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Efficacy of Synthetic and Biopesticides on Bacteria Leaf Streak Management and Influence of Cultivar and Environment on Epiphytic Bacteria Diversity on Wheat Seeds

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EFFICACY OF SYNTHETIC AND BIOPESTICIDES ON BACTERIA LEAF STREAK

MANAGEMENT AND INFLUENCE OF CULTIVAR AND ENVIRONMENT ON

EPIPHYTIC BACTERIA DIVERSITY ON WHEAT SEEDS

BY

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This thesis is approved as a creditable and independent investigation by a candidate for

the Master of Science degree and is acceptable for meeting the thesis requirements for

this degree. Acceptance of this does not imply that the conclusions reached by the

candidate are necessarily the conclusions of the major department.

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ABBREVIATIONS

ACE: Abundance-based Coverage Estimator

BLS: Bacterial leaf streak of wheat

EDTA: Ethylenediaminetetraacetic acid

FAO: Food and Agriculture Organization

MBC: Minimum bactericidal concentration

MIC: Minimum inhibitory concentration

NERF: North East Research Farm

OTU: Operational taxonomic units

PCoA: Principal coordinate analysis

USDA: United States Department of Agriculture

Xtpvu: *Xanthomonas translucens pv.undulosa*

ZOI: Zone of Inhibition.

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ABSTRACT

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Wheat (*Triticum aestivum*) is the most important staple food for over two billion people,

or 36% of the world population. The United States ranks 4th in the world for wheat

production. However, wheat production is faced by both biological and non-biological

constraints. Among the biological constraints, diseases play a big role in limiting wheat

production with estimated yield losses ranging from 20% to 40%. Bacterial leaf streak

(BLS) of wheat caused by *Xanthomonas translucens pv undulosa* (Xtpvu) is one of the

major bacterial diseases affecting the wheat production in most wheat growing regions in

the USA. In recent years, it has become more prevalent in the upper mid-west due to the

warm and humid summer conditions that highly favor its development. In our study, we

looked at various synthetic products and bio-pesticides and tested their efficacy in

managing BLS in both greenhouse and in the field. We also determined how the

epiphytic bacteria diversity is influenced by environment and cultivar. Lastly, we

determined the effects of various spices and plant extracts on the growth of Xtpvu invitro.

In the greenhouse study, we applied synthetic and biopesticide products that included:

Champ 2, Regalia, Badge SC, Cuproxat® FL, Sonata ® ASO, Biochar and Headline on

wheat (Brick). Plants were later inoculated and rated for disease severity by measuring

the percentage of the leaf area affected with disease. Under field conditions, we conducted the experiments at the North Research Farm (NERF) and Volga Research Farm with the same products that we used in the greenhouse study with plant extracts, and other synthetic chemicals. The wheat variety Select was used in the field due to its high susceptibility to BLS. The experiment was laid in a completely randomized block design with four replicates. We randomly picked 10 plants per plot and determined the percentage of leaf area affected by the disease. There was no significant difference among treatments in comparison to the check for the greenhouse study. For the field study, there were significant differences among the synthetic and biopesticides with Sonata ASO and Cuproxat having relatively low disease severity with mean log transformed percentage severities of 1.27 and 1.25 respectively. There were no significant differences in effects among the plant extracts when compared to the check in the field study. Yield and test weight were not significantly different among treatments. The most abundant genera identified on seeds were: *Sphingomonas, Pseudomonas, Pedobcater, Neorrhizobium, Microbacterium, Methylobacterium, Massilia, Hymenobacter, Kinecoccus, Muciliginabacter, Curtobacterium,* and *Chrysobacterium. Sphingomonas* had a higher relative abundance compared to other genera in both locations while the genera that could harbor potential wheat pathogens were *Pseudomonas* and *Xanthomonas*. Among all the plant extracts and spices tested, only tannic acid had possible bactericidal effects. This study demonstrates that some ethyl acetate extracts of plant products have a significant antimicrobial effect on Xtpvu in vitro. Further research is needed to test plant extracts and spices in the field for potential

integration into bacterial leaf streak management in conjunction with the current bacterial

leaf streak management practices.

Chapter 1

Literature review

1.2 Wheat as a food crop

Wheat (*Triticum aestivum*) is the most important staple food for over two billion people, or 36% of the world population (Plains Grains Inc., 2012). Worldwide, wheat provides nearly 55% of the carbohydrates and 20% of the food calories consumed globally (Breiman and Graur, 1995). The United States ranks 4th in the world for wheat production (FAOSTAT, 2017). Half of the wheat produced in the U.S. is exported (Plains Grains Inc., 2012; USDA-ERS). In the Great Plains Region of the United States, wheat is the major food crop, with four classes; hard red spring, durum, hard white, and hard red winter wheat (2004; National Agricultural Statistics Service, 2005). These wheat classes are grown on one-third of arable land in the Great Plains and they account for more than 60% of the United States' wheat production (National Agricultural Statistics Service, 2005). Forty-eight percent of hard red spring wheat and 40% of hard red winter wheat are used for pastry (Lin and Vocke, 2004; National Agricultural Statistics Service, 2005).

Yield losses globally are estimated to be between 20% and 40% among field crops attributed to pathogens, animals and weeds (Teng and Krupa, 1980; Oerke et al. 1994; Oerke 2006).   Crop losses can be direct as well as indirect and they can be short-term or long-term (Zadoks, 1967). In the United States, over 100 diseases that stem from biological or non-biological causes are constraints to wheat production and some are localized to specific areas while others may have a nationwide occurrence (Bockus et al., 2010). Biological wheat production constraints include bacterial diseases. The common

bacterial diseases that affect wheat include bacterial leaf streak of wheat (BLS) and bacterial leaf blight. Of the two, BLS is the most common and most important. Bacterial diseases are challenging to manage in any crop since use of antibiotics and other synthetic pesticides is not encouraged due to the negative effects such as high and acute toxicity, long periods required for their degradation and their accumulation in the food chain (Rahman, 2014). Bacterial diseases caused by pathovars of *Xanthomonas* are a problem among various plants; thus, there is a need to manage them effectively since they lead to considerable yield losses (Aktar et al., 2009).

1.3   Bacterial disease development in plants

Once pathogenic bacteria encounter a suitable plant host, they first acclimatize to live externally without necessarily entering the host (Pfeilmeier et al., 2016). Favorable environmental conditions play an important role in the infection process coupled with intracellular and community-level signal transduction pathways. A combination of the three aforementioned factors triggers responses in the bacteria population and thus pathogenic bacteria pathogens move from the external surfaces into the interior of the plant through chemotaxis and motility (Pfeilmeier et al., 2016). These bacteria overcome many physical and chemical barriers before getting into the plant's apoplast. Once the bacteria are inside the plant, phytotoxins and proteins are released by a collection of secretion systems as shown in Fig 1.1 (Melotto and Kunkel, 2013; Phan Tran et al., 2011).
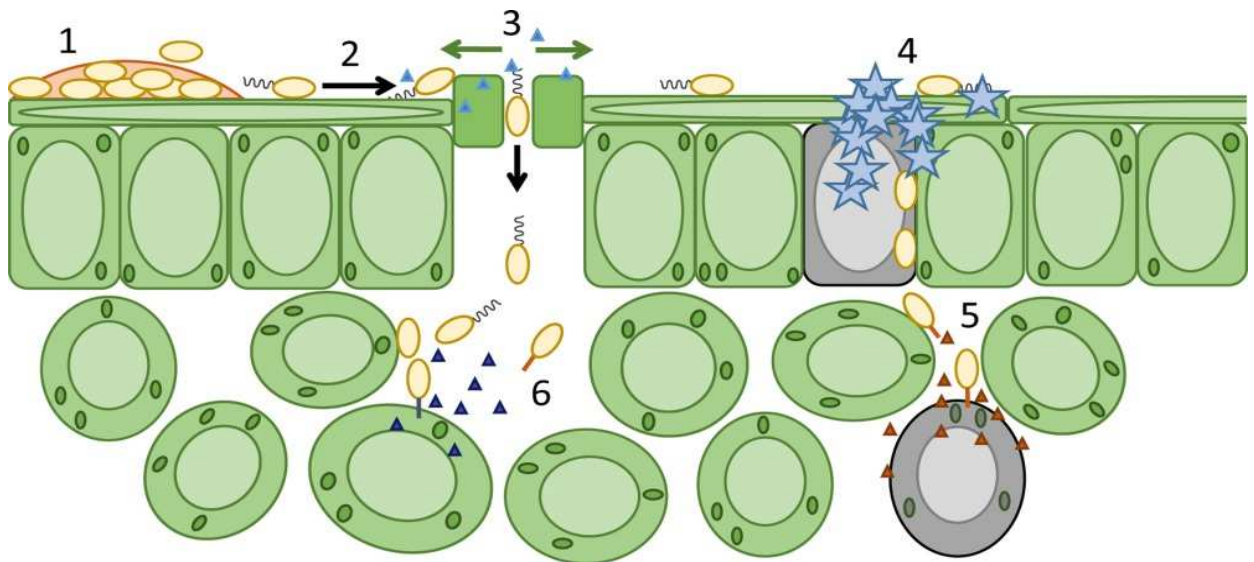
Figure 1.0.1: Plant bacterial infection process

1; Surface survival and biofilm formation. 2; Flagella/pili-driven migration across plant surfaces to apoplastic entry sites. 3; Release of phytotoxins to bypass stomatal closure. 4; Ice nucleation to damage plant surfaces. 5; Extracellular enzyme secretion to degrade cell walls and damage plant tissue. 6; Phytotoxin secretion to modify plant physiology, metabolism and immune responses (Melotto and Kunkel, 2013; Phan Tran et al., 2011).

1.4 Pathogenic bacteria epiphytic survival on plant leaves

Pathogenic bacteria are exposed to unfavorable conditions such as dehydration, adverse temperature changes, ultraviolet rays, and mechanical dislodging due to strong winds while on the plant leaf surfaces (Pfeilmeier et al., 2016). Even in the presence of these survival challenges, often epiphytic bacteria can reach densities of 10^6 to 10^7 cells/cm^2 of leaf surface (Andrews and Harris, 2000). Some of the unfavorable conditions such as cold, osmotic shock and dehydration trigger metabolic responses which eventually contribute to the survival of these epiphytic bacteria (Djonovic et al., 2013;

Freeman et al., 2013; Wu et al., 2012). For instance, survival and population maintenance of *Pseudomonas syringae* in the phyllosphere has been attributed to the osmoprotectant halose (Freeman et al., 2010). Additionally, *Xanthomonas* species can produce the exopolyscharride (EPS) that plays a key role in the epiphytic survival of the plant microbiome (Dunger et al., 2007 and Yu et al., 1999). Xanthan polymers (Dunger et al., 2007), such as alginate and levan (Freeman et al., 2013; Laue et al., 2006; Yu et al., 2013), facilitate plant surface colonization as well. Numerous plant interaction functions have been related to epiphytic survival which is highly associated with EPS molecules aiding the ability of these bacteria to resist desiccation, osmotic pressure and freeze-thaw (Wu et al., 2012, Chang et al., 2007; Freeman et al., 2013). EPS molecules also play a role in sustaining microbial populations (Dunger et al., 2007) and immune response evasion through promoting calcium signaling during the plant immune response (Aslam et al., 2008).

1.5   Bacteria migration across leaf surfaces and into the apoplast

 Upon achieving contact with leaf surfaces, plant pathogenic bacteria have traits that encourage survival until environmental conditions allow them to get into the apoplast (Pfeilmeier et al., 2016). When such conditions manifest, these bacteria use motility systems that enable them to move across the leaf surface to the stomata (Pfeilmeier et al., 2016). Chemotaxis is usually matched with flagella gene expression (Yu et al., 2013) and establishment of surface surfactant molecules that aid the bacteria in moving across the leaf surfaces (Burch et al., 2012). Pili are also necessary in bacterial biofilm formation,

adherence, motility and virulence in pathogens such as *Pseudomonas syringae* (Nguyen et al., 2012).

1.6 Bacterial leaf streak

Bacterial leaf streak (BLS) affects a number of crop species that belong to the Poaceae family such as barley, wheat, triticale and rye. Bacterial leaf streak of wheat is caused by *Xanthomonas translucens* pv.*undulosa (*Xtpvu) *(*Bragard et al*,* 1997, Vauterin et al., 1995). Several names have been given to this pathogen depending on the host it infects. Among the cereals that are closely related, pathogens that cause streak symptoms are grouped together under the group "translucens". A few years ago, there was an alteration of the pathogen's taxonomy based on its phytopathological relevance and clarifications that are more detailed have been made (Vauterin et al., 1995; Bragard et al., 1997). The name *Xanthomonas translucens* pv. *undulosa* is the accepted name for the pathogen that causes BLS.

Xtpvu is Gram negative, rod shaped and non-sporing. It is motile by a single polar flagellum. It grows as rods of 0.4 to 0.8μm x 1.0 to 2.5μm in singles and in pairs. In peptonized nutrient broth with 2% sodium chloride (NaCl), Xtpvu forms long non-motile chains (Jones et al., 1917; Dowson, 1939). There is no nitrate to nitrite reduction, and Kovacs' oxidase and arginine dihydrolase reactions are negative. According to Mohan and Metha (1985), hypersensitivity to tobacco is positive, there is no production of 2-ketogluconate and esculin hydrolysis is positive. The strains of *Xanthomonas translucens* do not hydrolyze starch and lactose (Schaad, 1987b).

Xtpvu is well-adapted to grow as an epiphyte on wheat (Azaad and Schaad, 1988). Thus, its existence as an epiphyte enables it to easily infect wheat and other related small grain crops (Leben, 1965). According to Duveiller (1994), some studies that were done in Mexico to monitor the Xtpvu populations in asymptomatic genotypes after a heavy downpour reported a reduction in the pathogen population, indicative of the pathogen's existence on wheat leaves before their permeation into the parenchyma.

BLS has two distinct symptoms; leaf lesions and black chaff. The disease first manifests as water-soaked lesions on the extremities or midvein of leaves. Under humid conditions, the streaks elongate rapidly following the parallel venation of the leaf, later resulting in necrotic and chlorotic leaves (Smith, 1919). Production of yellow-orange bacterial exudates (ooze) under humid conditions is observed, while in dry conditions the exudates solidify into easily detachable yellowish granules on the leaf surfaces (Smith, 1919). Black chaff refers to the incomplete or complete darkening of the glumes and peduncles in wheat plants that are infected with bacterial leaf streak pathogen (Smith, 1919). The severity of the symptoms in leaves and black chaff are independent, and cultivars that may be resistant to leaf symptoms may show darkening of the peduncle in infected plants (Tillman et al., 1996a).

BLS reduces the photosynthetic area and causes premature leaf death, resulting in limited grain fill, lighter test weights, and lower yields (Duveiller and Maraite, 1993). Bacteria leaf streak affects all classes of bread wheat. It is generally more damaging to spring wheat under humid conditions (Kandel et al., 2012). In some states like Idaho,

yield losses of about 40% were caused by Xtpvu (Duveiller and Maraite, 1993). Therefore, BLS is an economically important disease.

1.7   Life cycle of *Xanthomonas translucens pv. undulosa*

Xtpvu is a seed-borne pathogen (Wiese, 1987). There are differences in severity and incidences among different seed lots of the same cultivar planted under the same conditions (Milus E.A, unpublished data). Thus, it is suggested that the seed-borne inoculum could be the most important source of initial inoculum. Symptoms caused by Xtpvu are rarely seen before the heading stage, therefore an epiphytic phase early in the season is an important source of inoculum for infecting plants later in the season (Milus E.A, unpublished data). Xtpvu can also survive on volunteer cereals, wild hosts and on plant debris (Fig. 2). Previous studies indicate that Xtpvu survives on the alternative hosts and plant debris, but they are an insignificant source of inocula (Mehta et al., 1992). The survival of the bacteria in soil is poor, thus presence of stubble provides a suitable habitat for the survival of the pathogen (Boosalis, 1952). While Xtpvu may be on wild hosts, symptoms may not be visible. It is also suggested that the pathogen cannot live in the atmosphere for more than 14 days (Cunfer, 1988). The pathogen enters through the stomata and then later multiplies in the parenchyma cells, thus causing the elongated streaks that are observed (Cunfer, 1988).
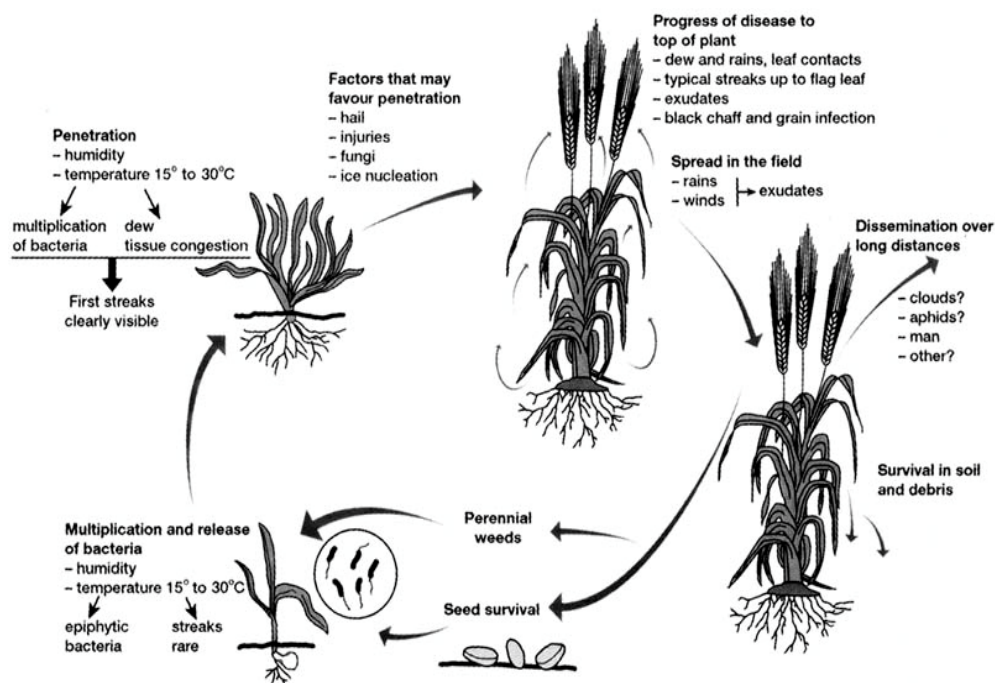
Figure 1.0.2: Schematic representation of the disease cycle of *Xanthomonas translucens pv. undulosa* and the mode of its spread (Duveiller and Maraite,1993).

1.8   Pathogen spread in the field and factors that promote

infection and disease development

Xtpvu spread amongst plants is via plant-to-plant contact, dew, insects that visit

the spikes such as aphids and splashing from rain or irrigation. Presence of moisture is a

key factor in BLS development (Kurt, 2011; Boosalis, 1952). Wind also spreads the

pathogen by circulating the bacterial exudates. Additionally, injuries by hail, wounds by

feeding insects and sand blasting also favor the penetration of the pathogen (Kurt, 2011;

Boosalis, 1952). Temperature is another condition that is key in BLS development, where

Xtpvu colonization is favored by temperatures between 15° C and 30° C (Duveiller et al.,

1991; Duveiller and Maraite, 1995) and its proliferation in the leaf tissue is largely

attributed to temperature. Symptoms due to pathogen infection manifest when the

temperatures permit the bacterial load to reach an estimated threshold of 10^8 colony forming units/leaf (Duveiller and Maraite, 1995), and its multiplication and disease progress is slowed down by low temperatures ($< 15^oC$) (Duveiller and Maraite, 1995).

1.9   Bacterial leaf streak management

Bacterial leaf streak management is through use of standard cultural control methods that include; crop rotation, tillage and weed control. Chemical management of the disease using synthetic bactericidal products is not recommended because of the persistent nature of the pathogen and the prolonged window of host susceptibility (Duveiller and Maraite, 1993). Seed treatments cannot be used to control Xtpvu due to the extreme harm to the seed (Kurt, 2011). Some treatments such as dry heat (Fourest et al., 1990), hot acidified cupric acetate (Forster & Schaad, 1988; Duveiller, 1989) and Guzatine plus (Metha and Bassoi, 1993) have demonstrated a considerable reduction of Xtpvu population in the field. Nevertheless, these treatments are limited to small seed lots and their efficacy is not 100% (Duveiller, 1994). Another management strategy of BLS is through use of moderately susceptible genotypes since there is no complete resistance to the pathogen. There are only few published studies on sources of resistance to the disease. Studies done in Mexico identified few BLS resistance genes and cultivars ('Pavon 76' and 'Mochis') (Duveiller et al., 1993). However cultivar 'Pavon 76' that showed resistance in Mexico was found to be very susceptible in Louisiana (Tillman et al., 1996b).Thus environment-specific studies may not be enough in attempting to find resistance genes (Kandel, 2012).

1.10   Mechanism of plant acquired immunity due to antimicrobial

compounds and pathogen presence.

Plant diseases are as a result of biotic factors such as microbial pathogens and
insect herbivores, or abiotic factors such as environmental influences which include too
low or high temperature, lack of or excess soil moisture, lack of oxygen, and nutrient
deficiencies to mention but a few (Agrios, 2005). Thus, plants have developed strategies
to overcome the environmental resistances they encounter during their growth to enable
their survival and propagation in diverse ecological landscapes (Rasmann *et al.,* 2012).
Even though plants have defensive strategies that are present all the time (constitutive),
most of them are because of exposure to stimulus such as pathogen infection (Frost *et al*.,
2008). Plant pathogens produce elicitors that are recognized by plants and thus stimulate
plants to elevate their defenses, such as production of active oxygen species (AOS),
phytoalexin biosynthesis, reinforcement of plant cell wall associated with phenyl
propanoid compounds, synthesis of defense enzymes and callose deposition (Hammond-
Kosack et al., 1996; Yang et al., 1997). Additionally, there could be accumulation of
pathogenesis-related proteins (PR) that have antimicrobial properties (Van loon and Van
strien, 1999). In a similar manner, there are also natural and synthetic elicitors similar to
those of the plant pathogens that induce plant defenses (Gómez-Vásquez et al., 2004).
Thus, for this study we tested both natural and synthetic compounds to determine their
effect on BLS with plant immunity induction in mind. The use of natural antimicrobial
compounds is further supported by the current global campaign to incorporate Integrated
Pest Management in agricultural production, and the ever-changing policies concerning
pesticide usage that seek to reduce the harm these chemicals may cause to the

environment and human health (Tiilikkala et al., 2010). According to the United States Environmental Protection Agency (2008), use of low risk, biological pesticides is an alternative to conventional chemical pesticides. These biological pesticides promote sustainable agriculture through agro-ecosystem diversification and soil carbon sequestration (Food and Agriculture Organization, 2009).

1.11   Biopesticides

Biopesticides are made from naturally occurring living things such as plants, animals and microorganisms like bacteria, fungi and viruses (Sengottayan, 2015). For this thesis we define a biopesticide as either a chemical natural product, or a living microorganism that can help control plant disease. Some of the biopesticides include wood vinegar, chitin, fulvic acid and plant extracts derived from moringa, soursop, tannic acid, wood vinegar, and spirulina. Spices such as onions, garlic, red pepper, black pepper, poppy seed and cinnamon are also plant extracts containing potential antibacterial compounds (Nabavi et al., 2015). Wood vinegar is a brown, flavorful liquid that is a result of pyrolysis. Pyrolysis is the process by which wood is heated in a closed vessel or airtight container leading to carbonization. During this process smoke is given off which is cooled, and a liquid is collected (Yang et al., 2016). When this liquid settles, three distinct layers form: an oily liquid occupies the top layer, the middle layer consists of a transparent, yellowish–brown liquid called raw wood vinegar; and the thick wood tar settles at the bottom. Burning different kinds of wood, for example eucalyptus (Amen-Chen et al., 1977; Pimenta et al., 2000), oak (Guillen et al., 2002), bamboo (Mu et al., 2004), mangrove (Loo et al., 2008; Loo *et al.,* 2007), coconut shell (Wititsiri et al., 2011) and apple trees in an airtight vessel can produce various kinds of wood vinegar. Many

wood-vinegar sources are identified as safe, natural inhibitors with numerous bioactive compounds that make them suitable for use in antifungal, termiticidal and repellent applications (Mu et al., 2004). Wood vinegar has antibacterial activities as well (Mu et al., 2004). Recently, advancement in the use of wood vinegar has earned much attention, especially in the agriculture industry (Tiilikkala et al., 2010). There are minimal negative effects associated with its usage to the environment as compared to the synthetic pesticides which can run off or leach to the ground water (Tiilikkala et al., 2010). On plants, wood vinegar has been used as a foliar spray and as a replacement for synthetic fertilizers and pesticides (Yatagai, 2000). However, there is no documented research done on the effects of wood vinegar on bacterial leaf streak development in wheat.

Biochar is another plant-based product produced from the process of pyrolysis that has anti-microbial properties. Biochar has been used as a soil amendment and there is evidence of improved crop response (Chan et al., 2007; Glaser et al., 2002; Lehmann et al., 2003; Steiner et al., 2007). According to Elad et al., (2010), biochar was able to suppress the growth of pepper powdery mildew; however, it was applied as soil amendment. It also suppressed symptoms caused by the broad mite on the pepper canopy. Biochar is comprised of residual tars, which house a multitude of individual organic compounds (Schnitzer et al., 2008; Schnitzer et al., 2007). Some compounds contained in biochar have been found to have antimicrobial properties (Amonette and Joseph, 2009).

Chitin has shown antibacterial, antiviral and antifungal properties, and it is used in many agricultural applications (El Hadrami et al., 2010). It is for example extracted from crab shells using 12 M hydrochloric acid. In nature, it is found in many internal structures of invertebrates and exoskeletons of arthropods and in the cell walls of fungi and yeast. It

is a hard, white, inelastic polysaccharide (Younes, and Rinaudo, 2015). It has been used to control diseases or minimize their spread: to chelate nutrients and minerals, thus hindering pathogens from gaining access to them; or to boost plant natural defenses. Chitin stimulates host defense responses in dicotyledons and monocotyledons, thus playing a key role in strengthening plant defenses (El Hadrami et al., 2010). Some of the plant defense responses are lignification in wounded (Pearce and Ride, 1982; Barber *et al.,* 1989), and intact wheat leaves (Moerschbacher et al., 1986) and in suspension-cultured wheat cells (Gotthardt and Grambow, 1992), ion flux variation, cytoplasmic acidification, membrane depolarization and protein phosphorylation (Felix *et al.,*1993,1998; Kikuyama et al.,1997; Kuchitsu et al.,1977). Chitin can cause chitinase and glucanase activation (Robby et al., 1986; Kaku et al., 1997), phytoalexin biosynthesis (Ren et al., 1992; Yamada et al., 1993), generation of reactive oxygen species (Kuchitsu et al., 1995) and biosynthesis of jasmonic acid (Nojiri *et al.,* 1996). It also promotes expression of unique early responsive and defense-related plant genes (Minami *et al.,* 1996; Nishizawa et al., 1999; Takai et al., 2001).

Fulvic acid is comprised of carbon, hydrogen, oxygen, nitrogen and sulphur. The size of fulvic acid is 800A and <3,500Da (Beckett et al., 1987 and Muscolo et al., 2007). Fulvic acid is one of the humic substances (HS), comprising 50 to 90% of natural organic compounds from lignites, sapropels, and peat coupled with non-living organic matter of the soil and water ecosystems (Thurman, 1985, Orlov, 1990; Swift, 1993). Humic substances are "a general category of naturally occurring heterogeneous organic substances that can generally be characterized as being yellow to black in color, of high molecular weight and refractory" (Thurman, 1985, Orlov, 1990; Swift, 1993).   Fulvic

acid is a novel antimicrobial molecule that has sometimes been shown to have antibacterial and antifungal properties (van Rensburg et al*.,* 2000).

Plants are also a source of natural compounds that may have antibacterial, antifungal and antiviral properties. These bioactive compounds may include peptides, glycosides, alkaloids, saponins, terpenoids and flavonoids (Janathan *et* al., 2003; Khan et al., 2001; Perez et al., 2003). Some of the plant extracts found to have antibacterial properties include those from *Spirulina platensis* (Spiriluna), *Moringa oleifera* and *Annona muricata* (Soursop). *Spirulina platensis* is a blue-green alga, also called a cyanobacterium or microalgae. It is multicellular, and its habitat is either seawater or fresh water. It contains chlorophyll, carotenoids, phenolics and flavonoids (Chu et al., 2010; Madhyastha et al., 2006; Sari, 2011). Spirulina produces a varied range of bioactive molecules (Borowitzka, 1995). Extracts from spirulina can potentially control plant-pathogenic bacteria, due their antimicrobial property that also exists in many other cyanobacteria (Usharani et al., 2015).

Moringa oleifera is among the best known and widely distributed and domesticated species of the Moringaceae family (Alemayehu and Serawit, 2014). The tree ranges in height from 5 to 10 m. It is grown throughout the plains, especially in hedges and house yards. Its bark is whitish, thick and corky; the crown is fragile and drooping and the leaves are tripinnate (Roloff *et al.,* 2009). *Moringa oleifera* has antibacterial compounds including; 4-(4'-O-acetyl-α- L-rhamnopyranosyloxy) benzyl isothiocy-anate, 4-(α-L-rhamnopyranosyloxy) benzyl isothiocy-anate,niazimicin, pterygospermin, benzyl isothiocyanate and 4-(α-L-rhamnopyranosyloxy) benzyl

glucosinolate (Jed and Fahey, 2005). In recent studies, leaves, roots, bark and seeds of

Moringa oleifera have shown in vitro antimicrobial activity against both Gram-negative

and Gram-positive bacteria using the disc diffusion assay technique (Nadkami et al.,

1985). Chemical constituents that are responsible for the anti-bacterial properties are;4-

(α-L-rhamnopyranosyloxy) benzyl isothiocy-anate, 4-(α-L-rhamnopyranosyloxy) benzyl

glucosinolate and pterygospermin (Nadkami et al., 1985). Soursop (*Annona muricata)*

originates from Mexico, Cuba, Central America and some parts of India. It is a flowering

evergreen tree. It belongs to the family Annonaceae. The tree is typical of the tropics and

bears heart-shaped edible fruits (de Feo, 1992). According to different authors, its leaf

extracts have been used at varying concentrations as an antibacterial agent against

pathogens. (Misas et al., 1979; Sundarrao et al*., 1993).

Apart from the plant extracts, there also other synthetic compounds that are used

in the control of plant-pathogenic bacteria. Some of these compounds are

ethylenediaminetetraacetic acid (EDTA), Champ 2 flowable, Badge and Cuproxat.

EDTA is a well-known metal chelating agent that has been used in many applications, for

example in the identification of extracellular DNA in the biofilms of *Xanthomonas citri*

subsp. citri (Sena-Vélez et al., 2016; and Percival, 2014). EDTA can chelate and

potentiate the cell walls of bacteria and destabilize biofilms by sequestering calcium,

magnesium, zinc, and iron, which makes it a suitable agent for use in the management of

biofilms. *Xanthomonas* species are among the Gram-negative bacteria that form biofilms

on plants. Biofilm formation facilitates the host infection process (Sena-Vélez et al.,

2016). Due to EDTA's excellent antimicrobial and anti-biofilm activity and considerable

synergistic and permeating ability, it could potentially control bacterial leaf streak of wheat.

Champ 2 flowable contains copper hydroxide as the active ingredient. It is used in vegetable crops and field crops to control both bacterial and fungal pathogens such as bacterial spot, and *Pseudomonas* blight. Its mode of action is unknown, though it is suggested that the highly charged copper ions get into the bacterial cell wall or fungal spores and causes damage, thus halting cell division (http://nufarmpoint.com). Cuproxat has copper sulfate as the active ingredient containing copper particles of less than 1 micron. It is used to control both bacterial and fungal diseases in field crops, ornamental crops and vegetable crops (http://nufarminsider.com/products/cuproxat/). Badge is a liquid formulation that contains copper hydroxide and copper oxychloride. Copper ions are released by copper hydroxide for immediate plant protection, while copper oxychloride slowly releases the copper ions for extended plant protection. It is used to control a broad range of both bacterial and fungal pathogens in field, ornamental and vegetable crops. (https://www.gowanco.com/products/badge-sc).

1.12 Biofungicides

Biofungicides are fungicides that contain a microorganism (usually a bacterium or fungus) as the active ingredient (Swain, 2014). Biofungicides can control an array of plant pathogens such as bacteria, fungi and water molds; however, each separate active ingredient may control a particular group of pathogens (Swain, 2014).The microorganisms incorporated in these biofungicides occur naturally in the soil or on plant surfaces. They have a compounded mode of action; for example, they can control pathogens through competition for critical nutrients, and/or physical space; and are

applied prior to disease onset (Swain, 2014). Additionally their mode of action could be through antibiosis; where the microorganism produces an antibiotic or a toxin that interferes with the pathogen. Induction of plant host resistance could also be another way these microorganism-based fungicides work; that is to say, they trigger a defense response in the plant, which makes the plant more resistant to pathogen attack (Swain, 2014). Sonata is a broad-spectrum, preventative product for the control or suppression of many important plant diseases. It contains *Bacillus pumilus* (QST 2808) and it is used to control bacterial spot, downy mildew and powdery mildew, white mold, fire blight, scab, early and late blight (www. ag.wilburellis.com). Regalia contains extract of knotweed (*Reynoutria sachalinensis*) as the active ingredient. It stimulates the plant's innate ability to fight against diseases. Its mode of action is through induced systemic resistance (ISR). ISR induces the plants to produce specialized proteins and other compounds such as phytoalexins, cell strengtheners, antioxidants, phenolics, and pathogenesis related (PR) proteins, which are known to inhibit fungal and bacterial diseases and improve plant health and vigor. ISR also builds defense in the treated plants and induces other biochemical pathways that reinforce the plant structure, which deters plant pathogens (https://www.evergreengrowers.com/regalia-biofungicide). Thus, it is a preventative biofungicide and can control a variety of bacterial and fungal diseases before they develop. It is used to protect various crops such as cereal grains, cotton, forage, peanuts and sorghum (https://marronebioinnovations.com/cg-products).

1.13 Justification of the study:

Wheat is an economically important food crop (FAO, 2017) in the world. Its nutritive value provides dietary fiber, vitamins, other minerals and carbohydrates.

However, there are constraints such as diseases and abiotic stresses that limit wheat yield

(USDA-National Nutrient Database for Standard Reference, Release 19, 2006;

FAOSTAT, 2017). Diseases are the major constraint to wheat production due to the

severe yield losses and reduction in quality (Goyal, 2010). Some of the pathogens that

affect wheat are biotrophic fungi causing powdery mildew and rust; nectrotrophic fungi

causing diseases like tan spot, and bacterial pathogens such as *Xanthomonas translucens*

*pv. undulosa (*Xtpvu) and *Pseudomonas syringae* that also affect wheat productivity

(Goyal, 2010). Xtpvu is the causal agent of bacterial leaf streak of wheat (BLS). It has

become more prevalent in the upper Midwest and occurs in states like North Dakota,

South Dakota, and Minnesota. Its occurrence is attributed to primary inoculum build up,

favorable environmental conditions and a high susceptibility of the current major

cultivars that are planted in these regions (Adhikari et al. 2012). To ensure high yield and

good quality, several disease management practices that can reduce the impact of BLS on

yield have to be adopted including tillage, use of resistant cultivars, crop rotation, and

chemical control. One of the essential tenets of an effective disease management program

is understanding source of inoculum. The role of environment and cultivar on Xtpvu

inoculum presence on seed in wheat is not well studied. Although there are a number of

products on the market that can provide control of bacterial diseases on other crops,

scanty information exists on whether such products may have an effect on Xtpvu.

Moreover, several biopesticides have not yet been evaluated for their efficacy against

BLS both in vitro and in vivo.

1.14 Study Objectives.

The objectives of the study were to;

i) Evaluate the efficacy of both synthetic and biopesticides in vivo on the management of *Xanthomonas translucens pv undulosa*.

ii) Determining the influence of environment and cultivar on epiphytic bacterial diversity on wheat seeds.

iii) Determining the antimicrobial activity of selected bio-pesticides on Xtpvu in vitro.

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Chapter 2

2.1 Efficacy of synthetic products and Biopesticides in the management of bacteria leaf streak of wheat

Abstract

Various fungal, viral and bacterial diseases affect wheat. These diseases affect yield, thus low profits are often realized. Bacterial diseases are among the plant diseases that have proven hard to control. Using antibiotics to control bacterial diseases is not recommended due to the risk of fostering antibiotic resistance and economic feasibility. Once a plant is infected with this pathogen, the leaves become necrotic resulting in a small photosynthetic area negatively impacting yield in turn. In the Midwest, BLS has become more prevalent due to the warm and humid conditions that often exist in this region.

In this study, we looked at the efficacy of an array of compounds including synthetic and biopesticides under the greenhouse and under field conditions. In the greenhouse study, we applied Champ® formula 2 flowable, Regalia, Badge® SC, Cuproxat® FL, Sonata ® ASO, Headline® and Bio-char on spring wheat cultivar (Brick).Wheat plants were later inoculated and rated for disease severity by measuring the percentage of the leaf area affected with disease. We analyzed the data using SAS software (Version 9.4) and there were no significant differences among treatments in comparison to the check. For the field study, the experiments were conducted at the North East Research Farm (NERF) and Volga Research Farm with the same products as in the greenhouse study in addition to plant extracts and other synthetic products. Hard red spring cultivar Select was used in the field. The experiment was conducted in a completely randomized block design with

four replicates. Ten plants were randomly selected per plot and rated for BLS severity as before. Only Sonata ® Aso and Cuproxat ® had relatively low disease. These results suggest that Sonata and Cuproxat could be used in the management of BLS in conjunction with other available disease-management practices.

2.2   Introduction

Wheat is a highly valuable crop worldwide providing food for the majority of the human race (Tiwari and Shoran, 2011). It is widely grown in various agro-ecological zones throughout the world. Its extensive consumption by humans stems from the range of food products that are derived from it (Tiwari and Shoran, 2011). There are various categories of wheat grown in the United States including winter wheat, spring wheat, and durum. These wheat categories are subdivided into five major classes; hard red winter, hard red spring, soft red winter, white and durum. In the Northern Great Plains of which South Dakota is part, 20% of total hard red spring wheat in the USA is grown (USDA, 2019). Like any other crop, wheat production in this region is constrained by a number of factors, among which plant diseases are the most limiting. Bacterial leaf streak in particular has been on the rise in recent years, and its occurrence is attributed to the frequent humid and warm conditions in this region (Adhikari et al. 2012). Current management practices include use of resistant varieties, which while effective and sustainable is a prolonged process, and currently very few commercial resistant varieties are available.

The high prevalence of bacterial leaf streak of wheat in the upper Midwest calls for preventative measures; these may range from planting pathogen-free seed, use of seed

treatments and application of foliar pesticides. Various foliar pesticides and antibiotic compounds have been tested in the field; however, sufficient efficacy data are lacking for recommendations to be made (McMullen and Adhikari, 2011). Some of the products that have been tested against bacterial diseases have not been evaluated for BLS management in wheat. In this study, we evaluated an array of foliar pesticides, a mixture of synthetically derived compounds and compounds from plant origin for their efficacy in controlling BLS.

2.3   Materials and Methods

2.3.1   Preparation of chitin

We prepared colloidal chitin using a modified protocol of Hsu and Lockwood (1975) (Murthy and Bleakley, 2012).   According to this method: "Crab shell flakes (Neptune's Harvest, MA, USA) were manually ground in a mortar and pestle for 5 minutes, then sieved through the top piece of 130 mm two piece polypropylene Buchner filter. Twenty grams of the sieved crab shell flakes were then treated with 150 ml of ~12M concentrated HCl (BDH Aristar) in a 1000 ml beaker. The HCl was added slowly with continuous stirring with the use of a glass pipette for 5 minutes, followed by stirring for 1 minute at an interval of every 5 minutes for 60 minutes in a chemical fume hood at room temperature (25 ° C). The chitin- HCl mixture was then passed through 8 layers of cheesecloth to remove large chitin chunks. The clear filtrate obtained (100 ml) was then treated with 2 liters of ice cold distilled water to allow precipitation of colloidal chitin."

2.3.2 Greenhouse study

Hard red spring wheat cultivar "Brick" was planted in the Plant Science greenhouse at South Dakota State University. The plot size was one row of 5 m in length. Products labelled to control bacterial diseases in vegetables such as Champ ® 2 flowable, Regalia, Badge ® SC, Cuproxat® FL, Sonata ® ASO, and Bio-char were applied at jointing growth stage using the recommended manufacturer's application rates. Headline SC fungicide, distilled water and non-inoculated and non-treated plants were included as checks. Treatments were allocated to plots in a completely randomized design with four replicates. Inoculum was prepared by streaking bacteria on modified Kings B agar media from cryovial stocks that were initially stored at -80 ^{0}C.

After incubating at 30^{0}C for 24 hours, four bacterial colonies were inoculated in fresh Kings B broth media, and incubated at 30^{0}C for a period of 24 hours with constant shaking at 150 rpm. Four days after product application, all plots except the non-treated non-inoculated check were inoculated with *Xanthomonas translucens pv. undulosa* using a carbon dioxide sprayer with 3×10^8 bacterial cells / ml.

2.3.3 Field study

Hard red spring wheat cultivar "Select" was planted at the Volga Research Farm and North East Research Farm (NERF) in the spring of 2018. Select is known for its high susceptibility to BLS in the field, and for this reason it was chosen to be used in the field study. Planting of seeds was done using a 7-row tractor-mounted small grain planter fitted with cone units at a seeding rate of 323 / m^2. The plot size measured 1.5m wide and

4.6m long. The following products were applied at the tillering growth stage: Cuproxat®, Regalia, Sonata ® Aso, Headline®, Champ ® formula 2 flowable, Badge ®, Biochar, Moringa (MAJU super foods, Amazon), Ginseng (Wisconsin Grown Ginseng), Spiriluna (MAJU super foods, Amazon) Wood vinegar (8030-97-5 Sigma Aldrich), Fulvic acid 95% (Supernal Sublime, via Amazon), Chitin (Neptune's Harvest, MA, USA), Soursop (ZOKIVA Nutritionals, Amazon), and EDTA (Sigma Aldrich). All plots except the untreated checks were inoculated with Xtpvu at $3x10^8$ CFU/ml using a back pack sprayer at 7 days after the products were applied. The non-inoculated non-treated plots were sprayed with water. This study was laid out in a completely randomized block design with four replications.

2.4   Data collection and analysis.

All plants were assessed for BLS severity by estimating the area of the flag leaf affected with disease 14 days post-inoculation in the greenhouse study and at 7 and 14 days for the field study. All plants were assessed in the greenhouse while 10 plants per plot were randomly selected and rated for the field experiments. In order to determine the effect of BLS severity on yield, the number of spikes per plant were counted for the different treatments at harvest for the greenhouse study. The number of seeds per spike were counted for all the plants that were rated. These plants were tagged with colors corresponding to different severity percentages. Field plots were harvested and yield determined for each plot. Severity data were log transformations to satisfy the normality assumption and to reduce variance. Data were analyzed using R software Version 3.5.1 to get the mean separations using Bonferroni least square

means using the model and to determine the correlation between BLS severity and yield.

2.5   Results

Disease severity assessments that were done in the greenhouse after application of different biopesticides revealed no significant differences (Table 0.1) compared to the check (BLS + water). However, there was a significant difference in severity between all the treatments and the non-treated control (non-inoculated + water).Yield was determined by counting the number of seeds per spike and the number of spikes produced per plant. There were no significant differences in the mean number of spikes among the different treatments as compared to the check (Table 2.1). The number of seeds per spike was negatively correlated (-0.14) to the BLS percentage severities.

In the field study, disease severity percentage assessments for the biopesticides that were done in Volga and NERF revealed no significant differences in the first ratings (Table 2.2) while there were significant differences in the second rating (Table 2.2). Sonata ASO and Cuproxat were significantly different from the check, with mean log transformed percentage severities of 1.27 and 1.25 respectively. In the organic trial at NERF and Volga, in the first rating, there were significant differences between the check with wood vinegar having log transformed mean percentage severity of 0.19, while the rest of the treatments did not have any significant differences when compared to the check (Table 2.3). In the second rating, there were no statistically significant differences among all the treatments (Table 2.3).

There were no significant differences in yield when different biopesticides were applied at both NERF and Volga (Table 2.4). In the same manner, there were no significant differences in yield when the different plant extracts were applied at both NERF and Volga (Table 2.4). The test weight for the different treatments did not significantly differ in comparison to the check (2.5).

2.6   Discussion

In this study, we evaluated the efficacy of potential synthetic and biopesticides under greenhouse and field conditions. In the greenhouse study there was no significant difference in disease severity when compared to the check. Possibly, because plant infection depended solely on the artificial inoculum, and there is a possibility that the bacterial inoculum did not penetrate the inner leaf tissues to cause significant infection to enable comparison among the different treatments used. According to Weindling (1947), the method of inoculation affects the bacteria infection process. Probably the inoculation method that would forcefully drive the bacteria into the wheat leaves to cause significant symptoms would have been more suitable for the greenhouse study. For some copper-based products that we used, there is also a possibility that the Xtpvu strain used might have had copper-resistance genes. Copper-resistance genes are acquired through horizontal gene transfer from other bacteria living in the phyllosphere (Sundin et al., 2016). Horizontal gene transfer is highly possible since it can occur even among bacteria that are phylogenetically dissimilar (Perry and Wright, 2013; Smilie et al., 2012).

Under field conditions, there were significant differences among the results for some of the synthetic pesticides, with Sonata Aso and Cuproxat having relatively low disease

severity with mean log transformed percentage severities of 1.27 and 1.25 respectively. *Bacillus pumilus* strain QST 2808 is the active ingredient of Sonata ASO ® and it may have produced bioactive molecules such as ribosomally synthesized peptides, lipopeptides, bacteriocins and siderophores which had an effect on Xtpvu.These bioactive molecules are characteristically produced by the genus *Bacillus* (Fira et al., 2018). Cuproxat to a certain extent controlled the severity of BLS severity compared to the rest of the treatments. This could be because disease under field conditions is mainly dependent on the natural inoculum and there is a possibility that multiple strains of Xtpvu might have caused BLS in conjunction with the artificially inoculated strain. Since various strains have variability in virulence, it is possible that Cuproxat controlled some strains of Xtpvu compared to other treatments, whereas other strains still survived to cause significant BLS symptoms since there was not complete control of the disease. The major active ingredient in Cuproxat is copper sulfate. Copper is known to be a vital micronutrient for normal bacterial growth, since it is a vital cofactor for many enzymes in respiration such as oxygenases and electron transport chains (Garcia-Horsman et al., 1994). However, beyond a certain concentration it becomes toxic, generating free radicals and damaging DNA and lipid membranes (Hoshino et al., 1999; Muller et al., 2000). This is one possible mode of action by which copper might have acted on Xtpvu.

From field experiments carried out at both Volga and NERF, some biopesticides and synthetic products had no significant control on BLS when compared to the non-treated plots and the check. The possible reasons for their lack of efficacy may be attributed to the likely quick degradation of plant-based extracts (no residual effect), thus little protection of leaves would be achieved. Therefore, the bacteria multiplied and caused

significant symptoms on the wheat plants. Additionally since Xtpvu cells are Gram-negative, their walls are multilayered which presents a barrier for many antibacterial compounds to penetrate to their inner layers, making the compounds less effective in controlling the bacteria. Additionally, the long window of infection exhibited by Xtpvu (Duveiller and Maraite, 1993) could have been another reason why all the plant extracts had no significant control on BLS. Additionally, the rapid cell division exhibited by bacteria in general allows their population densities to reach high numbers in very short periods under favorable environmental conditions, causing increased levels of infection and the potential to spread to new infection sites (Sundin et al., 2016). If this occurred, it might have contributed to less plant extract efficacy. Xtpvu is an epiphyte (Duveiller and Maraite,1995), and epiphytes normally do not show symptoms until a later time, which gives them the ability to attain sufficient population size that leads to disease which makes the products applied ineffective (Sundin et al., 2016). Similarly, when bacteria access the internal parts of the plant through the stomata or wounds, they become inaccessible to most spray chemicals and other naturally derived antibacterial compounds (Sundin et al., 2016) however, there is a possibility that bacterial growth on the leaf surfaces is inhibited. This could be another reason why some of the foliar spray products turned out to be ineffective in the field trials. Xtpvu's ability to survive on crop debris and alternative hosts might have resulted in high inoculum build up that later spread to the wheat plants, thus outweighing the products' efficacy.

Products such as biochar, moringa, ginseng, spirulina, wood vinegar, fulvic acid, chitin and soursop are not of standardized source composition or formulation. For example, the fulvic acid used in this study was obtained by hot water extraction of peat. Fulvic acid

obtained from a mineral soil by standard extraction methods (Lamar et al., 2014) could possibly yield different results. There is a chance that one or more of these products from other commercial sources could have more efficacy in disease control than the ones used in this study.

2.7 Conclusion

Cuproxat® and Sonata® demonstrated a significant control of BLS severity under field conditions, and they show potential to be integrated with other BLS management strategies that are currently available. Since the plant products used were from different commercial brands and are prepared differently, there is a possibility that some related products from other commercial sources may demonstrate efficacy in disease control in future studies.

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Table 0.1 Effect of different biopesticides and synthetic pesticides on BLS by assessing the disease severity on the flag leaf and determining the total number of spikes.

Treatment	Severity%	Treatments	Mean number of spikes
Champ ®2 Flowable	1.41 a	Regalia®	15.2 a
Regalia® Rx	1.29 a	Champ® 2 Flowable	15.0 a
Badge® SC	1.29 a	Biochar	14.6 a
Cuproxat®	1.18 a	BLS+water	14.6 a
Sonata ASO	1.09 a	Badge®	14.0 a
BioChar	1.05 a	Cuproxat®	14.0 a
BLS+Water	1.02 a	Sonata	13.2 a
Headline® SC	0.94 a	Headline®	13.0 a
Non-inoculated+water	0.0 b		

Least squared mean values after log transformation in Volga and NERF using "Select" as the Cultivar. For each treatment within a column, means with the same letter are not significantly different according to Bonfferoni least square means (P ≤ 0.05)

Table 0.2 Effect of different bio pesticides and synthetic pesticides on BLS severity on the flag leaf in Volga and NERF

Treatment	% Severity ($\log_{10}+10$)
Rating 1	NERF and Volga
Headline®	0.658 a
Cuproxat®	0.657 a
Sonata	0.638 a
BLS+water	0.626 a
Non inoculated +water	0.621 a
Regalia® RX	0.607 a
Champ® 2 Flowable	0.558 a
Badge® Sc	0.521 a
Rating 2	
BLS + water	1.47 a
Non inoculated + water	1.38 ab
Regalia® RX	1.35 ab
Headline ®	1.34 ab
Champ® 2 Flowable	1.34 ab
Badge®	1.30 ab
Sonata®ASO	1.27 b
Cuproxat®	1.25 b

Least squared mean values after log transformation in Volga and NERF using "Select" as the Cultivar. For each treatment within a column, means with the same letter are not significantly different according to Bonfferoni least square means ($P \leq 0.05$)

Table 0.3: Effect of different plant extracts and biopesticides on BLS severity on the flag leaf in NERF and Volga

Treatment	Severity% (log10+1)
Rating 1	
Wood vinegar	0.19 a
EDTA	0.14 ab
Biochar	0.14 ab
BLS + water	0.13 ab
Soursop	0.11 ab
Spiriluna	0.11 ab
Moringa	0.10 ab
Non inoculated + water	0.09 ab
Ginseng	0.08 ab
Fulvic acid	0.07 b
Chitin	0.04 b
Rating 2	
BLS + water	1.46 a
EDTA	1.43 a
Wood vinegar	1.41 a
Ginseng	1.39 a
Non inoculated + water	1.38 a
Spiriluna	1.38 a
Fulvic acid	1.37 a
Biochar	1.37 a
Chitin	1.36 a
Moringa	1.36 a
Soursop	1.35 a

Least squared mean values after log transformation in Volga and NERF using "Select" as the Cultivar. For each treatment within a column, means with the same letter are not significantly different according to Bonfferoni least square means t-test (P $\leq$ 0.05)

Table 0.4: Effect of different biopesticides and a fungicide on yield in NERF and Volga

Treatment	Yield(Kg/ha)	Treatment	Yield Kg/ha
Pesticides		Plant extracts	
	NERF and Volga		
Noninoculated+water	3289.28 a	BLS +water	3330.72 a
Champ 2 Flowable	3178.82 a	Chitin	3143.77 a
Cuproxat	3103.11 a	EDTA	3069.85 a
Badge	3097.53 a	Non inoculated+water	3053.85 a
Headline	3048.02 a	Biochar	3053.66 a
BLS +water	2915.85 a	Wood vinegar	2973.08 a
Sonata ASO	2801.94 a	Fulvic acid	2806.69 a
Regalia RX	2687.43 a	Spiriluna	2538.12 a
		Moringa	2440.32 a
		Soursop	2392.32 a
		Ginseng	2284.37 a

Least squared mean values of yield in Volga and NERF using "Select" as the Cultivar. For each treatment within a column, means with the same letter are not significantly different according to Bonfferoni least square means (P ≤ 0.05).

Table 0.5: Effect of different biopesticides and synthetic products on test weight in NERF and Volga

Treatment Biochemical pesticides	Test weight kg/m^3	Treatments Plant extracts and Synthetic products	Test weight Kg/m^3
Volga and NERF			
Non-inoculated+water	1300.273 a	Non-inoculated+water	1300.273 a
Headline®	1220.476 a	Biochar	1264.054 a
Badge®	1212.672 a	Chitin	1230.733 a
BLS +water	1136.560 a	EDTA	1201.985 a
Champ® 2 Flowable	1135.708 a	BLS +water	1111.105 a
Cuproxat®	1128.851 a	Wood vinegar	1136.560 a
Sonata ® ASO	1127.077 a	Moringa	1063.646 a
Regalia® RX	1092.548 a	Spirulina	1063.076 a
		Ginseng	1041.646 a
		Fulvic acid	1030.159 a
		Soursop	1014.029 a

Least squared mean values of test weights of wheat in Volga and NERF using "Select" as the Cultivar. For each treatment within a column, means with the same letter are not significantly different according to Bonfferoni least square means (P ≤ 0.05).

Chapter 3

3.1   Determining bacterial diversity on wheat seed as influenced

by different cultivar and environment.

Abstract

Seeds are sources of inocula and can facilitate the introduction of pathogens into the

field. It is therefore essential to determine the microbiota present on them to limit the

spread of plant pathogens. This study aimed to determine the bacterial diversity on wheat

seeds as influenced by wheat variety and location in South Dakota. Twenty grams of

seed from three replicates of eight wheat varieties obtained from Aurora and Volga sites

were suspended in and vortexed twice, then sonicated for four minutes in a phosphate

buffer suspension at pH of 7.4. Fifteen ml of the seed wash were used for DNA extraction

and sequencing, while 5 ml were stored for biochemical tests. Sequencing was done at

the University of Minnesota genomics laboratory with Primers 515Ff and 806rB targeting

the V4 region on the 16S rRNA. The resultant sequences were analyzed using Mothur

software and custom Perl scripts. Alpha diversity indices such as Chao1, Simpson, and

Shannon determined species richness and evenness, while beta diversity determined the

taxa shared between locations and among the eight varieties. The most abundant genera /

families identified were: *Sphingomonas, Pseudomonas, Pedobacter, Neorrhizobium,*

Microbacterium, Methylobacterium, Massilia, Hymenobacter, Kinecoccus,

Muciliginabacter, Enterobacteriaceae, Curtobacterium, and *Chrysobacterium.* The genus

Xanthomonas was also identified, but in very low abundance compared to other genera.

Sphingomonas had the highest relative abundance as compared to other genera in both

locations, while the detected genera that could harbor potential wheat pathogens were *Pseudomonas* and *Xanthomonas*. The results provide limited evidence that cultivar and environment influence bacteria diversity.

3.2   Introduction

Plants can sustain a multifaceted micro-ecosystem that is home to a diversity of bacteria. These bacteria can colonize roots, leaves, flowers, fruits, and seeds (Hallmann et al., 1997; Gray and Smith, 2005). Thus, the presence or absence of these bacteria can affect crop health through stimulation or suppression of tissue colonization by plant pathogens (Lindow and Brand, 2003).

Seeds are sexual reproductive organs of plants, which are also involved in vertical transmission of microorganisms from one generation to another. In turn, they serve as the primary source of inoculum for crops. Seeds germinate under favorable conditions to give rise to new offspring (Barret et al., 2015). Like roots (Philippot et al., 2013), leaves (Rastogi et al., 2013) and flowers (Shade et al., 2013), seeds have undergone evolution with various dissimilar microbial communities (Barret et al., 2015). A variety of plant-growth-promoting bacteria and phytopathogenic bacteria have been isolated from the seeds of various plant species (Baker et al., 1966; Nelson et al., 2004). Pathogen spread in new fields has been attributed to seed (Baker et al., 1966). Internal transmission, external spread through contact of seed with residue, and floral transmission are some of the pathways of pathogen spread from plant to seed (Maude, 1996). Both endophytic and epiphytic bacteria inhabit seeds; some of these can be beneficial to plants, while others can be pathogenic.

Epiphytic bacteria are capable of existing and reproducing on plant surfaces like leaves, roots, and seeds (Junk, R. et al., 2011) and their colonization is controlled by biological factors such as the host plant's growth and the life cycle of epiphytes (Brandl, 1998; Karamanoli, 2000). Epiphytes do not inhabit broad ecological niches because they exist at the plant-atmosphere interface, and any discrepancy in climatic conditions like moisture, humidity, temperature, wind speed, radiation, and rainfall may influence epiphyte diversity (Hirano, 1983). The epiphytic microbiota includes synergistic, commensal, and potentially pathogenic microbes that play a central role in plant health and susceptibility to disease (Hashidoko, 2005; Critzer and Doyle, 2010). Among the bacterial epiphytes that are supported on the aboveground parts of plants, some are phytopathogenic (Hirano and Upper 1983, Hirano and Upper, 1990). There is also spatial and temporal variability in epiphytic bacterial populations. They frequently vary significantly on a given plant species at different times of the growing season, often being relatively low on immature plant parts and increasing with plant age (Fryda et al., 1978; Gross et al., 1983; Lindow et al., 1978; Hirano et al., 1991; Hirano et al., 1989; Jacques et al., 1995; Lindow, 1982.).

Additionally, it is also expected that organisms that are naturally found on seed surfaces are similar to seed-associated and foliar endophytes, and may be selected by the environment for commensal, mutualistic and synergistic purposes. Thus, there is a possibility that different seed varieties may harbor different bacterial biodiversity, and environment may be playing a big role in determining bacterial diversity. Knowledge of bacterial diversity associated with different seed varieties and different environments is invaluable, because it can be a basis for choosing quality seed stocks. Quality seed stocks

are of utmost importance in the agricultural sector and serve as the basis for the next plant generation. Consequently, healthy and high-quality seeds are very vital to sustain food supply and to improve and uplift farmers' livelihoods (Links et al., 2014). Use of high-throughput-sequencing technology is a useful tool in determining the identity of microorganisms associated with different plant surfaces, due to its ability to detect even unculturable microorganisms. The use of sequencing technology is projected to lead to improved plant health, due to the possibility of identifying microorganisms that can inhibit the presence of plant-pathogenic bacteria (Tikhonovich and provorov, 2011).

3.3   Microbial diversity measurements

Microbial diversity on seed is measured using α and β diversity (Lozupone, and Knight, 2008). Alpha diversity refers to diversity within a given community, and is comprised of species richness, which is the total number of species within the community, and species evenness, which is the relative abundances of the species. Beta diversity involves apportioning biological diversity among communities. It describes the number of species shared between communities (Lozupone and Knight, 2008).

Table 0.1: Classes of diversity measurements (Lozupone and Knight, 2008)

	Measurement of diversity within a single community (α diversity)	**Measurement of diversity shared among communities (β diversity)**

Only presence/absence of taxa considered	**Qualitative α diversity**(Richness)
	Species-based:
	  Chao 1
	  ACE
	  Rarefaction
	Divergence-based:
	  Phylogenetic Diversity (PD)
		Qualitative β diversity
		Species-based:
		  Sörensen index
		  Jaccard index
		Divergence-based:
		  Unweighted UniFrac
		  Taxonomic Similarity (Δ_S)

Additionally accounts for the number of times that each taxon was observed	**Quantitative α diversity**(Richness and/or Evenness)
	  Species-based:
	    Shannon's index
	    Simpson's index
	  Divergence-based:
	    Theta
		Quantitative β diversity
		Species-based:
		  Sörensen quantitative index
		  Morisita-Horn measure
		Divergence-based:
		  Weighted UniFrac
		  F_{ST}
		  DPCoA

For this study, we tested the hypothesis that seed-associated epiphytic bacteria from different environments would differ noticeably.

3.4   Materials and Methods

To assess the epiphytic microbiota on wheat seeds, 20 g of seed samples from various cultivars (SD4148, Select, Brick, Faller, Forefront, Samson, Prevail, SD4011) obtained from South Dakota State University Aurora and Volga Research Farms. The seeds were washed for 1 minute with sterile phosphate buffer comprised of 5.27 g of potassium per liter and dihydrogen phosphate and 10.73 g monopotassium hydrogen phosphate per liter at pH 7.4. The seeds were then vortexed for 4 minutes in 20 ml of phosphate buffer, and then sonicated by using an ultrasonic bath (Branson 2510) for a duration of 4 minutes at a constant frequency of 47 kHz ± 6% at 25 º C, and then vortexed again for 4 minutes. Fifteen ml of seed wash were frozen (-20 C) and then shipped overnight on ice to a separate laboratory for molecular identification of the bacterial community. Additionally, 100 microliters of seed wash from the different cultivars were pipetted and spread on Petri plates containing Kings B agar media and incubated at 30 º C for a period of 48 hours. Colonies with different morphology and pigmentation were randomly selected from each plate and streaked on fresh Kings B media plates to observe their growth characteristics. For biochemical tests and Gram staining, we carried out biochemical tests for traits such as motility, indole utilization, triple sugar utilization (glucose, lactose and lactose) and Gram stain reaction with cultured bacteria from three replicates of cultivars Select and Brick.

3.4.1   DNA extraction and Sequencing of Amplicons

The DNA extraction was done using the Qiagen (DNeasy PowerSoil Pro) as per manufacturer's protocol. DNA quality and concentration were measured using a NanoDrop 1000 spectrophotometer (Thermo Scientific). DNA amplification was done

following the protocol of Ul-Hasan et al., (2019). PCR Primers 515Ff and 806rB (Apprill et al., 2015; Parada et al., 2016) that target the V4 hypervariable region of bacterial and archaeal 16S SSU rRNA gene sequences were ordered from Integrated DNA Technologies, Inc. (Coralville, IA). Each sample of the DNA extracted from three replicates of the eight varieties were amplified in a 25-µl PCR reaction under the following conditions: 94°C for 3 minutes, 72°C for 10 minutes, 94°C for 60 seconds, 50°C for 60 seconds and 72°C for 105 seconds.

The amplified triplicates were then pooled together into a single volume of 75 µl. The amplicons of each sample were electrophoresed on 2% of agarose gel, for 20 minutes and a 300 bp band size was produced. The amplicons were quantified with Quant-itPicoGreen dsDNA Assay kit (ThermoFischer/Invitrogen cat.no.P11496) as per the manufacturer's instructions. An equal amount of amplicon of 240 ng was combined into a single tube. The amplicon pool was cleaned using MoBio UltraClean PCR Clean-Up Kit (Cat.no.12500) following the manufacturer's instructions. The amplicon DNA concentration was measured and then sequenced using forward and reverse primers with sequences of 5′GTGYCAGCMGCCGCGGTAA3′ and 5′GGACTACNVGGGTWTCTAAT3′ at University of Minnesota Genomics Center using the MiSeq Illumina 2 × 300 bp chemistry.

3.4.2   Data processing of sequences

Raw bacterial 16S SSU rRNA V4 amplicon sequences that were processed were analyzed using custom Perl scripts unless stated differently. Overlapping pair-end reads from the same flow cluster were stitched together to make contigs using Mothur software.

Reads were screened based on the following conditions: Intact 515Ff (forward) and 806rB (reverse) primer nucleotide sequences with the length between 400 and 500 base pairs and a minimum quality threshold of no more than 1% of nucleotides with a Phred-like algorithm quality score not lower than 63. Screened reads were aligned, and then Operational Taxonomic Units (OTUs) were generated at a genetic distance cut-off of 15% dissimilarity (Opdahl et al., 2018). A commonly used clustering cut-off of 3% for the V4 or V4-V5 variable regions of the 16S rRNA was used. DNA artifacts were screened from the OTUs following these methods: Chimeric sequences were singled out using chimera.uchime and chimera. Slayer commands were acquired from Mothur software (Schloss et al., 2009).

3.4.3 Analysis of Beta and Alpha diversity

The Aurora and Volga data sets were randomly rarefied to 17000 reads with 50 subsamples, and these were used to create 'shared'-type-formatted files using custom Perl scripts. All the proceeding steps were performed using Mothur commands (Schloss et al., 2009). Chao1, Simpson and Shannon indices were determined from the 'shared' files using using Mothur software. A visual heatmap (Figure 3.1) was produced using Idep.81, an online tool (http://bioinformatics.sdstate.edu/idep). We used the Mothur command PCoA and generated Bray-Curtis distances based on OTU compositional dissimilarity (Figure 0.1).

3.4.4 Statistical analysis

An independent t-test was conducted to compare relative abundances of bacterial taxonomic groups between Aurora and Volga. Data were analyzed using GraphPad

Software, (https://www.graphpad.com/quickcalcs/ttest1.cfm). We then normalized data

and compared the combined relative abundancies of the different varieties from both

Aurora and Volga.   Data were analyzed using R statistical software version 3.6.0

3.5   Results

From the biochemical tests done on the sampled varieties, we found 90% of the bacteria

to be Gram negative and motile, while 10% were Gram positive. All of them fermented

glucose, lactose and sucrose while one bacterium was positive for indole utilization.

After sequence data processing, high quality 1, 802,437 reads free of chimera were used

for analysis with an average of 39,949.43 ± 12210.96 reads per sample from Aurora and

3,352 ± 985.74 reads from Volga. *Sphingomonas* was the most abundant genus in Volga

and Aurora with average relative abundances of 34.16% and 15.40% respectively across

all the 8 wheat varieties; followed by *Masilia* (25.52%) and *Neorrhizobium* (12.17%) in

both locations. Additionally, other genera in varying abundances found on the wheat

seeds in both Volga and Aurora included *Pedobacter, Chryseobacterium,*

Methylobacterium, , Hymenobacter, Pseudomonas, Mucilaginibacter, Ramilibacter and

Xanthomonas , and are shown in Figures 3.2 and 3.4. Generally, both Aurora and Volga

locations had similar bacterial diversity and the same applies to all the 8 wheat varieties.
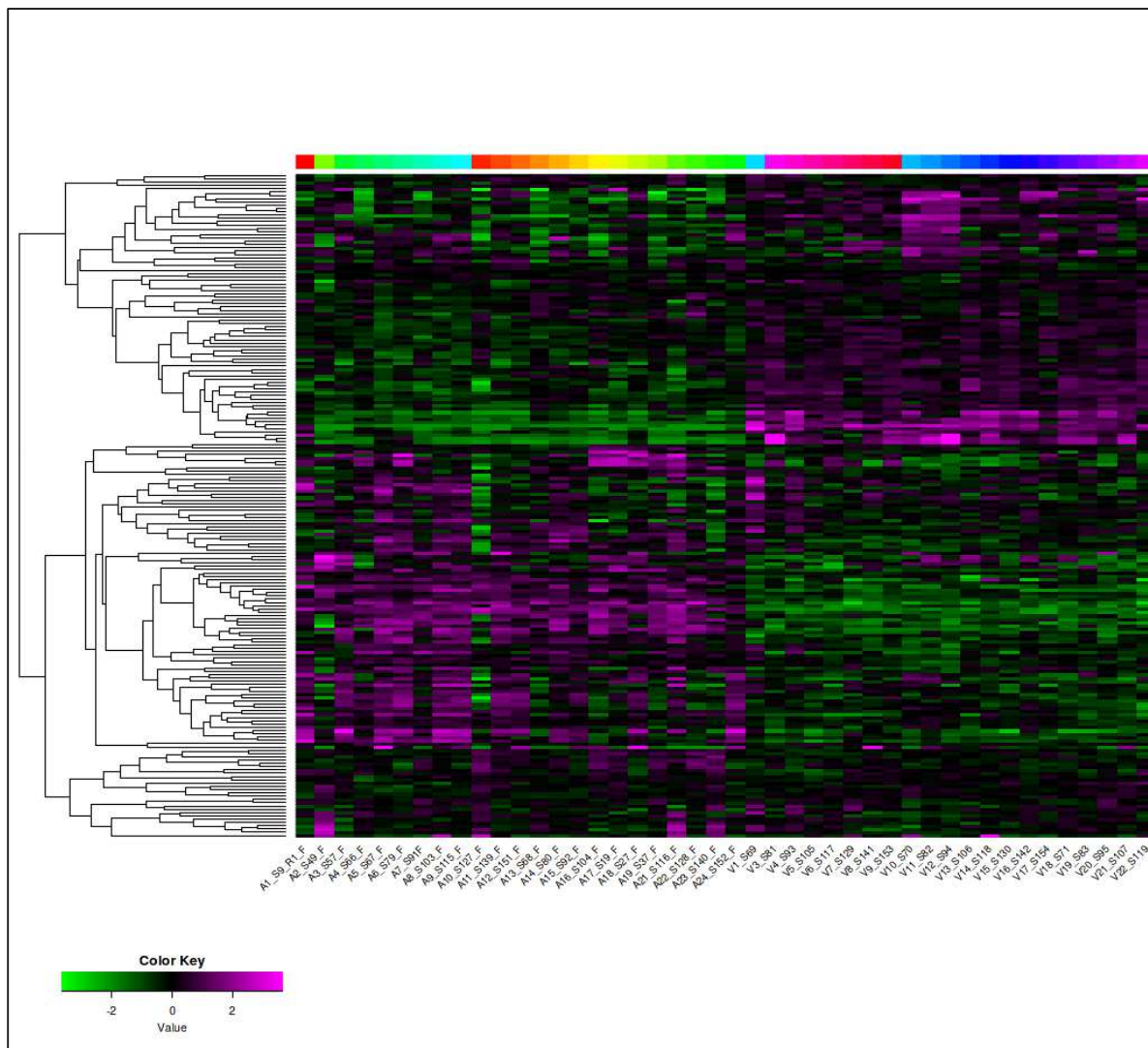
Figure 0.2: Hierarchical cluster analysis based on 2453 OTUs found on wheat seeds in both Volga and Aurora. The trees indicate the OTUs while the labels indicate the different wheat varieties with three or two replicates per variety,21 columns and OTUs are rows.
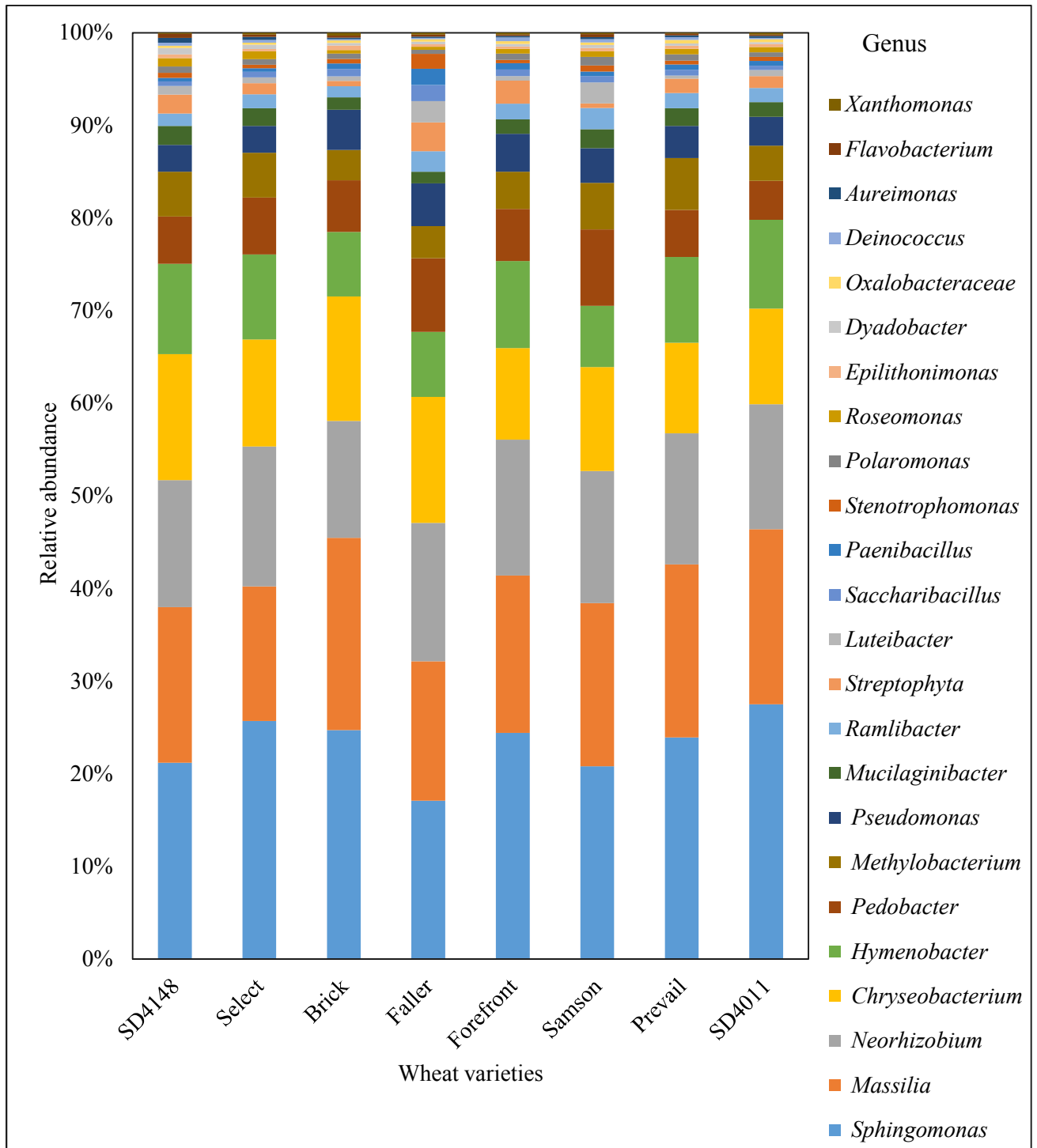
Figure 0.3: Relative abundance of 34 most abundant bacterial genera on different wheat seeds varieties in Volga, some genera not on graph for clarity.
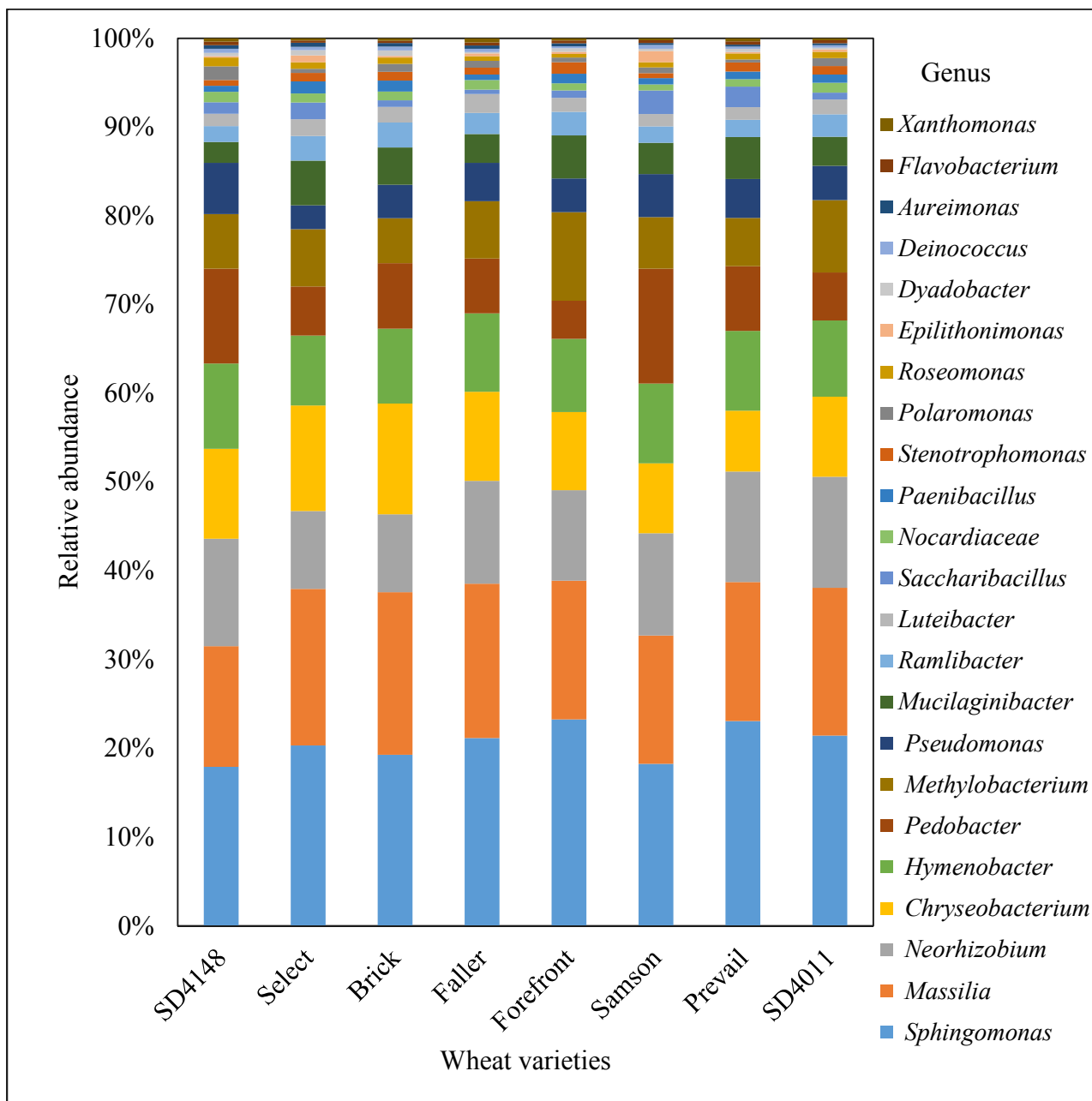
Figure 0.4: Relative abundance of 34 most abundant bacterial genera on different wheat seeds varieties in Aurora, some genera not on graph for clarity.
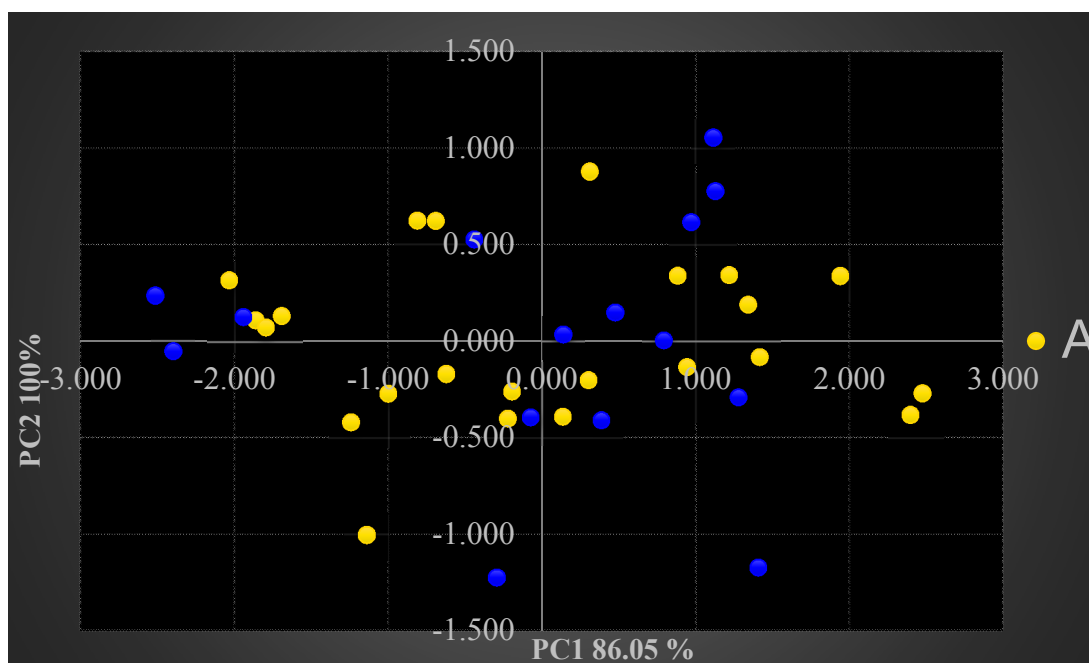
Figure 0.5: Comparison of wheat seed bacterial communities from two locations using Principle Coordinate Analysis. The x and y axes correspond to principle component PC 1 and PC 2, explaining the highest level of variation.

3.6   Discussion

A total of 2453 OTUs were associated with the eight wheat varieties in both Aurora and Volga. The most abundant genera found on the wheat seeds were *Sphingomona*s, *Masilia, Neorrhizobium, Pedobacter, Chryseobacterium, , Methylobacterium, Hymenobacter, Pseudomonas, Mucilaginibacter* and *Xanthomonas.* All eight varieties shared the same microbiota in both Aurora and Volga, which implies that the existence of the microbiota across all varieties is not attributed to contamination. Instead, different varieties of wheat select for these bacteria and are intimately associated with the seeds. These results are consistent with previous studies done on Triticum seeds and Brassicae in Canada, where seeds from different geographical locations were found to have the same epiphytic bacterial microbiota (Links et al., 2014). Many of the seed epiphytic bacteria identified, for example the genus *Neorrhizobium,* are also found in the soil, which suggests a

possible linkage between bacterial soil microbiota and seedborne bacterial microbiota (Links et al., 2014). There is also a possibility that some epiphytic bacteria associated with the different wheat-seed varieties could be airborne bacteria. Some of the genera found on the wheat-seed surfaces were *Xanthomonas* and *Pseudomonas*, which can include wheat pathogens such as *Xanthomonas translucens pv. undulosa* and *Pseudomonas syringae*. Another taxon that was found on wheat seeds which has some potential plant pathogens is the *Enterobacteriaceae,* which contains *Erwinia* species, which affect many other crops. The presence of the *Sphingomonas* genus in relatively high abundance compared to the rest of the genera may be directly associated with the relatively low abundances of other genera that are known to include plant pathogens, because certain *Sphingomonas* species have been found to control some bacterial pathogens of many plants. For example some *Sphingomonas* species suppressed the populations of *X. campestris* pv. c*ampestris* in *A. thaliana* , and many studies have indicated that *Sphingomonas* species are effective against many foliar plant pathogens (Buell, 2002).

3.7   Conclusion

The results from this study provide limited evidence that epiphytic bacterial diversity is influenced by cultivar and environment. Although for this study we used only a few cultivars and considered two locations, it provides valuable information that reveals presence of seedborne taxa that include some wheat bacterial pathogens as members. It also demonstrates presence of bacterial genera such as *Sphingomonas* that includes species which have the ability to suppress growth of some plant pathogens. These findings call for future work to culture and test the effect of these *Sphingomonas* species

on *Xanthomonas translucens pv.undulosa* (Xtpvu*)*. There is a possibility that

Sphingomonas species could include some biocontrol agents that can antagonize Xtpvu.

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Tabe 0.2: Alpha diversity indices from 8 wheat seed varieties from Aurora and Volga

	Volga	Aurora	P value
Observed OTUSs	553.476 ± 39.61	595.04 ± 145.46	0.2124
Chao1	784.77 ± 66.73	899.65 ± 410.03	0.2021
Ace	879.36 ± 123.37	1048.09 ± 797.03	0.3326
Shannon	4.096 ± 0.11	4.27 ± 0.18	0.00045
Simpson	0.034 ± 0.003	0.029 ± 0.0039	4.76E-05

Table 0.3: Combined bacterial taxonomic relative abundances from different wheat seed varieties from both Volga and Aurora.

Variety	Relative abundance	
Samson	0.01211631	a
Faller	0.01208900	a
SD4011	0.01202759	a
SD4148	0.01202477	a
Prevail	0.01202354	a
Select	0.01200964	a
Forefront	0.01191849	a

Least squared mean values of combined bacteria taxonomic relative abundances on wheat seed varieties from Volga and Aurora. For each variety within a column, means with the same letter are not significantly different according to Bonfferoni least square means (P ≤ 0.05).

Chapter 4

4.1   Antimicrobial activity of selected ethyl acetate extracts of

plant products and spices against *Xanthomonas pv. undulosa*

in vitro

Abstract

Plant pathogenic bacteria in the genus *Xanthomonas* can affect a majority of crops all

over the world, and they can decrease quality and quantity of produce. Many extracts

obtained from plants are sources of antibacterial agents, which interfere with bacterial

growth. These antibacterial agents are mainly secondary metabolites that plants produce

to protect themselves against abiotic and biotic stresses. In this study, we screened ethyl

acetate extracts of ginseng, tannic acid, moringa, spirulina, soursop and spices such as

onion, garlic, sage, poppy seed, black pepper, red pepper, sesame and rosemary against

the plant pathogenic bacterium *Xanthomonas translucens pv. undulosa* that causes

bacterial leaf streak of wheat. We used crude ethyl acetate extract concentrations of

285.7mg/ml for all other plant products and spices apart from sage, onion and garlic that

had concentrations of 100 mg / ml. Streptomycin sulfate (10 µg / ml) was used as the

positive control, while 1% methanol was the negative control. Ethyl acetate extract of

tannic acid had a zone of inhibition (ZOI) of 28.26 ± 5.0 mm with a minimum inhibitory

concentration (MIC) and minimum bactericidal concentration (MBC) of 71.43 mg / ml.

Ethyl acetate extracts of moringa and spirulina had a range of 19.57 ± 3.62 mm and 15.12

± 4.08 mm respectively with MICs of 100 mg / ml. Ethyl acetate extract of garlic and

sage had ZOI of 26.67 ± 4.85 mm and 20.09 ± 4.08 mm respectively, while onion had

ZOI of 19.54 ± 3.94 mm with MICs of 100 mg / ml. Among all the products tested, only

tannic acid was found to possibly have bactericidal effects. This study demonstrates that ethyl acetate extracts of some plant products and spices have a significant effect on Xtpvu in vitro, and could be integrated into bacterial leaf streak management in conjunction with current bacterial leaf streak management practices.

4.2   Introduction

Xanthomonas translucens pv. undulosa is prevalent in the Midwest, especially in the wheat-growing regions (Adhikari et al., 2012). Currently, there are no known bactericides to control the pathogen, which calls for testing of various antibacterial products to determine their effect against Xtpvu. In vitro testing of various products serves as a basis for identifying products that may have significant control of this bacterial pathogen in the field.

There is growing evidence that some plants can be a rich source of antimicrobial substances that are part of their natural defense mechanisms to protect them against biotic and abiotic living stresses (Nabavi *et al.,* 2015). Different plant parts may contain biologically active compounds, and these can be extracted using organic solvents such as ethyl acetate, methanol, ethanol, hexane and cyclohexane. These biologically active compounds can be a source of new ecofriendly pesticides to fight against plant pathogenic bacteria (Gan-Mor and Matthews, 2003). Many spices and other plant extracts are a source of antimicrobial substances due to the presence of secondary metabolites, and are not detrimental to either animals or the environment (Nabavi *et al.,* 2015).

4.3   Chemical composition of selected spices and plant products

Garlic (*Allium sativum L.*) contains a major biologically active substance called allicin (diallyl-thiosulfinate). Cavallito *et al*., (1944) first noted its potent antimicrobial activity. Allicin is not present in raw garlic, but it is rapidly produced by the action of CS-lyase (allinase) on alliin. Allinase is activated by crushing or cutting the garlic cloves (Block *et al.,* 1985; Stoll *et al.,* 1949). Sage (*Salvia officinalis*) belongs to the *Lamiaceae* (mint) family that includes about 900 species (Nikavar et al., 2008; Itani et al., 2008). The plants of the mint family have a characteristic aromatic odor and are perennials. The Mediterranean region is home to many *Salvia* species, including *Salvia officinalis*. It is an angiosperm and a dicotyledonous evergreen with purple flowers (Kesmati *et al.,* 2015). Sage oil contains major components such as α-thujone, β-thujone, borneol, and viridiflorol 1, 8-cineole, and camphor (Hamidpour *et al.,* 2014).

Tannnic acid is derived from tannins that can be found in the buds, bark, galls, fruits, roots and the leaves of many plants (Kraus et al.,2003). They have two major subdivisions, hydrolysable and condensed tannins. Hydrolysable tannins form gallo tannins due to the esterification between polyol and gallic acid (Smith *et al.,* 2003), while flavonol polymers make up condensed tannins (Schofield *et al.,* 2001). Plants can manufacture many products from these basic structures. The plants use the manufactured products as a defense against many pathogens and herbivores (Scalbert, 1991). Tannins can inhibit microbial growth (Chung *et al.,* 1998; Funatogawa *et al.,* 2004, Scalbert, 1991) by tannin-metal complexation, cell membrane interference and metal chelation (Smith *et al.,* 2005). Tannins can complex with different metals, including copper

(Andrade *et al.,* 2005), iron (South and Miller, 1998; Lopes *et al.,* 1999), and zinc (McDonald et al., 1996; Cruz et al., 2000).

In this study, we evaluated the in vitro efficacy of various plant extracts and spices on *Xanthomonas translucens pv. undulosa* (Xtpvu), a bacterial pathogen that causes bacteria leaf streak of wheat. The effect of many of these plant extracts and spices has been studied on human bacterial pathogens in vitro, and they have demonstrated significant growth inhibition of both Gram-negative and Gram-positive bacteria. We hypothesized that these products would also have a significant inhibition effect on Xtpvu.

4.4   Materials and Methods

We purchased dry or powder forms of plant products (ginseng (Bulk Supplements), tannic acid (Sigma Aldrich T0200), moringa (MAJU super foods, Amazon), spirulina (MAJU super foods, Amazon, Soursop) from Amazon and spices (black pepper, red pepper poppy seed, sage, rosemary, sesame (McCormick Gourmet via Walmart). Onion and garlic were purchased as fresh vegetables from a local store. Ten-gram mass of each product were weighed except for sage and rosemary where 5.0 g were used. All the products were individually transferred into 50 ml Erlenmeyer conical flasks and 100 ml of ethyl acetate was added. The conical flasks were then placed on a rotary shaker at 150 rpm at 25 $^\circ$C room temperature for 48 hours. The extracts were later filtered through layers of cheesecloth in a chemical fume hood, and the filtrate was concentrated in a rotary evaporator for 2 hours. The filtrates were then left in the fume hood to allow removal of excess ethyl acetate solvent. The resultant residue after evaporation was re-weighed. Only 2 g per product were weighed except for sage, garlic and onions whose

weight was each 0.5g and all were dissolved in 7 ml of 1% methanol. The concentrations

obtained were 285.7 mg / ml except for sage, garlic and onion that each had a

concentration of 100 mg / ml. Streptomycin sulfate was prepared according to the

standardized method. These products were then filter-sterilized using a 0.02 µm filter.

The suspensions were then stored in centrifuge tubes at 4ºC for further use.

The antibacterial activity of these products were evaluated using the agar well

diffusion method described by Logan (1996). Four colonies of Xtpvu were grown on KB

broth media amended with 5 g of glucose instead of glycerol for 6 hours. An absorbance

of 0.063 at 600 nm was measured using a spectrophotometer, which was the equivalent to

a 0.5 McFarland standard. Then 100 µl of bacterial suspension were spread on KB agar

media modified with glucose. Five wells were aseptically made in the agar using a 15

mm cork borer; three wells had the plant products or spices while the other two wells

contained streptomycin sulfate and 1% methanol respectively. A volume of 25 µl of each

product and spice were added per well. The experiment was a completely randomized

design with 100 replications for each plant product or spice. The assay was repeated three

times. The zones of inhibition in mm were measured using a ruler and data were analyzed

using R statistical software Version 3.5.1 to obtain the mean separations.

4.4.1   Minimum Inhibitory Concentration (MIC)

To determine the minimum inhibitory concentration (MIC), we used a modified

method by Khaleel et al. (2016). Only the products that showed inhibitory effects on

Xtpvu were used to determine the MIC. Concentrations of 285.7 mg/ml were used for

moringa, tannic acid, soursop and spirulina; and 100 mg / ml of onion, garlic, and sage

were the starting concentrations for making dilutions. Twelve test tubes labelled (K_1-K_{12})

for each plant extract and spice were filled with 1 ml of modified Kings B broth. A series

of serial dilutions was made for each by dispensing 1 ml of the plant extract or spice to

the respective tubes K_1 to K_{10} but not K_{11}. A 100 µl volume of the standardized bacterial

suspension was dispensed into all tubes. Tube K_{11} was the negative control having broth

and the bacteria suspension. Then 50 µl of 10 mg / ml of 2, 3, 5

Iodophenylnitrotretrazolium violet was added to all the test tubes and they were

incubated at 30 ^{O}C for 24 hours in darkness. After 24 hours, the test tubes that did not

have any color change were used to determine the MIC based on unaided eye visual

inspection.

4.5   Results

The results presented here are only for those products that showed inhibition effects on

Xtpvu. Based on Table 4.1 and 4.2, there was a significant difference among all the plant

extracts and spices used. Streptomycin had the largest zone of inhibition with a range of

35.05 ± 4.54 mm; followed by crude ethyl-acetate extracts of tannic acid with a zone of

inhibition of 28.26 ± 5.01 mm and ginseng with a range of 20.09 ± 5.32 mm. The

moringa and spirulina had a range of 19.57 ± 3.62 mm and 15.12 ± 4.08 mm respectively.

As shown in Table 4.1, tannic acid had a MIC of 71.43 mg / ml while moringa, spirulina

and ginseng had a MIC of 142.85 mg / ml. All the spices had a MIC of 100 mg / ml.

Tannic acid had a MBC of 71.43 mg / ml, while moringa, ginseng, spirulina, onion, sage

and garlic did not have a bactericidal effect on Xtpvu.
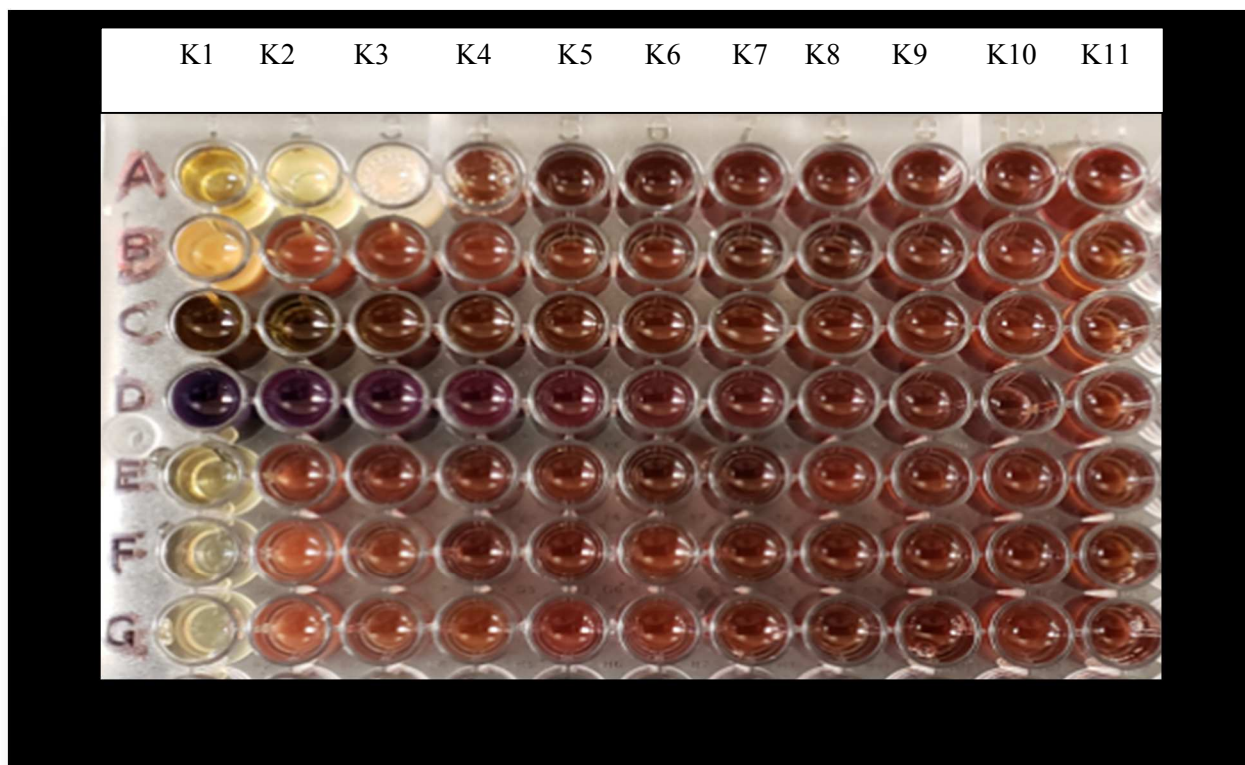
Figure 0.1: Minimum Inhibitory Concentration (MIC) of ethyl acetate plant extracts and spices against *Xanthomonas translucens* pv *undulosa*;

A (tannic acid), B (ginseng), C (Moringa), D (Spirulina) and spices E (sage), F (onion), G (garlic). K1A=71.43 mg / ml, K1B =142.85 mg / ml, K1C =142.85 mg / ml, K1F =142.85 mg / ml, K1E =100mg / ml, K1F = 100 mg / ml, K1G = 100 mg / ml. The contents from the test tube were transferred into a micro titer plate for the photography purposes.

4.6   Discussion

Among the plant extracts, tannic acid was the most effective in inhibiting the growth of Xtpvu in vitro with a zone of inhibition of 28.26 ± 5.01 mm; but it did not inhibit Xtpvu growth better than the known antibacterial agent streptomycin sulfate, which had a zone of inhibition of 35.05 ± 4.54 mm. There have been studies regarding the antimicrobial properties of tannic acid on various human pathogens and a few plant pathogens. Studies have shown that tannins are the primary components of tannic acid.

Their antimicrobial activity (Chung et al., 1998; Funatogawa et al., 2004; Scalbert, 1991) is due to their ability to bind proteins (Adamczyk, 2008) and scavenge free radicals (Kraus et al., 2003). Related studies suggest that the antimicrobial activity is due to tannin-polymer complexation, membrane disruption, and chelation of metal ions (Smith et al., 2005). Tannins can also suppress many enzymes, such as glycosyltransferases (Naziri, 2012) which are essential enzymes in the biosynthesis of complex carbohydrates and glucoconjugates that are fundamental in bacterial processes such as adhesion, cell wall biosynthesis and signaling (Drickamer, 1998). In studies of human pathogens, tannic acid has inhibited biofilm formation in both Gram-negative bacteria and Gram-positive bacteria such as *Escherichia coli* and *Staphylococcus aureus* (Payne et al., 2013; Dong et al., 2018). In a study done by Wu et al. (2013), tannic acid inhibited beta-ketoacyl-ACP reductase (FabG), which is an essential enzyme in bacterial fatty acid synthesis. Therefore, the ability of tannic acid to inhibit the growth of Xtpvu could be attributed to one or more of these mechanisms. The results were further supported by an MBC value of 71.43 mg / ml; at this concentration, there was no Xtpvu growth, which indicates that tannic acid has not only inhibitory effects but may have bactericidal properties.

Similarly, ethyl acetate extract of ginseng showed an average zone of inhibition of 20.09 ± 5.32 mm. Different researchers have demonstrated ginseng's inhibitory effect on various Gram-negative human pathogens such as *Pseudomonas aeruginosa*. Several studies have also shown its ability to reduce biofilm formation through interfering with quorum sensing signals in vitro (Wu et al., 2014; Song et al., 2010). Ginseng extracts have also been associated with the inhibition of cell adhesion, growth and urease activity

(Yang et al., 2012). Ginseng contains ginsenosides, which are bioactive saponins (Braga et al., 2011). This could possibly explain why the growth of Xtpvu was inhibited in vitro.

Spirulina was the least effective compared to the rest of the plant extracts in inhibiting the growth of Xtpvu, with a zone of inhibition of 15.12 ± 4.08 mm. Its antibacterial properties are attributed to the presence of compounds such as phlorotannins, peptides, sterols, aromatic organic acids, terpenes, shikimic acid ,fatty acids, alcohols and alkenes and halogenated furanones (Blunt et al., 2015; Mayer et al.,2013; Chojnacka et al.,2015). Some of the antibacterial properties of algal cell constituents like phlorotannins can inhibit oxidative phosphorylation and have the ability to bind to bacterial proteins and the cell membrane and promote lysis. Studies have shown that the OH groups of phlorotannins bind to the -NH groups of bacterial proteins by hydrogen bonding and hydrophobic interactions (Heldt and Piechulla, 2010; Wang et al., 2009). Other algal cell constituents like proteins and peptides have antibacterial properties associated with the ability to bind with polar and non-polar sites on bacterial cytoplasmic membranes due to their amphipathic nature. In turn, they are able to interfere with various cellular processes. (Nguyen et al., 2011; Pimenta and Lebrun 2007; Lordan et al., 2011). Terpenes, like diterpene-benzoate, are also constituents of blue green algae, and they can also inhibit bacterial growth in-vitro (Lane et al. 2009). In similar studies related to the study of the antibacterial effect of spirulina on human pathogens, many Gram-negative bacteria and Gram-positive bacteria such as *Escherichia coli, Pseudomonas aeruginosa* and *Bacillus cereus* were inhibited by various extracts of spirulina (Kumar et al. 2006). Therefore, the various phytochemical constituents of

spirulina that have antibacterial properties may help explain why there was Xtpvu growth inhibition.

There was a significant difference among the spices when compared to the positive control and the negative control, with garlic having the largest zone of inhibition of 26.67 ± 4.85 mm. Streptomycin sulfate, a known antibacterial agent, had a zone of inhibition of 35.05 ± 4.55 mm. Studies done on the antibacterial properties of garlic on various human bacterial pathogens have revealed that garlic's effect on bacteria is due to its major active ingredient allicin, which partially inhibits DNA and protein synthesis, and totally inhibits RNA synthesis which is the major target (Eja et al., 2007). Other studies have also discovered that the presence of organosulfur compounds and phenolic compounds contributes to the antibacterial properties of garlic (Griffiths et al., 2002) which further supports our findings.

In the same manner, sage ethyl acetate extract showed antibacterial effects on Xtpvu, with a relatively smaller zone of inhibition of 20.09 ± 4.08 mm, and a MIC of 100 mg / ml when compared to garlic extract and streptomycin sulfate. In previous studies, Pierozan et al. (2009) attributes the antibacterial activity of sage extracts against Gram-negative and Gram-positive bacteria to the presence of 1, 8-cineole, α-thujone and camphor. Additionally, the essential oils in sage could have inhibited the growth of Xtpvu due to their potential to inhibit microorganisms, because of their hydrophobic nature. They apportion themselves into the phospholipid bilayer of the cell membrane, which becomes more permeable thus causing leakage of vital cell components (Burt et al., 2004). Later cell death sets in due to loss of membrane differential permeability (Burt et al., 2004; Generalić et al., 2012).

The onion ethyl acetate extract had the smallest zone of inhibition of 19.54 ± 3.94 mm and an MIC of 100 mg / ml compared to all the spices tested. Bakht et al. (2013) reported that onion ethyl acetate extracts inhibited the growth of Gram-positive and Gram-negative bacteria affecting humans. Onion antibacterial effects on bacterial human pathogens are directly related to the effects on plant bacterial pathogens, since Xtpvu is a Gram-negative bacterium with a similar cell wall structure. Onion extracts have also been found to inhibit the growth of *Escherichia coli* and *Kleibsiella pneumonia* (Bakht et al., 2013), which are Gram-negative bacteria that are similar to Xtpvu.

4.7   Conclusion

Plant compounds such as tannic acid, moringa, sage, garlic and onions have the potential to be used to manage bacterial leaf streak of wheat. However, they may need some modifications to have more efficacy under field conditions. These products could be applied with stickers or spreaders to stick on the leaves more for a prolonged protection before these products degrade or are washed off.   It is possible that if different commercial sources were used to obtain the substances tested here, that experimental results may have differed somewhat from those reported here, due to variability in the sources and processing of these natural products.

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Table 0.1 Means (n = 300) of inhibition zone diameter (mm) of ethyl acetate plant

extracts against *Xanthomonas translucens pv.undulosa*. Within rows, values with

different letters differ significantly.

Moringa	Spiriluna	Ginseng	Tannic acid	Streptomycin	1% Methanol
19.57 ± 3.62 [c]	15.12 ± 4.08 [c]	20.09 ± 5.32 [d]	28.26 ± 5.01 [b]	35.05 ± 4.54 [a]	0 ± 0 [e]

Between columns, means with the same letter are not significantly different according to
Bonfferoni least square means ($P \leq 0.05$).

Table 0.2: Means (n = 300) of inhibition zone diameter (mm) of ethyl acetate Spice

extract against *Xanthomonas translucens pv. undulosa*.

Sage	Garlic	Onion	Streptomycin	1% Methanol
20.09 ± 4.08 [c]	26.67 ± 4.85 [b]	19.54 ± 3.94 [c]	35.05 ± 4.55 [a]	0 ± 0 [e]

Between columns, means with the same letter are not significantly different according to
Bonfferoni least square means ($P \leq 0.05$).

Table 0.3: Minimal inhibitory concentration (MIC) and minimum Bactericidal

Concentration (MBC) of plant extracts and spices against *Xanthomonas translucens pv*

undulosa.

Plant products	Minimum Inhibitory concentration(MIC) mg/ml	Minimum Bactericidal concentration(MBC) mg/ml
Plant extracts		
Moringa	142.85	-
Spiriluna	142.85	-
Ginseng	142.85	-
Tannic acid	71.43	71.43
Spices		
Sage	100.00	-
Garlic	100.00	-
Onion	100.00	-

Chapter 5

5.1   General Conclusions and Recommendations

Some copper-based bactericides like Cuproxat and the bio- pesticide Sonata
showed a significant control of BLS in the field. These can be used in conjunction with
the available BLS management practices. These products however should be applied
before the plants develop symptoms in order to control the epiphytic population of Xtpvu
early on in the season, and may be applied more than once to ensure maximum protection
of the leaves.

In the study to determine the effect of environment on bacterial diversity, seed
lots in both locations had similar bacteria microbiota but there were variations in relative
abundances. Through sequencing, we were able to identify different genera / families
(*Xanthomonas, Pseudomonas and Enterobacteriaceae*) to which some plant pathogens
belong. Presence of the genera that include plant pathogens, especially those that affect
wheat, calls for measures that can reduce their presence on wheat-seed surfaces,
especially for seed lots that are going to be used for planting in locations where these
plant pathogens have never existed in order to reduce their spread. It is also vital that seed
tests are done to reduce the risk associated with seedborne bacterial pathogens.

We were also able to detect the presence of one genus (*Sphingomonas*) that has
species that can suppress plant pathogens. The presence of this genus calls for further
culturing of these bacteria to test if they can antagonize Xtpvu in vitro. There is a
possibility that they may be useful as commercial biological control agents.

From the in vitro study, the plant products that had a large zone of inhibition such as tannic acid and garlic need to be further tested under field conditions to determine their efficacy. There is a possibility that combining these plant products may be useful and efficacious. Perhaps they could have a synergistic effect on Xtpvu that exceeds the effects of each individual product. Various concentrations of these products can be made to get the best concentration that can control Xtpvu without necessarily being phytotoxic to the wheat plants, and this is mainly applicable to tannic acid.

As overall recommendations, further studies on the efficacy of plant products, biopesticides and synthetic bactericides should be done. These products can be applied at the different stages of wheat growth, and later the severity of BLS can be determined. Any new approaches to control BLS would be valuable, especially if they are environmentally friendly.