rotation on *P. brassicae* spore populations and disease severity

To determine if a year of rotation with non-hosts crop is a recommended length to suppress the resting spores of *P. brassicae* in the soil, susceptible canola (cv. L233P) was planted after the first year of non-host rotation on the same ground that has previous crops of soybean, wheat, field pea, flax, and canola (Table 5.1). The estimated spore populations "at planting" and "60 days after planting" in each plot was then compared. An increase in spore concentrations was observed in all treatments. However significant increase between the two time points were observed in treatments of canola-canola-canola from 1.68 $\times 10^5$ g/soil to 8.17 $\times 10^5$ g/soil, canola-wheat-canola from 1.38 $\times 10^5$ g/soil to 9.52 $\times 10^5$ g/soil, and canola-flax-canola from 1.32 $\times 10^5$ g/soil to 9.99 $\times 10^5$ g/soil at $P \leq 0.05$. While the increase in spore concentrations observed in other treatments having field pea and soybean as previous crops was insignificant (Table 5.3). Canola-field pea-canola treatment had an increase from 1.55×10^5 to 7.63×10^5 and canola-soybean-canola had an increase from 1.33×10^5 to 6.0×10^5.

The average clubroot disease index (DI%) indicated there was no significant differences between each treatment. However, the lowest DI% were found in canola-soybean-

canola (23.5%) and canola-field pea-canola (24.7%) treatments, while the highest average disease index was found in canola-canola-canola (44.5%) and canola-wheat-canola (34.25%) (Table 5.3).

ANOVA performed on the pH data collected from the soil samples collected "at planting" and "60 days after planting" from each treatment indicate that no significant different between the two time points in each treatment individually (Table 5.3).

Table 5.3. Effect of host interval rotation (planted after the first year of non-host rotation) on *P. brassicae* spore populations, disease severity (DI%) and soil pH parameters, with non-host crops and resistant check as previous crops.

Crop rotation pattern	**Average disease index (%)**	**Average spore concentrations (g/soil)**		**Fold increase (%)**	**Average soil pH**	
		At planting	**60DAP**		**At planting**	**60DAP**
canola-soybean-canola	23.5 $^{n.s}$	1.13 $\times 10^5$ $^{n.s}$	6.0 $\times 10^5$ $^{n.s}$	436	6.1 $^{n.s}$	6.2 $^{n.s}$
canola-canola-canola	44.5 $^{n.s}$	1.68 $\times 10^5$ *	8.17 $\times 10^5$ *	384	6.1 $^{n.s}$	6.2 $^{n.s}$
canola-wheat-canola	34.25 $^{n.s}$	1.38 $\times 10^5$ *	9.52 $\times 10^5$ *	586	6.2 $^{n.s}$	6.3 $^{n.s}$
canola-flax-canola	31.5 $^{n.s}$	1.32 $\times 10^5$ *	9.99 $\times 10^5$ *	654	6.0 $^{n.s}$	6.1 $^{n.s}$
canola-field Pea-canola	24.75 $^{n.s}$	1.55 $\times 10^5$ $^{n.s}$	7.63 $\times 10^5$ $^{n.s}$	390	6.1 $^{n.s}$	6.1 $^{n.s}$

DAP = days after planting; n.s= non-significant; * = significance obtained based on the Tukey-Kramer's test at $P \leq 0.05$.

5.5. Discussion

Clubroot disease is rapidly becoming a threat to canola production in North Dakota. Currently, available management practices such as soil amendment and soil drench fungicide application are not practical for canola production (Hynes and Boyetchko 2011; Donald and Porter 2014). Furthermore, single genetic resistance have been quickly eroded in clubroot resistant (CR) cultivars due to high variability of the clubroot pathogen, *Plasmodiophora brassicae* (Hwang et al. 2014; Strelkov et al. 2016a). The resting spores of *P. brassicae* is the most important source of

primary inoculum to determine clubroot severity (Murakami et al. 2002). Therefore, rotation of CR canola cultivars that carry different sources of resistance (Hwang et al. 2014) or cultivation of non-host crops may eliminate or reduce the population of the resting spores in the soil (Ikegami 1975). In this study, we investigated the suitability of the non-host crop, hard red spring wheat (HRSW) in comparison with other commonly cultivated non-host crops (soybean, flax, and field pea) as the suppressant rotation crop of *P. brassicae* population in the soil for two consecutive years and showed that field pea or soybean planted after canola may have the best potential to suppress *P. brassicae* resting spores in the soil. We also showed that one year of non-host rotation in clubroot infested fields is not an ideal practice for clubroot management in North Dakota. This research aids to help canola growers find a suitable non-host crop to rotate with CR canola cultivars to delay or prevent genetic resistance breakdown in North Dakota.

In the analysis of two years non-host rotation, suppression of spores was mainly observed in canola-field pea with 9% decrease in spore concentrations and in canola-soybean with 32% increase in spore concentrations between the two sampling points (Figure 5.1). The decrease in canola-field pea indicate that the roots exudates from field pea did not favor the germination of *P. brassicae* resting spores in the soil. This is consistent with study done by Hwang et al. (2015), where, 2-years non-host crop rotation of field pea showed a significant reduced clubroot resting spore concentration and severity in the soil, as compared to a continuous cropping of either resistant or susceptible canola. Although a 32% increase was observed in the canola-soybean, however, this increase was shown to be insignificant between the spore concentrations of the two sampling points. This also indicated that soybean as a previous crop suppressed the rapid increase of spore concentrations. Yang et al. (2020) in a study done in China showed that soybean as the

previous crop had the potential to reduce incidence rate and disease index of clubroot of canola by 50% and 40% respectively.

In contrast, a significant increase in spore concentrations was observed in canola-canola and canola-wheat treatment by 63% and 68% respectively (Figure 5.1). The significant increase in canola-canola treatment served as a susceptible check and this showed that continuous planting of susceptible canola can rapidly increase the soil inoculum of *P. brassicae*. However, the significant increase in canola-wheat was quite interesting as wheat is a non-host crop of *P. brassicae*. Although, previous study by Ahmed et al. (2011) in Canada reported a slightly reduction of spore concentrations of *P. brassicae* following two cycles of wheat. However, in this study, wheat did not prove as a suitable non-host crop to decrease the resting spores. In the field, other factors asides the crop choice such as soil pH, temperature, moisture, and nutrient status may affect *P. brassicae* resting spore populations (Gossen et al. 2013, 2014) which could have led to the increase. On the other hand, it is possible that wheat produce root exudates that mimic those from the *Brassica* family, thereby, stimulating an increase in spore germination. Furthermore, *P. brassicae* have been shown to germinate and initiate root hair infection but are unable to complete this primary stage infection in non-host crops (Friberg et al. 2006; Liu et al. 2020).

One-way analysis of variance (ANOVA) performed on the soil pH in all treatments between the two sampling time points for two years individually showed that no significant difference was observed (Table 5.2). This indicates that the non-host and susceptible check rotation had no influence on the soil pH. Studies have shown that clubroot development and incidence is favored by acidic soils (Tewari et al. 2005), and a maximum clubroot disease infestation have been observed at pH 6.6 (Palm 1963). Although, the soil pH value in each year averaged between acidic pH of 6.0- 6.6 which is favorable for the germination of spores (Table 5.2). However, spore's

suppression observed in canola-field pea and canola-soybean treatments, or spore increase observed other treatments may have been attributed to an influence by the non-host crop rather than the soil pH.

Congruent with several studies that have shown that a longer rotation break from Brassica hosts is effective against clubroot severity due to the longevity of the resting spores of *P. brassicae* (Rempel et al. 2014; Peng et al. 2014; Yang et al. 2020), planting of susceptible canola after 1 year of non-host rotation in this study showed an increase in spore populations after 60 days of planting in all treatments (Table 5.3). Spore concentrations had a 380-600-fold increase across all treatments. However, this increase was insignificant in canola-fieldpea-canola, and canola-soybean-canola treatments further validating the potential of field pea and soybean to suppress *P. brassicae* in North Dakota clubroot infested fields. In addition, the lowest disease index (DI%) of 24.75% and 23.5% was seen in treatments of field pea and soybean as previous crops respectively, as compared to continuous canola cropping or other non-host crops.

In conclusion, this study aimed to determine the suitability of commonly grown non-host crops in North Dakota, including HRSW, soybean, flax and field pea to effectively reduce the spore populations of *P. brassicae* in the soil, influence the acidic soil pH and determine the required rotation length. The two-year non-host rotation after a susceptible canola plant, showed a higher potential of field pea and soybean to reduce *P. brassicae* inoculum in the soil than HRSW. However, they did not have the ability to influence the soil pH from acidic to alkalinity. Furthermore, for suppression of *P. brassicae* resting spores that could significantly affect clubroot severity, more than one-year continuous non-host crop rotation is required.

5.6. References

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