Exploring fungi associated with the perennial grain crop Intermediate Wheatgrass

by

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B.S., Tribhuvan University, 2018

A THESIS

submitted in partial fulfillment of the requirements for the degree

MASTER OF SCIENCE

Department of Plant Pathology
College of Agriculture

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2023

Approved by:

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Abstract

Perennial crops, such as intermediate wheatgrass (IWG), have been recognized as a valuable component of sustainable agriculture, providing ecosystem benefits while minimizing adverse effects of conventional farming on the environment. Domestication of intermediate wheatgrass as a grain crop began in 1980s, and grain harvested from it is marketed as Kernza®. As adoption of this crop increases, farmers have expressed concerns about potential disease and pest issues and mycotoxin contamination of the grain. Though IWG has been used extensively as a donor of disease resistance genes to annual crops like wheat, there may still be diseases that cause detrimental effects to food and feed. To address these concerns, this study aims to characterize the fungal and viral populations from the grain samples. Head samples were collected from farmers in Kansas, Minnesota, North Dakota and Montana, and leaf samples were obtained from an IWG nursery in Olathe, Kansas. The head samples were sterilized, cultured, single-spored, and DNA was extracted. Polymerase chain reaction (PCR) was performed using ITS and Tef-1α primers and then sequenced. The results from the head samples confirmed the presence of diverse Fusarium species such as, *Fusarium graminearum, Fusarium incarnatum-equiseti* species complex, *Fusarium armeniacum, Fusarium verticillioides, Fusarium asiaticum, Fusarium boothii, Fusarium sporotrichioides,* and *Fusarium lacertarum.* The analysis of mycotoxins in representative grain samples revealed presence of regulated mycotoxins including DON, fumonisin, zearalenone, and T-2. DNA sequencing of leaf samples showed the presence of important leaf disease causing fungal pathogens including *Pyrenophora tritici-repentis* which causes tan spot, *Bipolaris sorokiniana* which causes spot blotch, and *Parastagnospora nodorum* which causes leaf blotch. The nanopore sequencing of leaf samples revealed the presence of

wheat streak mosaic virus, soil borne wheat mosaic virus, and barley yellow dwarf virus. The information gathered from this study is valuable for farmers and researchers who seek to understand disease diversity in domesticated IWG and develop effective breeding and disease management strategies. While the presence of some disease-causing pathogens has been confirmed in IWG, further research is needed to understand other pathogens, host-pathogen interactions, and their localization in the crop to obtain a comprehensive knowledge of crop pathology.

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Acknowledgements

I want to thank my advisor, Dr. Jessica Rupp, for consistently guiding and supporting me throughout my academic journey. Your unwavering support and motivation have been instrumental in my academic and career development. I am confident that I can always count on you to be available for me, even after I have completed my studies. I would like to thank Dr. Chris Little and Dr. Kelsey Andersen Onofre for serving on my committee and providing me with helpful feedback to make my research better.

I am grateful to Dr. Kathryn Turner from The Land Institute of Salina for her continuous effort to make sure I received samples from growers and advising me and updating me with Kernza® related information. This project would not have been possible without your involvement. I would like to express my gratitude to Dr. Myron Bruce for imparting valuable knowledge about molecular techniques to me. I appreciate your patience in teaching me these techniques during my learning process, I also thank you for your help in troubleshooting DNA sequencing issues.

Thank you to Dr. Fellers' lab for allowing me to use the nanodrop, to Dr. Akhunov's lab for providing access to the Tissuelyser and to Dr. Fritz's lab for permitting me to capture the gel image. I extend my thanks to Mark Davis for sharing his expertise on disease phenotyping and inoculation techniques, as well as for assisting with my transportation to the fields.

I want to extend my gratitude to my previous lab mates, Dylan Mangel and Nar Ranabhat for sharing their research knowledge, advising me with courses and for always being willing to help me. I would also like to acknowledge Lawrence Tidakbi for his support in the lab work. I would like to thank all members of the Department of Plant Pathology, including the Department

head, faculties, office team, and graduate students for contributing to my wonderful experience throughout this journey.

I express my utmost gratitude to both of my families in Nepal, including my parents, brother, relatives, dear friends, and all those who have influenced me into the person I am today, whether directly or indirectly.

Dedication

I want to dedicate this thesis to my beloved parents, Tikaram Dahal and Yasoda Dahal, whose love, encouragement, and sacrifices have played an integral part in shaping the person that I am today. I am grateful for everything they have done. As I complete this chapter in my academic journey, I cannot help but miss my parents' physical presence. Their love and support have been the cornerstone of my success.

I would also like to dedicate this thesis to my loving husband, Gaurav Neupane. I cannot thank him enough for his support and understanding throughout my academic journey. He has been my rock, patiently listening to my frustrations and providing me with encouragement and motivation. Despite my occasional outbursts and moments of stress, he has always stood by my side, providing a calming presence and reminding me of my capabilities. I feel incredibly lucky to have such a caring and supportive partner who believes in me. His presence has been invaluable for achieving my academic goals.

Chapter 1 - Literature Review

Intermediate Wheatgrass

Intermediate wheatgrass (IWG) is a perennial grass with grains marketed under the trade name Kernza® (DeHaan and Ismail, 2017). It is a species that originated from Eastern Europe and Mediterranean region (Tsvelev, 1983). The plant grows vegetatively and requires a vernalization temperature between 0°C and 10°C for a period of six weeks for successful flowering and grain fill. In spring, as the days get longer, the stem elongates in mid-May usually. In most regions, where IWG is grown, flowering occurs in early July. Following maturation, IWG is ready for harvest in mid-July to early August (The Land Institute, 2019).

In the 1980s, efforts towards domestication of IWG began at the Rodale Research Center, and later, breeding germplasm was further developed by the Land Institute in Salina. The Land Institute's breeding program for intermediate wheat grass, under the guidance of Dr. Lee DeHaan, commenced in 2003. The first IWG planting was initiated in 2010 on 30 acres of land and seeds were made available for growers after the registration of Kernza® trademark in 2011. Currently, 150 farmers grow IWG on 3,951 acres of land (The Land Institute, 2021). Kernza® is used in milling, baking and brewing (University of Minnesota, 2021). It has a potential to serve as a nutritious grain similar to wheat while also offering ecological benefits such as protecting soil from erosion and preventing nutrient leaching into groundwater through its year-round soil cover (Becker et al., 1992). With roots that can reach over 3m long, it aids in carbon sequestration, ecosystem nutrient retention, water infiltration and uptake efficiency (DeHaan and Ismail, 2017). It enhances soil microbial activity and increases organic matter content, promoting improved soil and crop health (Asbjornsen et al., 2014). This perennial crop can adapt to

changing climates and can be used to improve the productivity of marginal land. By not requiring yearly planting, farmers can produce grain and forage with reduced labor and input costs (Becker et al., 1992).

Intermediate wheatgrass (Thinopyrum intermedium, (Host) Barkworth and D.R. Dewey (2n=6x=42) (syn. Agropyron intermedium (Host) Beauvoir; syn. Elytrigia intermedia (Host) Nevski) is an allohexaploid grass (Li and Wang, 2009; Mahelka et al., 2011; Wang et al., 2015). This grass species belongs to the tribe Triticeae (family Poaceae), the same family as wheat, which is why hybridization is possible with wheat (Mahelka et al., 2011). IWG has been utilized to transfer various disease resistant genes, such as Lr38 for leaf rust resistance, Sr43, Sr44 for stem rust resistance, YrYU25 for stripe rust resistance (Luo et al., 2010) Pm40, Pm43 for powdery mildew resistance, Bdv2, Bdv3 for barley yellow dwarf resistance, and Wsm1 for wheat streak mosaic resistance, to wheat (Li and Wang, 2009). This crop has also been found to have quantitative trait loci (QTL) for Fusarium head blight and bacterial leaf streak (Bajgain et al., 2019). Therefore, IWG serves as a valuable genetic reservoir of resistance.

Although IWG possesses disease resistant genes that allow it to withstand various fungal and viral diseases (Cox et al., 2005), it is not completely immune to pathogen attacks. A study conducted by (Fulcher et al., 2022) has found four common annual system fungal diseases: ergot, Fusarium head blight, leaf blotch, and tar spot that are associated with IWG which are common annual system pathogens. IWG is susceptible to fungal infections and interspecies transmission of the pathogen between annual and perennial hosts has been identified (Fulcher et al., 2022).

Several fungal pathogens like FHB, ergot, tan spot, leaf blotch, spot blotch, tar spot (Fulcher et al., 2022, Mangel et al., personal communication) and viral pathogens like wheat streak mosaic virus, soil borne wheat mosaic virus and barley yellow dwarf virus have been identified. Growers are concerned about the disease, pest in this crop (Wayman et al., 2019) and they are worried about grain rejection from commercial supply chain because of mycotoxin accumulation by some of these pathogens (R. Magnusson, personal communication). Perennial crops aim to restore ecosystem benefits and reduce the environmental impact of conventional agriculture (Becker et al., 1992). However, managing diseases in these crops is challenging since there are no herbicides or pesticides recommended and crop rotation is not viable (Wayman et al., 2019). The only viable option is to rely on host resistance. So, it is important to develop disease resistant plants to address the disease management issues in this crop.

The genetic makeup of annual wheat and IWG is similar, and they exhibit similar head morphology (Smith et al., 1960). As they both belong to the Triticeae tribe and have similar genetics (Mahelka et al., 2011), it suggests they may share common pathogens. Additionally, Fusarium head blight, which is a significant disease of wheat with economic importance, has already been identified in association with IWG (Turner et al., 2013; Fulcher et al., 2022).

Mycotoxin production

Mycotoxins are toxins produced by fungi as a secondary metabolite (Bennett and Bentley, 1989). The predominant fungal genera that produce these toxic substances are Aspergillus, Penicillium, and Fusarium, which can contaminate grains, fruits, and nuts (Magan et al., 2011). Fusarium species are a common type of pathogenic fungus that infects cereal crops and can produce toxins in the grains (Bottalico, 1998). The Fusarium genus produces a variety of

mycotoxins such as fusaric acids (FA), fumonisins (fumonisin B1), beauvericin (BEA), enniatin (ENN), moniliformin (MON) and trichothecenes (Ismaiel and Papenbrock, 2015). Trichothecenes, Zearalenone and Fumonisins are the most concerning toxic group of Fusarium mycotoxins due to their regulatory status. Trichothecenes can be divided into two types based on their functional group, Type A (T-2, HT-2 toxin, diacetoxyscirpenol (DAS) and monoacetoxyscirpenol (MAS)) and Type B (deoxynivalenol (DON), nivalenol (NIV), 3-acetylDON, 15-acetylDON and fusarenone X (FUS)) (Bottalico, 1998; Ferrigo et al., 2016). T-2, HT-2 toxin, DON, NIV and DAS are among the most poisonous trichothecenes (Nayakwadi et al., 2020). These Mycotoxins have a significant effect on the health of humans and animals, leading to symptoms such as vomiting, feed rejection, and even teratogenic effects particularly in DON (Khera et al., 1986; Magan et al., 2011). Certain mycotoxins like DON, zearalenone and fumonisins are subjected to regulation (FDA, 2001, 2010; Authority (EFSA) et al., 2017). Recommended maximum levels of mycotoxin in food and feed vary between the USA and Europe. In the USA, the recommended maximum DON level is 1ppm for finished wheat products, 10ppm for cattle older than four months and 5ppm for those younger than four months, and less than 10ppm for chickens not exceeding 50% of their diet (FDA, 2010). Meanwhile in Europe, the recommended level for DON is 8ppm in cereal products, 12ppm for maize products, 0.9ppm for pig feeds and 5ppm for other feeds (Regulations for Europe). The recommended maximum levels for fumonisins in the USA are 2ppm for degermed corn products, 4ppm for whole corn and 3ppm for popcorn corn (FDA, 2001), while in Europe, the levels should not exceed 6ppm in maize and maize products and 5ppm in feed (Regulations for Europe). Only European countries have regulations for zearalenone and T-2/HT-2 toxins. The recommended maximum levels of zearalenone are 2ppm in cereal and cereal products, 3ppm in maize products,

0.1ppm in pig feeds and 0.5ppm in cattle feeds, and for T-2/HT-2 toxin, the recommended maximum levels are 2ppm for oat feeds and 0.5ppm for others (Regulations for Europe). Food and feed items contaminated with mycotoxins are not suitable for consumption. The presence of mycotoxin in our food supply poses a significant threat to global food security and food and feed safety.

Fusarium graminearum

Fusarium graminearum, a member of the family Nectriaceae, is a type of filamentous, ascomycetous, homothallic fungus (O'Donnell et al., 2000). This fungus has two different forms: the anamorph, which is known as *F. graminearum* and the teleomorph, which is called *Gibberella zeae* (Goswami and Kistler, 2004). *F. graminearum* is a prevalent fungal pathogen responsible for causing fusarium head blight in wheat, oat, and barley and ear rot in maize (Wang and Miller, 1988; Trail, 2009). The organisms responsible for causing FHB are categorized under the group called the *F. graminearum* species complex (FGSC), which is the subdivision of larger lineage known as the *F. sambucinum* species complex (Valverde-Bogantes et al., 2020). The FGSC includes the following species: *F. acaciae-mearnsii, F. aethiopicum, F. asiaticum, F. austroamericanum, F. boothii, F. brasilicum, F. cortaderiae, F. gerlachii, F. graminearum, F. louisianense, F. meridionale, F. mesoamericanum, F. nepalense, F. ussurianum,* and *F. vorosii* (Valverde-Bogantes et al., 2020). *F. graminearum* is the most common species found in the United States causing FHB (Xu et al., 2008). This fungus produces a dense mycelium of varying color, forms a red pigment in the culture and produces mostly uniform 5-6 septate macroconidia from sporodochia (Leslie and Summerell, 2006a). The macroconidia are thick-walled and moderately curved to straight, with a straight ventral surface and smoothly arched dorsal side, as described by Leslie and Summerell (2006).

F. graminearum overwinters on infested crop residues (Xu and Nicholson, 2009). It produces asexual spores (macroconidia) there, which get dispersed to plants by rain splash or wind (Schamale III and D.G. G.C. Bergstorm, 2003). The fungus develops its sexual stage (*Gibberella zeae*) in warm, humid and wet weather on infested plant debris, and forms bluish black perithecia that release ascospores (sexual spores) into the air to be carried over 2.8-8.5mm distance horizontally (Fernando et al., 1997; Schamale III and D.G. G.C. Bergstorm, 2003). During anthesis, the fungus infects the susceptible wheat head through the anthers, resulting in bleached symptoms in one or more spikelets with pink or salmon color in some glumes and later produces diseased kernels that appear shriveled and wilted, commonly referred to as tombstone (Aaron G. (Aaron Guy) Johnson 1880- and James G. (James Geere) Dickson 1891-, 1921). Additionally, there will be production of highly regulated mycotoxin DON (Deoxynivalenol) on the infected grains. Apart from DON, this fungus also produces zearalenone, nivalenol, fusarenone-x, which are of great concern in cereal grains (Bottalico, 1998).

Fusarium incarnatum-equiseti species complex

The *Fusarium incarnatum-equiseti* species complex (FIESC) is a group of saprophytic fungi that do not cause disease symptoms in the infected plants. However, they are capable of depositing toxins in the grains (Lu et al., 2021), including trichothecenes, fusaric acid and zearalenone (Matic et al., 2020). Despite being closely related *Fusarium* species and consisting of cryptic speciation, FIESC cannot be differentiated into separate species (Castellá and Cabañes, 2014). While FIESC has been reported to cause fusarium head blight in wheat, ear rot of foxtail millet and leaf diseases in leafy vegetables (Zheng et al., 2019; Matic et al., 2020; Leyva-Mir et al., 2022), it was not previously known to cause significant plant diseases (Villani et al., 2016). FIESC is composed of two important clades: the *Equiseti* clade and the *Incarnatum* clade. The

Equiseti clade includes 19 phylogenetic species (FIESC 1 to 14, FIESC 30, FIESC 31, FIESC 33, FIESC 34 and FIESC 35), while the *Incarnatum* clade has 18 phylogenetic species (FIESC 15 to 29, FIESC 32, FIESC 36, FIESC 37, FIESC 38) (Avila et al., 2019; Lu et al., 2021). Three phylogenetic species in the *Equiseti* clade have been given Latin binomials name: *F. equiseti* (FIESC 14), *F. lacertarum* (FIESC 4), *F. scirpi* (FIESC 9) (O'Donnell, 2009; Villani et al., 2016) by using multilocus sequencing technology through genealogical concordance of phylogenetic species recognition (GCPSR) (O'Donnell, 2009). Given that FIESC fungi have been found to compromise kernel grains with mycotoxin deposition, their presence should be carefully surveyed and studied.

Fusarium verticillioides

Fusarium verticillioides, previously referred to as *F. moniliforme*, is a member of *F. fujikuroi* species complex (FFSC) (Blacutt et al., 2018a). It is a filamentous fungus that belongs to the family Nectriaceae (Schoch et al., 2020). This heterothallic fungus is primarily an endophytic pathogen of maize, which leads to the development of ear and stalk rot (Kant et al., 2017). It is a significant fungal pathogen that also poses a threat to wheat, rice and sorghum (Blacutt et al., 2018b). The teleomorph of this fungus is known as *Gibberellla fujikuroi*, mating population A (Kant et al., 2017). The fungus displays diverse pigmentation in agar, ranging from no pigmentation or grayish orange to violet gray, dark violet or dark magenta, and some strains also produce blue-black sclerotia, while its macroconidia are slim and slightly curved or straight with thin walls and 3-5 septations, and its microconidia arise from monophialides that are oval to club-shaped with a flattened base and no septation (Leslie and Summerell, 2006b).

Through seeds or infection of wounds and silk, the fungus can enter the plant's system and spread throughout (Leslie and Summerell, 2006b). Seeds that germinate in *Fusarium*

infested soil may exhibit either symptomatic infection resulting in rot and blight or an endophytic infection that remains asymptomatic (Blacutt et al., 2018a). *Fusarium verticillioides* can infect maize stalks and cause rot when they are wounded by mechanical damage or insect feeding (Blacutt et al., 2018a). During the silking stage, the fungus can colonize maize kernels by passing through the stylar canal, resulting in a starburst pattern on the kernels (Duncan and Howard, 2010). The feeding activities of the larvae of European corn borer moth on maize leaves, stalk, ear and collar tissues create an opportunity for *F. verticillioides* to infect these tissues, causing ear and stalk (Sobek and Munkvold, 1999). Even after harvest, the fungus can survive and produce windborne microconidia, which serves as a source of inoculum for subsequent infections (Blacutt et al., 2018a). Maize growers are seriously affected by Fusarium ear rot and fumonisin contamination (Bush et al., 2004). 95% of the diagnosed mycotoxicosis in midwestern United States is due to fumonisins (Haschek and Voss, 2013). Fumonisin B1 (FB1) is commonly isolated mycotoxin from the contaminated grains that causes disease to pig and horses and suspected to be a human carcinogen (Gelderblom et al., 1988). Changing climatic conditions can cause an increase in fumonisin accumulation, which can result in severe outbreaks of fumonisin related diseases in both animals and humans (Haschek and Voss, 2013).

Fusarium armeniacum

Fusarium armeniacum was formerly known as *Fusarium acuminatum* ssp. *armeniacum* (Leslie and Summerell, 2006b). It has similar macroconidial morphology like *Fusarium acuminatum* and was classified as a sub species of *F. acuminatum* (Burgess et al., 1993). Macroconidia have a distinct foot-shaped base and are long, tapered, curved and commonly 5-septate (Leslie and Summerell, 2006b). *F. armeniacum* is characterized by a more pronounced foot-shaped base that is not prominent in typical *F. acuminatum* (Leslie and Summerell, 2006b).

Later based on chromosomal karyotype differences, it was designated as a separate species and was found to be more similar to *F. sporotrichioides* (Nagy and Hornok, 1994). In the past, *F. armeniacum* was classified as saprophytes, but more recently, it has been identified as a pathogen that causes root rot and seed rot in soybean (Ellis et al., 2012), as well as Fusarium head blight in wheat (Fulcher and Bergstrom, 2020; Krone et al., 2021). *Fusarium armeniacum* strains contain Tri5 gene which codes for trichodiene synthase, producing a precursor for trichothecenes production (Fekete et al., 1997). It is highly toxigenic and produces type A trichothecenes, especially T-2, HT-2 and neosolaniol (Nichea et al., 2015).

T-2 toxin is a potent mycotoxin that can cause toxicities in humans and animals (Janik et al., 2021). In animals, it can cause feed refusal, vomiting, clotting disorders and reproductive problems (Haschek and Voss, 2013). Although T-2 toxin outbreaks are rare in the United States, it has caused outbreak of alimentary toxic aleukia (ATA) in humans in Russia (McLean, 1996). HT-2 toxin comes together with T-2 toxin and is considered precursor of T-2. Neosolaniol is another mycotoxin produced by this species whose toxicity is not known.

Fusarium acuminatum

Fusarium acuminatum, a fungal species, which prefers cooler temperatures has a global distribution and is commonly found in regions with temperate climates (Marín et al., 2012). This species has also been referred to by several other names such as *F. acuminatum* ssp. *acuminatum*, *F. scirpi* var. *acuminatum*, *F. scirpi* ssp. *acuminatum*, *F. gibbosum* var. *acuminatum* (Leslie and Summerell, 2006b). It is morphologically similar to *Fusarium avenaceum* and can be distinguished from the shape of macroconidia which are large, curved with thick walls, 5-septate and possesses a distinct foot cell (Leslie and Summerell, 2006b). *F. acuminatum* produces trichothecenes, which includes diacetoxyscirpenol (DAS),

monoacetoxyscirpenol (MAS), neosolaniol (NEO), T-2 and HT-2 toxin (Logrieco et al., 1992; Adejumo et al., 2007). It is commonly a soil saprophyte (Wing et al., 1993) but reported to cause root and crown disease of wheat in the USA (Hill et al., 1983) as well as infects fruits (Visconti et al., 1989; Logrieco et al., 1992).

Fusarium sporotrichioides

Fusarium sporotrichioides was formally known as *F. tricinctum, F. sporotrichiella* var. *sporotrichioides*. Macroconidia are mostly 3-septate, falcate to almost lunate with a curved and tapered apex and a basal cell that is not distinctly foot-shaped or notched (Leslie and Summerell, 2006b). During the late wet harvest, cereal crops frequently come into contact with *F. sporotrichioides*, which has low pathogenicity to these crops (Leslie and Summerell, 2006b). However, this fungus is capable of producing the highly concerning toxigenic mycotoxin T-2 and diacetoxyscirpenol, which causes alimentary toxic aleukia in humans (Visconti et al., 1989). As a result of contaminated grains, they are rejected in the commercial human food chain and are often fed to the animals resulting in significant mycotoxicosis issues in animals (Abramson, 1999).

Fusarium lacertarum

Fusarium lacertarum belongs to the *Fusarium incarnatum-equiseti* species complex (O'Donnell, 2009), which can cause FHB disease complex (Beacorn and Thiessen, 2021). In particular, *F. lacertarum* causes FHB on sorghum, resulting in red lesions on the infected panicle and necrosis (Beacorn and Thiessen, 2021) and damping-off in *Casuarina equisetifolia* (Favaretto et al., 2018). They also cause rot symptoms in plants (Santiago et al., 2018). In culture, they produce white mycelium with orange-brown to red pigmentation (Beacorn and Thiessen, 2021), and their macroconidia are elongated with foot-shaped basal cells and a hooked

apex. Although mycotoxins produced by *F. lacertarum* have not been fully identified, they have the potential to produce toxigenic mycotoxins like trichothecenes (Adejumo et al., 2007; Lincy et al., 2011). The chararacterized FHB mycotoxins are trichothecenes such as deoxynivalenol, nivalenol, and their acetyl derivaties, and zearalenones (Jang et al., 2019).

Fusarium asiaticum

Fusarium asiaticum is a pathogen responsible for causing FHB in wheat, rice (Lee et al., 2010; Xu et al., 2021), and it belongs to *Fusarium graminearum* species complex. This fungal species is commonly found in Asia (Suga et al., 2008). Despite sharing similar physical characteristics, *F. graminearum* and *F. asiaticum* can be differentiated based on the type of mycotoxin they produce (Jang et al., 2019). *F. asiaticum* produces nivalenol, a mycotoxin that is more toxic than deoxynivalenol (Ryu et al., 1988) produced by *F. graminearum* with daily intake limit of nivalenol stricter than that of deoxynivalenol (Schothorst and van Egmond, 2004; Jang et al., 2019).

Fusarium boothii

Fusarium boothii is a member of FGSC (Lee et al., 2016) and infects grain crops mainly maize, wheat and barley (Gryzenhout et al., 2016). This fungus is designated in lineage 3 of *F. graminearum* clade (Xu and Nicholson, 2009) and is typically found in regions with warm temperatures, high precipitation and drier flowering periods (Backhouse, 2014). It produces 15-ADON trichothecene chemotype (Wegulo et al., 2018; Valverde-Bogantes et al., 2020) which is a harmful mycotoxin. This fungus is known to cause Gibberella ear rot (GER) in maize and infects barley (Boutigny et al., 2011). It is specifically adapted to infecting maize and can thrive in areas with low humidity (Backhouse, 2014). Despite its prevalence in crops, there is still limited information available about this fungus.

Rationale for study

Comprehensive knowledge about diseases and their associated pathogens is essential for successful crop establishment and commercialization, particularly for the crop that is still in its developmental stage. However, for IWG crop development there is limited understanding of potential diseases and pests, which pose a significant challenge in terms of yield. *Fusarium* species known to cause widespread fungal diseases such as Fusarium head blight, produce mycotoxins that are regulated in food and feed. The presence of mycotoxins could lead to grain rejection and hinder growers, ultimately affecting the crop's commercialization.

IWG is grown alongside other annual crops, it could serve as a reservoir for pathogens that migrate to those crops. Therefore, understanding the range of pathogens that may be harbored by IWG is critical for developing effective disease management practices. This study aims to understand the *Fusarium* species that colonize IWG heads and produce mycotoxins that can compromise the quality of the grain. The findings of this study will help develop strategies to ensure food and feed safety while commercializing IWG.

This study aimed to investigate fungi and fungal pathogens present in head samples of IWG. The samples were obtained from growers and isolated in the Applied Wheat Pathology Laboratory at Kansas State University for culture. Additionally, toxin measurement was carried out at the Land Institute of Salina to compare the pathogen identified with the toxin produced.

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Chapter 2 - Characterization of *Fusarium* species from the collected head samples

Abstract

Kernza® is a type of grain harvested from intermediate wheatgrass (IWG) (Thinopyrum intermedium), a wild relative of wheat and grown commercially primarily in Montana, Kansas, Minnesota and North Dakota. Wheat is the third most important field crop in the United States, following corn and soybean, but it is highly susceptible to Fusarium head blight (FHB), a severe disease that affects wheat crops in all growing regions causing 18,101 million bushels yield loss (Friskop et al., 2022). As IWG is a wheat relative, it could potentially serve as a host for FHB. Also, planting IWG in close proximity or into the stubble of susceptible wheat varieties could increase the disease in this more resistant crop. The objective of this project was to identify fungi and fungal pathogens associated with IWG, and to confirm the presence of *Fusarium graminearum*. To identify these pathogens, head samples were contributed from on-farm and research trial locations. Spikelets were used to isolate the fungi using morphological methods. Later, single-spored fungal cultures were obtained, and DNA was extracted. Sequencing with ITS1 and ITS4 primers, 57 of the 79 samples were confirmed to belong to the *Fusarium* genus. Ef1 and Ef2 primers were then used to sequence *Fusarium* confirmed samples. Our analysis identified the presence of multiple *Fusarium* species including, *F. graminearum, F. incarnatum-equiseti* species complex, *F. armeniacum, F. verticillioides, F. asiaticum, F. boothii, F. sporotrichioides* and *F. lacertarum*. The detection and characterization of *Fusarium* species present in IWG can help in the development of disease-resistant varieties, improved management of FHB, and mycotoxin contamination that pose a threat to food and feed safety. The outcomes

of this research have significant implication for the crop's increased adoption and commercial viability.

Introduction

Perennial crops are a vital component for sustainable agriculture and play an important role in maintaining soil health. With their extensive root systems, perennial crops like intermediate wheatgrass help to stabilize the soil, provide year-round ground cover, sequester carbon and reduce farm inputs (Wayman et al., 2019). As they do not need to be replanted each year, they are a more efficient and cost-effective choice than annual crops (Becker et al., 1992; DeHaan et al., 2013). Perennials are better equipped to withstand disease and pests because of their tolerance and adaptation to the environment, leading to a robust food supply to address global food security (Glover et al., 2007). When comparing to annual crops, the perennial system has the potential to be more favorable, provided that it can produce equivalent yields and grain size (Zhang et al., 2011).

There exists a multitude of perennial species in nature that have been utilized to create annual species. The available annual crop species are the result of natural hybridization and crosses of perennials (Wagoner and Schaeffer, 1990). Intermediate wheatgrass (*Thinopyrum intermedium* (Host) Barkworth and Dewey) is a perennial grass species that originates from Eastern Europe and the Mediterranean region. This grass species holds a great potential for being developed into perennial grain crop, due to its advantageous features such as large seed size, moderate spike fragility, and good threshability, along with a high level of biomass and remarkable fodder quality (Wagoner and Schaeffer, 1990). Today, the grain produced from the domesticated intermediate wheatgrass is marketed under the brand name Kernza® (DeHaan and Ismail, 2017). At present, IWG is grown by 150 farmers on 3,951 acres of land (TLI, 2023).

Perennial crops remain in the field for multiple years and can potentially harbor diseases over an extended period. The majority of plant diseases are caused by plant pathogenic fungi (Arie, 2019). The *Fusarium* genus is a well-known group of fungi that causes multiple plant diseases, such as rots, cankers, wilts and leaf diseases (Leslie and Summerell, 2006a). In addition, *Fusarium* species are notorious for producing mycotoxins such as trichothecenes (deoxynivalenol (DON), nivalenol (NIV), T-2), zearalenone, fusaric acids and fumonisins (Ismaiel and Papenbrock, 2015). Fusarium Head Blight (FHB) is the most devastating plant disease in wheat crops and produces trichothecene mycotoxin deoxynivalenol in the grains (Wood et al., 1999; Turner et al., 2013).

Intermediate Wheatgrass (IWG) has been recognized and evaluated for the typeII FHB resistance which limits the spread of infection from the initial inoculated point to other spikelets on the spikes (Han et al., 2003; Turner et al., 2013). A recent study conducted in New York found *Fusarium graminearum* in IWG samples, and the presence of DON was also reported in grain samples (Fulcher et al., 2022). As traditional disease management practices for annual crops are not available for organic perennial cropping systems (Cox et al., 2005), identifying specific disease and pests is crucial for effective disease management. Therefore, understanding the fungi and fungal pathogens associated with IWG will help in developing and implementing disease management practices in a timely manner to increase crop yield and reduce mycotoxin contamination in the grain. Given the limited information on IWG diseases, this study aimed to examine fungal pathogens in head and leaf samples, determine the diversity of *Fusarium* species in the heads, and detect potential mycotoxins to ensure food and feed safety.

Methods

IWG head samples

A total of 79 head samples were collected from producer fields and research sites from Kansas, North Dakota, Minnesota, and Montana in 2021 and 2022. The dried matured head samples were maintained in paper bags at room temperature until sample processing in the Applied Wheat Pathology Laboratory at Kansas State University.

Sample isolation and pure culture

Fungi were isolated from the head samples of IWG collected from growers from 12 counties in four states of the United States (Fig 2.1, 2.2, 2.3, 2.4). The individual spikelets of IWG were separated from the head. 20 spikelets with disease symptoms were chosen from each sample for sterilization. The sterilization was performed under a laminar flow hood (Contamination Control Inc., Lansdale, Penna, 19446) with 10% bleach solution. Selected spikelets were first placed in 10% bleach solution using sterile forceps and allowed to soak for 5 min. Spikelets were then transferred to a plate containing sterile distilled water for 5 min to rinse residual bleach from the samples. Then, the sterilized samples were transferred to Spezieller Nahrstoffarmer Agar (SNA) (0.5g potassium dihydrogen phosphate, 0.5g potassium nitrate, 0.25g magnesium sulfate heptahydrate, 0.25g potassium chloride, 0.1g glucose, 0.1 g sucrose, 10g agar, 500ml water) media plate. Five replications for each sample, each containing four sterilized spikelets were grown in the growth chamber (Powers Scientific, Inc, Pipersville, PA) for 4-5 days. The growth chamber was on a 20°C/19°C day/night regime, with a supplemental light regime of 16hr days using L9T8SE240-G 4000K lights (MaxLite, West Caldwell, NJ). Distinct fungal colonies were sub-cultured by cutting a 10mm × 10mm plug from the leading edge of growth and transferred to a new SNA plate to obtain a pure culture. Two subcultures were prepared to obtain a pure culture

of the fungus for all samples. One plate was selected as a representative plate for the sample from among the replications after visual observation.

Single spore

To produce a single spore, mung bean broth was prepared in the lab. One liter(L) of distilled water was boiled in a cylindrical flask by placing the flask over a Bunsen burner (Fisher burner, merek type) in a tripod iron ring stand (Fisher Stand, 12 × 20 cm). 20 gm of mung beans were poured into 1L boiling water in the cylindrical flask and boiled for 5 min. The flame was turned off and the flask was transferred into a hot plate stirrer (Benchmark, Hotplate Stirrer) and let cool for 2 min. After that, the clear broth was collected in a 1000ml graduated cylinder by sieving out the mung beans with cheesecloth. The final volume was adjusted to 1L by adding distilled water. 75 ml of broth was poured into each of six (250 ml) flasks. These flasks were autoclaved (liquid cycle, 121°C for 20 min) and cooled at room temperature for 10-12 hours. 4 small plugs (5mm × 5mm) of mycelium were cut from the sample representative subculture plate with sterilized forceps and dropped into the labeled mung bean broth flask for each sample. The flask was put on the shaker table with lights on for 14 hours per day. After 10 days, 5 µl of the sample was pipetted onto the glass slide, covered with a covered slip, and observed under the compound microscope (Carl Zeiss Microscopy GmbH, Jena, Germany) for conidia at 100× magnification. Samples with conidia were diluted to 1000 conidia/ml by counting in the hemocytometer (Phase Counting Chamber Hausser Scientific, Horsham, PA) and cultured in the 2% water agar (20g agar, 1000ml water) for single spore isolation. After letting them grow for 12 hours on water agar plates, plates were observed under the dissecting microscope (Carl Zeiss Microscopy GmbH, 37081 Gottingen) to observe the germinated single spore. The location of the single spore was marked outside the plate and the plug (1mm × 1 mm) with the single spore was cut

under the laminar flow hood. The plug was transferred to SNA media and incubated for 4-5 days inside the growth chamber to produce single-spored fungal mycelium.

DNA Extraction

The single-spored fungal mycelium was used for the DNA extraction of samples producing conidia and pure culture was used for the DNA extraction of samples that failed to produce conidia. Cetyltrimethylammonium bromide (CTAB) extraction protocol (Murray, M. G., & Thompson, W. F. (1980)) was used for the DNA extraction of the samples with some modifications. 70-80mg of fungal tissue was scraped and placed in a 1.5ml tube with 4.5mm galvanized steel pellets (Daisy Outdoor Products, Rogers, AR) and 100µl of CTAB to put in the bead mill for 1 minute.14µl of 2-mercaptoethanol and 600µl of CTAB were added after tissue disruption. The mixture was incubated at 60°C for 30 min followed by 15 min of ice cooling on ice. 500µl of 24:1 chloroform: isoamyl alcohol was then added and samples were thoroughly mixed. Samples were placed in a centrifuge (Eppendorf Centrifuge,Germany) for 5 minutes at 5000rpm. The upper aqueous phase was transferred to a clean 1.5ml tube and DNA was precipitated using 0.8 volumes of 2-propanol. Samples were centrifuged for 5 min at 5000rpm to pellet the DNA. The supernatant was discarded, and the pellet was washed twice in 70% ethanol. After briefly drying the pellet, it was resuspended in 100µl of high-performance liquid chromatography (HPLC) water. The obtained clean DNA sample was stored in a 4°C freezer before measuring the concentration in the nanophotometer (Implen C40, Munich, Germany). After the measurement, the DNA was diluted to 20ng/µl using HPLC water for polymerase chain reaction (PCR).

Molecular characterization

The ribosomal internal transcribed spacer (ITS) region was amplified using ITS1(TCCGTAGGTGAACCTGCGG) and ITS4 (TCCTCCGCTTATTGATATGC) primers

(White, T.J., T. Bruns, S. Lee, and J.W. Taylor. 1990). by performing polymerase chain reaction (PCR) of 25µl reactions were prepared using 9.5µl of HPLC water, 12.5µl of supermix (GoTaq® Green Master Mix, 2X), 2µl of ITS1 and ITS4 (10nm) primers and 1µl of fungal DNA template (20ng). Samples were loaded into a thermocycler (Labnet, Multigene Optimax, Edison, NJ) with the following conditions: 95°C for 6 min and then 35 cycles of 95°C for 30 sec, 60°C for 40 sec, 72°C for 40 sec and a final 72°C extension for 5 min. The amplified DNA samples were sent for sanger sequencing (MCLAB, South San Francisco, CA) for sequence generation using ITS1 and ITS4 as sequencing primers. Results obtained were processed through pregap4 and gap4 Staden packages (version: 2.0.0b11-2016) to generate a consensus sequence which was compared by BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) with sequence in National Center for Biotechnology Information (NCBI) database.

Fusarium-specific primers M13F(-20)-EF-1(GTAAAACGACGGCCAGATGGGTAAGGARGACAAGAC) and M13R-EF-2 (CAGGAAACAGCTATGACGGARGTACCAGTSATCATGTT) with M13 tail was used for samples that were confirmed Fusarium with ITS primers to amplify translation elongation factor region (TEF1α) for verifying species (O'Donnell, Kerry, H. Corby Kistler, Elizabeth Cigelnik, and Randy C. Ploetz. 1998). These primers underwent PCR of 25µl reactions in the thermocycler (Labnet, Multigene Optimax, Edison, NJ) with the following conditions: 94°C for 5 min and then 35 cycles of 94°C for 30 sec, 49°C for 45 sec, 72°C for 45 sec and a final 72°C extension for 5 min and sent for sanger sequencing using M13F (-20) and M13R as sequencing primers. A consensus sequence was generated using the pregap4 and gap4 Staden packages (version:2.0.0b11-2016) and compared to Fusarium Multilocus Sequence Typing (MLST) database (https://fusarium.mycobank.org/) for species identification.

Results

Fungal Distribution

Nine different groups of fungi, namely, *Fusarium*, *Alternaria*, *Bipolaris*, *Rhizopus*, *Talaromyces*, *Epicoccum*, *Phlebiopsis*, "uncultured fungus" and "fungal sp." from the head samples were isolated. ITS1 and ITS4 primers (Table 2.1) were used as the sequencing primers for the genus differentiation in the beginning. Among the seventy-nine sequenced samples, fifty-five were confirmed to be *Fusarium* spp., seven were *Alternaria*, one was *Bipolaris*, three were *Rhizopus*, one was *Talaromyces*, four were *Epicoccum*, one was *Phlebiopsis*, five were uncultured fungus and two were listed as "fungal sp." (Fig 2.5) when sequences were compared to NCBI database. Further confirmation of *Fusarium* spp. and "fungal sp." were carried out by amplifying the Tef-1α gene using Ef1 and Ef2 primers (Table 2.1), which allowed us to confirm ten different species of *Fusarium* among the fifty-five *Fusarium* confirmed samples and two "fungal sp." samples (Table 2.3, 2.4, 2.5, 2.6). The sequences were compared to Mycobank database (https://fusarium.mycobank.org/).

Unfortunately, one sample (18CKKS030) from the *Fusarium* confirmed samples did not generate the consensus sequence, which might be because of the poor DNA quality and was not included in the list of *Fusarium* species confirmed (Table 2.3). The most abundant species was *F. graminearum* (n=21, 37.5%) followed by *F. incarnatum-equiseti* species complex (n=17, 30.35%), *F. armeniacum* (n=5, 8.92%), *F. verticillioides* (n=4, 7.14%), *Fusarium sp.* (n=3, 5.35%), *F. acuminatum* (n=2, 3.57%), *F. boothii* (n=1, 1.78%), *F. lacertarum* (n=1, 1.78%), *F. asiaticum* (n=1, 1.78%) and *F. sporotrichioides* (n=1, 1.78%) (Fig 2.6).

Among the 44 samples from Kansas (Table 2.3), the most dominating species isolated was *F. graminearum* (n=15, 34.09%), followed by *F. incarnatum-equiseti* species complex (n=14,

31.81%), *F. armeniacum* (n=5, 11.36%), *F. verticillioides*(n=4, 9.09%), *Fusarium* sp.(n=2, 4.54%), *F. asiaticum*(n=1, 2.27%), *F. boothii*(n=1, 2.27%), *F. acuminatum*(n=1, 2.27%) and *F. lacertarum*(n=1, 2.27%).

In addition, six samples were received from Minnesota (Table 2.5) and most commom *Fusarium spp.* isolated was *F. incarnatum-equiseti* species complex (n=3, 50%), *F. graminearum*(n=1, 16.66%), *F. sporotrichioides*(n=1, 16.66%) and *Fusarium* sp.(n=1, 16.66%) Likewise, all five samples received from North Dakota (Table 2.4) contained *F. graminearum* and one sample from Montana (Table 2.6) harbored *F. acuminatum*.

Similarly, leaf samples from Olathe nursery in Kansas were analyzed using nanopore sequencing and DNA sequencing (N. Ranabhat, Personal communication, D. Mangel, Personal communication) which confirmed the presence of wheat streak mosaic virus, soil borne wheat mosaic virus, barley yellow dwarf virus, *Pyrenophora tritici-repentis*, *Bipolaris sorokiniana* and *Parastagonospora nodorum.*

Mycotoxin presence

The Land institute of Salina conducted mycotoxin testing using quantitative lateral flow test kits from Prognosis Biotech, USA. A total of 18 samples were sent to North Dakota State University for testing (Table 2.7). The analysis identified various mycotoxins such as DON, fumonisin, T-2/HT-2 and zearalenone, which were produced by Fusarium spp. isolated from the samples. The concentration of mycotoxins detected in each tested sample is presented in table 2.7 (K. Turner, *Personal communication*).

Discussion

IWG is in the early phases of development and is a potential crop for sustainable agriculture. Research on this emerging crop is primarily focused on variety development and

quantifying ecosystem services (Law et al., 2022). The presence of ergot, Fusarium head blight, leaf blotch by *Parastagonospora nodorum*, and tar spot by *Phyllachora graminis* has already been reported based on the presence of fruiting bodies and DNA sequencing (Fulcher et al., 2022). One of the main concerns is Fusarium head blight because it reduces grain yield (Treffer in preparation for publication) and produces toxin that can reduce the value of the grain. Farmers potentially could face grain rejection from the commercial supply chain because *Fusarium* species produce mycotoxins. Some of these mycotoxins like DON, fumonisins, zearalenone, T-2/HT-2 toxins are regulated in food and feed (van Egmond et al., 2007).

Plants commonly exhibit multiple diseases associated with *Fusarium* (Leslie and Summerell, 2006b). The result of this study found abundance of *Fusarium* species in the head samples, while other fungi such as *Alternaria*, *Rhizopus*, *Bipolaris*, *Epicoccum*, *Talaromyces*, *Phlebiopsis* were also found in lower amounts. *Fusarium* was confirmed in 72% of the total samples. Among the *Fusarium* spp. identified, there were *F. acuminatum*, *F. armeniacum*, *F. asiaticum*, *F. boothii*, *F. graminearum*, *F. incarnatum-equiseti* species complex, *F. lacertarum*, *F. sporotrichioides*, *Fusarium* sp., and *F. verticillioides*.

The causal agents for FHB are grouped within the *Fusarium graminearum* species complex (FGSC) which is part of the broad *Fusarium sambucinum* species complex lineage (Valverde-Bogantes et al., 2020). The FGSC includes the following species: *F. acaciae-mearnsii*, *F. aethiopicum*, *F. asiaticum*, *F. austroamericanum*, *F. boothii*, *F. brasilicum*, *F. cortaderiae*, *F. gerlachii*, *F. graminearum*, *F. louisianense*, *F. meridionale*, *F. mesoamericanum*, *F. nepalense*, *F. ussurianum*, and *F. vorosii* (Valverde-Bogantes et al., 2020). Our findings identified three species from FGSC: *F. asiaticum*, *F. boothii*, *F. graminearum*. *Fusarium*

graminearum is the predominant pathogen to cause Fusarium head blight in cereals in the United States (Xu et al., 2008).

Fusarium incarnatum-equiseti species complex (FIESC) are reported to cause Fusarium head blight in wheat, ear rot of foxtail millet and leaf diseases in leafy vegetables (Zheng et al., 2019; Matic et al., 2020a; Leyva-Mir et al., 2022). *Fusarium lacertarum* is the member of FIESC, which has been resolved by the multilocus sequencing technology of EF-1α gene, ITS+LSU 28s rRNA, RPB2 and CAM (O'Donnell, 2009) through genealogical concordance of phylogenetic species recognition (GCPSR) while there are other species in the complex which are still cryptic. *Fusarium verticillioides* is a member of *Fusarium fujikuroi* species complex (FFSC) (Qiu et al., 2020) and has been found to cause ear rot and blight in maize (Blacutt et al., 2018). *F. lacertarum* and *F. armeniacum* are reported to cause FHB symptoms on susceptible wheat and sorghum heads (Wegulo et al., 2018; Fulcher and Bergstrom, 2020; Beacorn and Thiessen, 2021; Krone et al., 2021). *F. acuminatum* causes root and crown diseases in wheat (Hill et al., 1983). *F. sporotrichioides* causes ear rot in maize (Wang et al., 2020), root rot in soybean (Abdelmagid et al., 2021) and stem wilt in sea buckthorn (Xia et al., 2021). Three samples were identified as *Fusarium* sp. Two of the samples had accession number MH582442, while other sample had the accession number GQ505596 (Table 2.4, 2.6). According to existing literature, these accession numbers correspond to *F. nanum* (O'Donnell, 2009; O'Donnell et al., 2018). *F. nanum* has been reported to cause fruit rot in muskmelon (Zhang et al., 2023) and root rot in soybean (Okello et al., 2020).

Along with causing disease in plants, these *Fusarium* spp. also deposit toxin in the grain as a secondary metabolite (Bottalico, 1998). Mycotoxin accumulation will be higher in severely diseased crops and vice-versa (Spanic et al., 2019). Thus, it is a measure for aggressiveness of

the pathogen (Spanic et al., 2019). In several non-host crops as well, mycotoxin has been a virulence factor for *Fusarium* spp. (Harris et al., 1999). *Fusarium graminearum* produces zearalenone and type B trichothecenes (DON, NIV, 3-ADON, 15-ADON (Ferrigo et al., 2016; Chen et al., 2019; Laraba et al., 2021). *Fusarium incarnatum-equiseti* species complex isolates produce trichothecenes, zearalenone, apicidin, beauvericin, butanolide, enniatins, equisetin, fusarochromanone and fusaric acid (Krogh et al., 1989; Phillips et al., 1989; Haese et al., 1993; Singh et al., 1996; Logrieco et al., 1998; Hestbjerg et al., 2002; O'Donnell et al., 2018; Avila et al., 2019; Villani et al., 2019; Matic et al., 2020b). *Fusarium verticillioides* produce fumonisin. *Fusarium armeniacum* produces neosolaniol, T-2 and HT-2 toxin (Fekete et al., 1997; Nichea et al., 2015). *Fusarium acuminatum* produces DAS, MAS, neosolaniol, T-2 and HT-2 toxin (Logrieco et al., 1992; Adejumo et al., 2007). *Fusarium sporotrichioides* produce T-2 and DAS (Visconti et al., 1989). *Fusarium lacertarum* produces deoxynivalenol, nivalenol, and their acetyl derivatives, and zearalenones (Adejumo et al., 2007; Jang et al., 2019). *Fusarium asiaticum* produces nivalenol (Ryu et al., 1988). *Fusarium boothii* produces 15-ADON (Wegulo et al., 2018; Valverde-Bogantes et al., 2020).

The mycotoxin testing done from The Land Institute, Kansas in some representative samples of the study revealed the presence of DON, fumonisin, T-2/HT-2 toxin, and zearalenone (K.Turner, *Personal communication*) (Table 2.7). These identified mycotoxins are harmful for human and animal health. DON is immunotoxic, infects the upper respiratory tract in children, causes vomiting, and feed refusal in animals (Murphy et al., 2006). In plants, DON slows down the root growth, prevents the growth of young plants and their ability to regenerate, and also hinders the production of kernel storage proteins (Miller, 1989; Rocha et al., 2005).

Fumonisin is a potential carcinogen for human health (Haschek and Voss, 2013). It can cause esophageal cancer, kidney damage, liver damage and neural tube defects in infants as well as it causes leukoencephalomalacia (necrotic lesions in cerebrum) in horses and pulmonary edema in swine (Murphy et al., 2006). It affects seedling emergence, inhibits root elongation and causes necrosis and wilting in plants (Abbas and Boyette, 1992; Doehlert et al., 1994).

T-2 toxin is the most poisonous mycotoxin which can be absorbed directly through skin and acts as an immunosuppressant. It slows down the production of protein and nucleic acid in the body, weakens immune function in human and causes alimentary toxic aleukia (ALT), affects pigs, horses, poultry causing intestinal hemorrhages and death (Adhikari et al., 2017). In addition, it inhibits root and shoot growth in plants, reduces the chlorophyll content and cell division (Wakulinski, 1989; Vesonder et al., 1992; Rahman et al., 1993).

Zearalenones are estrogenic in nature and is suspected to reduce the fertility in males in both human and animals and could potentially increase number of human breast cancer cells (Ekwomadu et al., 2021). Also, zearalenone and its metabolites have the ability to bind to plant starch, which can be broken down by α-amylase in the saliva and small intestine of both human and animals upon consumption (Zill et al., 1990; McLean, 1995).

It may not be economical to test for every mycotoxin, but it is necessary to have knowledge of mycotoxins produced by isolated *Fusarium* spp. from the head samples and conduct tests for the regulated ones. This will be very useful in ensuring that the grains produced meet regulatory requirements, protect farmers from grain rejection in the commercial supply chains for food and feed safety. Growers and researchers will be more cautious to implement disease management strategies and post-harvest practices to decrease the risk of mycotoxin contamination.

The study was helpful to get valuable information about head pathogens, and leaf pathogens of IWG. It provided a comprehensive understanding of mycotoxins and their hazardous impact on both human and animal health. In the future, research could be focused on collecting samples from various states and utilizing DNA sequencing and mycotoxin testing to verify the presence of pathogens. Multi locus sequencing with various available primers can be done, along with a deeper examination of the *equiseti-incarnatum* species complex to distinguish them from one another. The identification of other pathogens such as bacteria and viruses, and their localization within the crop should also be investigated. Gene expression analysis in healthy and diseased crops can be done and it will be valuable for molecular work. Root pathogens such as *Rhizoctonia* and *Pythium* are common problems in no-till systems (Cox et al., 2005). Given that root system plays a critical role in the success of perennial agricultural system, studying root pathogens is also essential.

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Figure 2.1. Head samples collected from counties in Kansas.

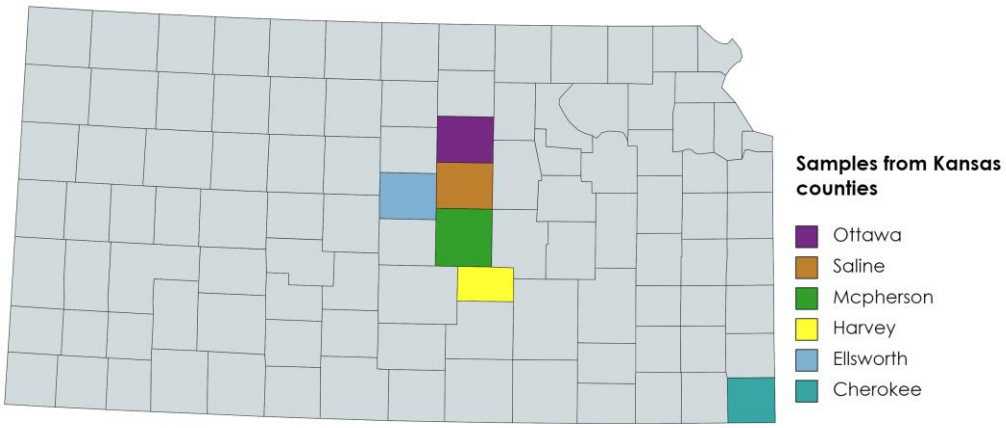


Figure 2.2. Head samples collected from counties in Minnesota.

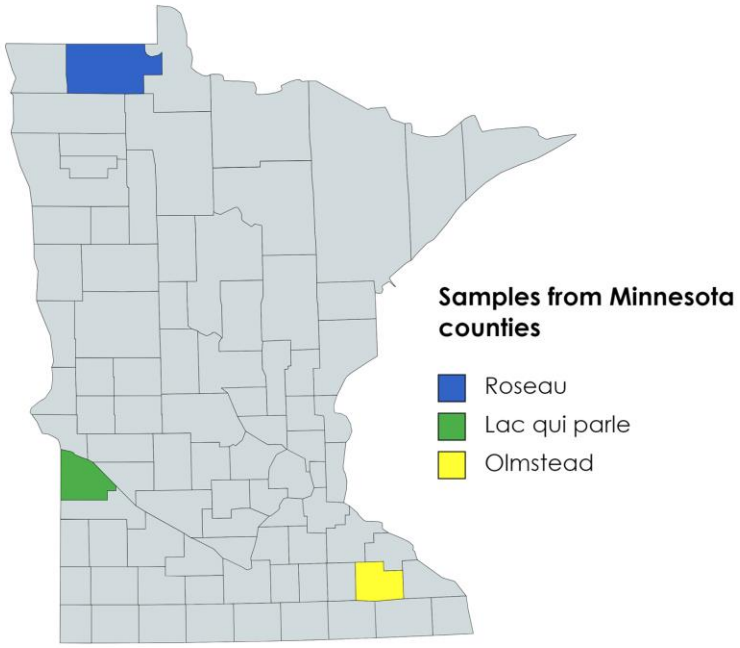


Figure 2.3. Head samples collected from counties in North Dakota.

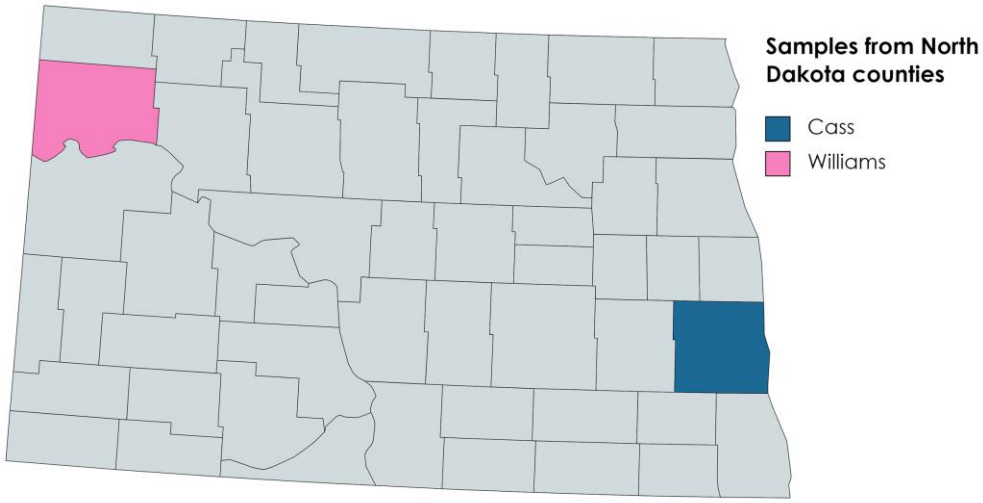


Figure 2.4. Head samples collected from a county in Montana.

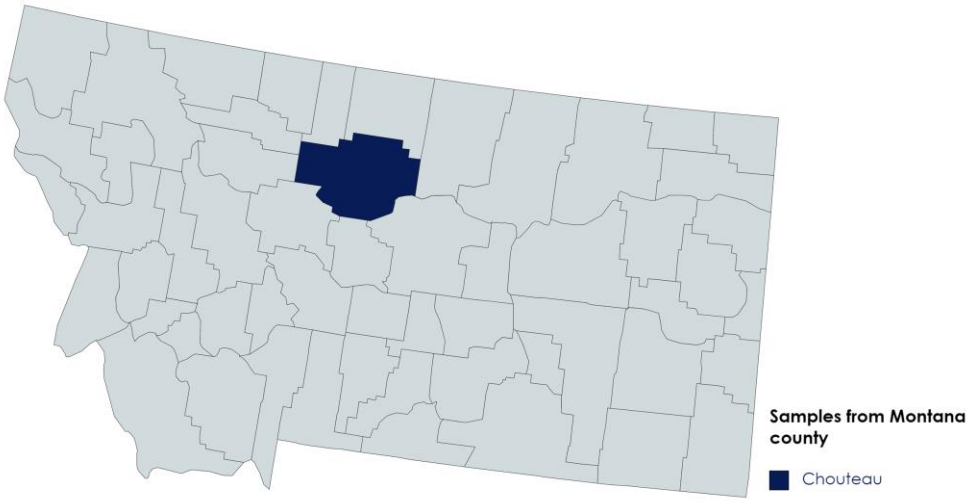


Figure 2.5. Barplot of fungi confirmed from the head samples.

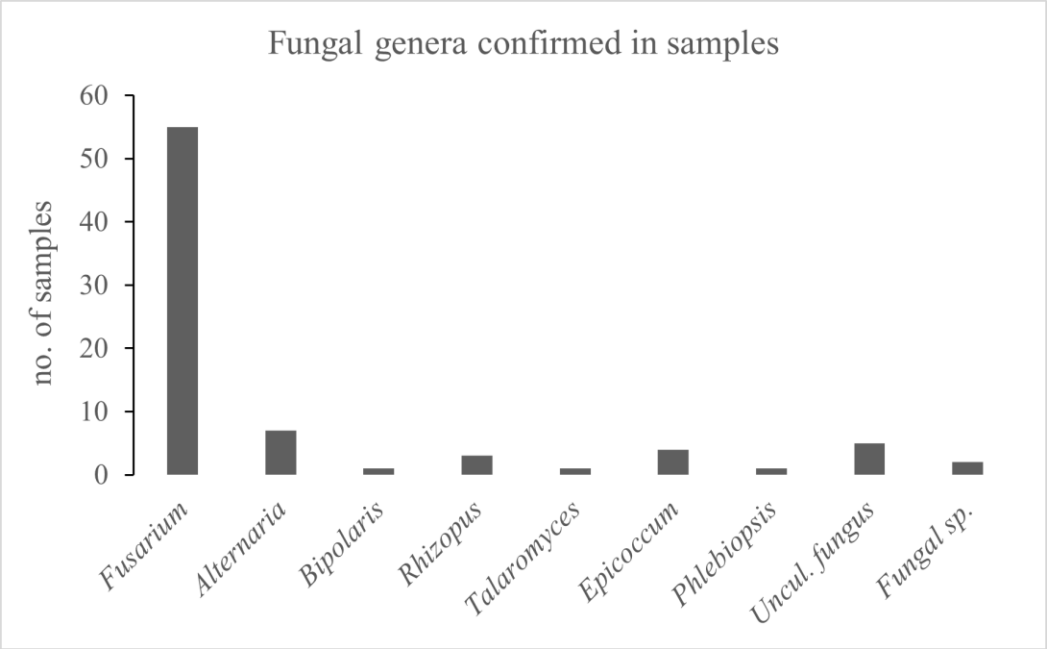


Figure 2.6. Pie-chart of diverse *Fusarium* spp. in the *Fusarium* confirmed samples.

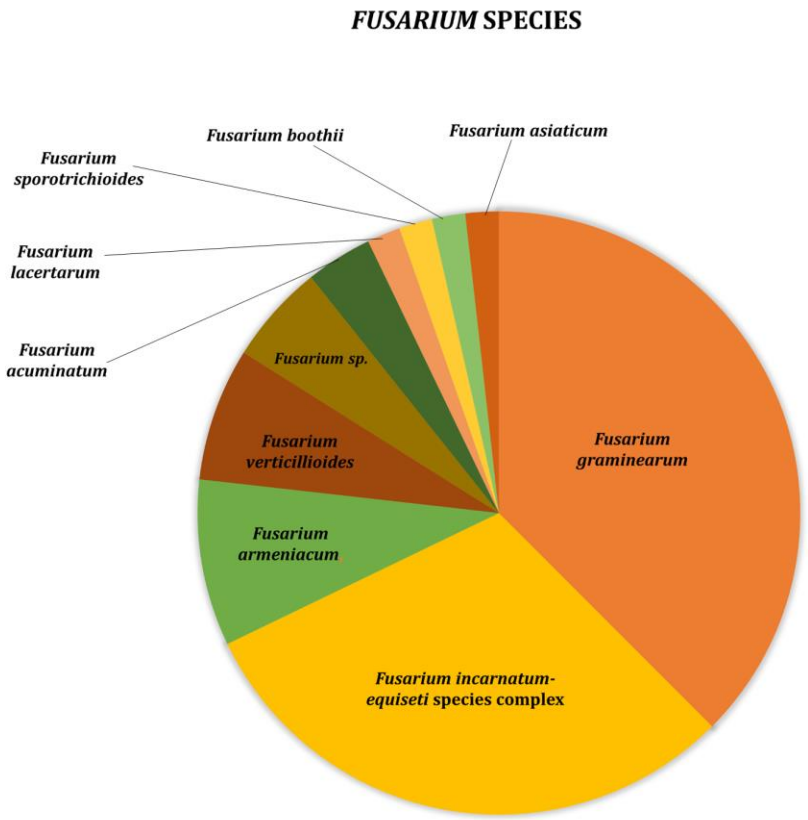


Table 2.1. List of all PCR primers used in this study.

Use	Primer Name	Primer sequence (5' to 3')	Tm(°C)	Expected amplicon length	Source
Fungal differentiation	ITS1 ITS4	TCCGTAGGTGAACCTGCGG TCCTCCGCTTATTGATATGC	63.4 60.4	600	White et al.,1990
Fusarium species confirmation	M13F(-20)-EF1 M13R-EF2	GTAAAACGACGGCCAGATGG GTAAGGARGACAAGAC CAGGAAACAGCTATGACGGA RGTACCAGTSATCATGTT	53.7 54.9	550-710	O'Donnell et al.,1998

Table 2.2. List of fungi confirmed by using ITS primers.

S.N.	Sample ID[A]	*Fusarium* check with ITS Primer	NCBI Database	Percent Identity	Accession number
1.	19RSMN002	Negative	*Alternaria* sp.	99.79%	OQ947366
2.	19OTKS010	Positive	*Fusarium* sp.	100%	MT560375
3.	18MCPKS011	Positive	*Fusarium* sp.	99.42%	OM955951
4.	18HVKS012	Positive	*Fusarium* sp.	99.06%	OQ561201
5.	19SLKS013	Positive	*Fusarium* sp.	100%	OQ938729
6.	18CKKS014	Positive	*Fusarium* sp.	100%	OQ658849
7.	20OTKS015	Positive	*Fusarium* sp.	100%	OQ938729
8.	20WLND016	Positive	*Fusarium* sp.	100%	0Q658849
9.	20OLMN017	Positive	*Fusarium* sp.	99.53%	MN452572
10.	19SLKS018	Negative	*Talaromyces* sp.	99.63%	MK805486
11.	19SLKS019	Positive	*Fusarium* sp.	99.80%	OQ658849
12.	19SLKS020	Positive	*Fusarium* sp.	100%	MT558570
13.	19SLKS021	Positive	*Fusarium* sp.	100%	MH333079
14.	19SLKS022	Positive	*Fusarium* sp.	100%	OK017465
15.	19SLKS023	Positive	*Fusarium* sp.	100%	OQ658849
16.	19SLKS024	Negative	*Rhizopus* sp.	99.83%	MT540020
17.	18CKKS025	Positive	*Fusarium* sp.	100%	MW341496
18.	18CKKS026	Positive	*Fusarium* sp.	99.79%	MF120225
19.	18CKKS027	Positive	*Fusarium* sp.	100%	MT393872
20.	18CKKS028	Negative	*Bipolaris* sp.	100%	HQ631050
21.	18CKKS029	Positive	*Fusarium* sp.	99.61%	OQ658849
22.	18CKKS030	Positive	*Fusarium* sp.	99.81%	OQ658849
23.	18MCPKS031	Positive	*Fusarium* sp.	100%	OQ658849
24.	18MCPKS032	Positive	*Fusarium* sp.	100%	OQ711830
25.	18MCPKS033	Positive	*Fusarium* sp.	100%	MT563420
26.	18MCPKS034	Positive	*Fusarium* sp.	100%	MT393872

27.	18MCPKS035	Positive	*Fusarium* sp.	100%	OQ947371
28.	18MCPKS036	Positive	*Fusarium* sp.	100%	MT393872
29.	17EWKS037	Negative	*Epicoccum* sp.	99.41%	MT573479
30.	17EWKS038	Positive	*Fusarium* sp.	99.82%	OM956058
31.	17EWKS039	Positive	*Fusarium* sp.	98.99%	KT318586
32.	17EWKS040	Positive	*Fusarium* sp.	100%	MN718934
33.	17EWKS041	Positive	*Fusarium* sp.	99.81%	MK621018
34.	17EWKS042	Positive	*Fusarium* sp.	100%	OQ561201
35.	17EWKS043	Positive	*Fusarium* sp.	100%	MH388451
36.	17EWKS044	Negative	*Alternaria* sp.	98.14%	MF141013
37.	17EWKS045	Positive	*Fusarium* sp.	98.10%	GQ352485
38.	21SLKS046	Positive	*Fusarium* sp.	98.46%	MG649271
39.	21SLKS047	Negative	*Epicoccum* sp.	100%	MT573479
40.	21SLKS048	Positive	*Fusarium* sp.	98.33%	ON712460
41.	21SLKS049	Positive	*Fusarium* sp.	100%	MT636465
42.	21SLKS050	Negative	*Epicoccum* sp.	99.80%	MT573479
43.	21SLKS051	Positive	*Fusarium* sp.	99.08%	OM956063
44.	18LQPMN052	Negative	*Epicoccum* sp.	100%	MT573479
45.	19LQPMN053	Positive	*Fusarium* sp.	100%	MT563420
46.	20LQPMN054	Positive	Fusarium sp.	99.80%	MT560375
47.	20OTKS055	Negative	*Rhizopus* sp.	98.01%	LC514332
48.	20OTKS056	Positive	*Fusarium* sp.	98.54%	MG649271
49.	20OTKS057	Negative	*Rhizopus* sp.	99.31%	MT279284
50.	20OTKS058	Negative	Uncultured fungus	99.54%	MT236592
51.	20OTKS059	Negative	Uncultured fungus	99.34%	MT236592
52.	20MCPKS060	Negative	Uncultured fungus	98.98%	MT236592
53.	20MCPKS061	Positive	*Fusarium* sp.	98.33%	OM514896
54.	20MCPKS062	Positive	*Fusarium* sp.	100%	MN718970

55.	20MCPKS063	Positive	*Fusarium* sp.	99.80%	OQ658849
56.	20MCPKS064	Negative	Uncultured fungus	99.28%	MT236592
57.	20MCPKS065	Positive	*Fusarium* sp.	99.41%	ON971221
58.	21WLND066	Positive	*Fusarium* sp.	99.79%	MZ089678
59.	21MCPKS067	Positive	*Fusarium* sp.	100%	OP782319
60.	21MCPKS068	Positive	*Fusarium* sp.	100%	MW369580
61.	21MCPKS069	Positive	*Fusarium* sp.	98.70%	GQ352485
62.	21SLKS070	Positive	*Fusarium* sp.	100%	MT598163
63.	21LQPMN071	Positive	*Fusarium* sp.	98.9%	KT318586
64.	21MCPKS072	Positive	*Fusarium* sp.	100%	OQ947371
65.	21LQPMN073	Positive	*Fusarium* sp.	98.05%	GQ352485
66.	21LQPMN074	Positive	*Fusarium* sp.	99.13%	MN748937
67.	21OTKS075	Positive	*Fusarium* sp.	98.3%	MH108127
68.	20SLKS076	Positive	*Fusarium* sp.	98.78%	OM514897
69.	21CHMT077	Positive	*Fusarium* sp.	100%	MH277491
70.	22SLKS078	Negative	*Alternaria* sp.	98.21%	MF422130
71.	22SLKS079	Negative	*Alternaria* sp.	98.18%	MF422133
72.	22SLKS080	Negative	*Alternaria* sp.	98.87%	MF422130
73.	22SLKS081	Negative	*Alternaria* sp.	98.39%	MF422130
74.	22SLKS082	Negative	*Alternaria* sp.	98.89%	ON712479
75.	22CSND083	Positive	Fungal sp.	99.67%	MG515308
76.	22WLND084	Positive	*Fusarium* sp.	98.73%	MW369580
77.	22WLND085	Negative	*Phlebiopsis* sp.	100%	MT561719
78.	22WLND086	Negative	Uncultured fungus clone	98.98%	MT236592
79.	22WLND087	Positive	Fungal sp.	99.51%	MG515308

[A]Sample Id is the unique identification number given to the collected samples. In the third column above, samples that were identified as *Fusarium* when compared to the NCBI database are called positive otherwise negative.

The sequencing data was compared to the NCBI database, ensuring a minimum of 98% similarity, in order to identify the species, present in the samples. The table above provides details on the identified fungi, including their corresponding accession numbers and the percentage of identity to the reference sequences sourced from the NCBI database.

Table 2.3. List of *Fusarium* spp. confirmed from Kansas samples.

S.N.	Sample ID[A]	MycoBank Database	Percent Identity	Accession number
1.	19OTKS010	*Fusarium incarnatum-equiseti* species complex	100%	GQ505636
2.	18MCPKS011	*F. verticillioides*	98.05%	MH582324
3.	18HVKS012	*F. verticillioides*	99.34%	MH582324
4.	19SLKS013	*F. verticillioides*	99.10%	MH582327
5.	18CKKS014	*F. graminearum*	99.51%	MH582244
6.	20OTKS015	*F. verticillioides*	99.51%	MH582324
7.	19SLKS019	*F. graminearum*	99.24%	MH582245
8.	19SLKS020	*F. incarnatum-equiseti* species complex	99.66%	GQ505612
9.	19SLKS021	*F. asiaticum*	98.37%	MH582232
10.	19SLKS022	*F. graminearum*	99.13%	MH582235
11.	19SLKS023	*F. graminearum*	98.65%	MH582240
12.	18CKKS025	*F. boothii*	98.71%	MH582231
13.	18CKKS026	*F. graminearum*	99.73%	MH582244
14.	18CKKS027	*F. armeniacum*	98.73%	MH582290
15.	18CKKS029	*F. graminearum*	100%	MH582235
16.	18MCPKS031	*F. graminearum*	100%	MH582235
17.	18MCPKS032	*F. graminearum*	100%	MH582235
18.	18MCPKS033	*Fusarium sp.*	100%	GQ505596
19.	18MCPKS034	*F. armeniacum*	100%	MH582288
20.	18MCPKS035	*Fusarium sp.*	99.85%	MH582442
21.	18MCPKS036	*F. armeniacum*	100%	MH582288
22.	17EWKS038	*F. acuminatum*	100%	GQ505420
23.	17EWKS039	*F. graminearum*	99.70%	MH582244
24.	17EWKS040	*F. armeniacum*	100%	MH582288
25.	17EWKS041	*F. incarnatum-equiseti* species complex	100%	GQ505636
26.	17EWKS042	*F. lacertarum*	99.41%	GQ505593
27.	17EWKS043	*F. incarnatum-equiseti* species complex	100%	GQ505636

28.	17EWKS045	*F. incarnatum-equiseti* species complex	100%	GQ505636
29.	21SLKS046	*F. incarnatum-equiseti* species complex	100%	GQ505636
30.	21SLKS048	*F. incarnatum-equiseti* species complex	100%	GQ505636
31.	21SLKS049	*F. incarnatum-equiseti* species complex	99.85%	GQ505612
32.	21SLKS051	*F. incarnatum-equiseti* species complex	100%	GQ505636
33.	20OTKS056	*F. incarnatum-equiseti* species complex	99.85%	GQ505672
34.	20MCPKS061	*F. incarnatum-equiseti* species complex	100%	GQ505636
35.	20MCPKS062	*F. armeniacum*	100%	MH582288
36.	20MCPKS063	*F. graminearum*	100%	MH582235
37.	20MCPKS065	*F. graminearum*	100%	MH582244
38.	21MCPKS067	*F. graminearum*	100%	MH582235
39.	21MCPKS068	*F. graminearum*	100%	MH582235
40.	21MCPKS069	*F. incarnatum-equiseti* species complex	100%	GQ505636
41.	21SLKS070	*F. graminearum*	100%	MH582244
42.	21MCPKS072	*F. incarnatum-equiseti* species complex	99.71%	GQ505636
43.	21OTKS075	*F. graminearum*	100%	MH582244
44.	20SLKS076	*F. incarnatum-equiseti* species complex	100%	GQ505636

Table 2.4. List of *Fusarium* spp. confirmed from North Dakota samples.

S.N.	**Sample ID**[A]	**MycoBank Database**	**Percent Identity**	**Accession number**
1.	20WLND016	*F. graminearum*	99.84%	MH582248
2.	21WLND066	*F. graminearum*	100%	MH582247
3.	22WLND083	*F. graminearum*	100%	MH582235
4.	22WLND084	*F. graminearum*	100%	MH582244
5.	22WLND087	*F. graminearum*	100%	MH582235

Table 2.5. List of *Fusarium* spp. confirmed from Minnesota samples.

S.N.	Sample ID[A]	MycoBank Database	Percent Identity	Accession number
1.	20OLMN017	*F. incarnatum- equiseti* species complex	100%	GQ505636
2.	19LQPMN053	*Fusarium sp.*	100%	MH582442
3.	20LQPMN054	*F. incarnatum- equiseti* species complex	99.85%	GQ505636
4.	21LQPMN071	*F. graminearum*	100%	MH582235
5.	21LQPMN073	*F. incarnatum- equiseti* species complex	99.85%	GQ505636
6.	21LQPMN074	*F. sporotrichioides*	100%	MH582277

Table 2.6. List of *Fusarium* spp. confirmed from Montana samples.

S.N.	Sample ID[A]	MycoBank Database	Percent Identity	Accession number
1.	21CHMT077	*F. acuminatum*	100%	GQ505420

Table 2.7. Mycotoxin result for IWG head samples in parts per million(ppm).

S.N.	Sample ID[A]	DON	Zearalelone	Fumonisin(B1)	T-2/HT-2
1.	19RSMN002	28.8	1.4	0	
2.	19OTKS010	0.1	0	0	
3.	18MCPKS011	0.3	0	0	0.166
4.	18HVKS012	0.5	0	0	
5.	19SLKS013	0.4	0.1	0	
6.	18CKKS014	0.1	0	0.0027	
7.	20OTKS015	0.3	0	0	
8.	20WLND016	0.1	0.6	0.00.8	
9.	20OLMN017	7.5	6.9	0.0029	0.151
10.	21WLND066	0.4	0	0.0017	
11.	21MCPKS068	0.7	0	0.0017	
12.	21SLKS070	0.3	0	0.0052	

13.	21LQPMN071	0.1	0	0	
14.	21MCPKS072	0.4	0	0	
15.	21LQPMN073	0	0	0.0079	
16.	21LQPMN074	0	0	0	
17.	22SLKS078	8.8	0.1	0.004	
18.	22WLND084	0	0	0	

Chapter 3 - Grower Disease Guides as an Extension Resource

Abstract

Farmers require guidance on identifying, preventing, and managing crop diseases. A tangible product, such as a grower disease guide can be useful to stakeholders. These guides enable farmers to visually recognize symptoms of a disease and identify it early on, leading to cost-effective management and increased productivity. Moreover, using these guides can contribute to food and feed safety by preventing mycotoxin contamination. The project aims to create pocket-sized disease cards to provide farmers with a convenient and concise way to access disease management information regarding the emerging crop Kernza®, referring to the grain harvested from domesticated intermediate wheatgrass. The disease data will rely on surveys, notable visual symptoms compared to available extension resources, and results obtained from DNA and RNA sequencing. The cards will feature at least two pictures of the confirmed disease on the front and provide information on the symptoms, factors that facilitate disease development, and other important facts about the disease on the back. The disease lists will be divided into fungal, bacterial, and viral categories. To ensure the cards are easily accessible and organized, they will be bound together with a metal ring, with a hole punched on the top-left corner of each card. As information regarding the diseases affecting IWG becomes available, more cards may be added at a later date. This material will be useful to increase awareness and knowledge about the various diseases and their management that affect intermediate wheatgrass among farmers, breeders and researchers.

Introduction

Research aims to disseminate research findings to growers, breeders, and other researchers as one of its important goals. Valuable insights on plant pathogens aid in planning disease management for both annual and perennial cropping systems and in improving breeding practices (Jeger et al., 2021). Communication through Extension services holds great importance in transferring knowledge, expertise promoting sustainable practices, and technology adoption among growers (Baig and Aldosari, 2013). This approach effectively bridges the gap between researchers and farmers (Rackham, 1961).

Growers can be reached through various means, such as workshops, field days, farm visits, demonstrations, agricultural conferences, exhibitions, digital platforms, online resources, mobile technology, SMS, agricultural extension services, and print materials. After considering different options, we have decided to create specialized grower disease guides specifically for IWG growers. These guides aim to provide convenient and reliable resources for early disease identification in the field. Designed to be user-friendly and practical, the guides are compact in size, allowing growers to easily carry them in their pockets while working. They are also water-resistant, ensuring durability even in challenging weather conditions. These grower disease guides are a valuable tool that combines research-based insights from researchers with practical applications for farmers.

Results and Discussion

The following figures represent the diseases that will be included in their visual format.

Figure 3.1. Fusarium head blight of IWG.

Figure 3.2. Ergot of IWG.

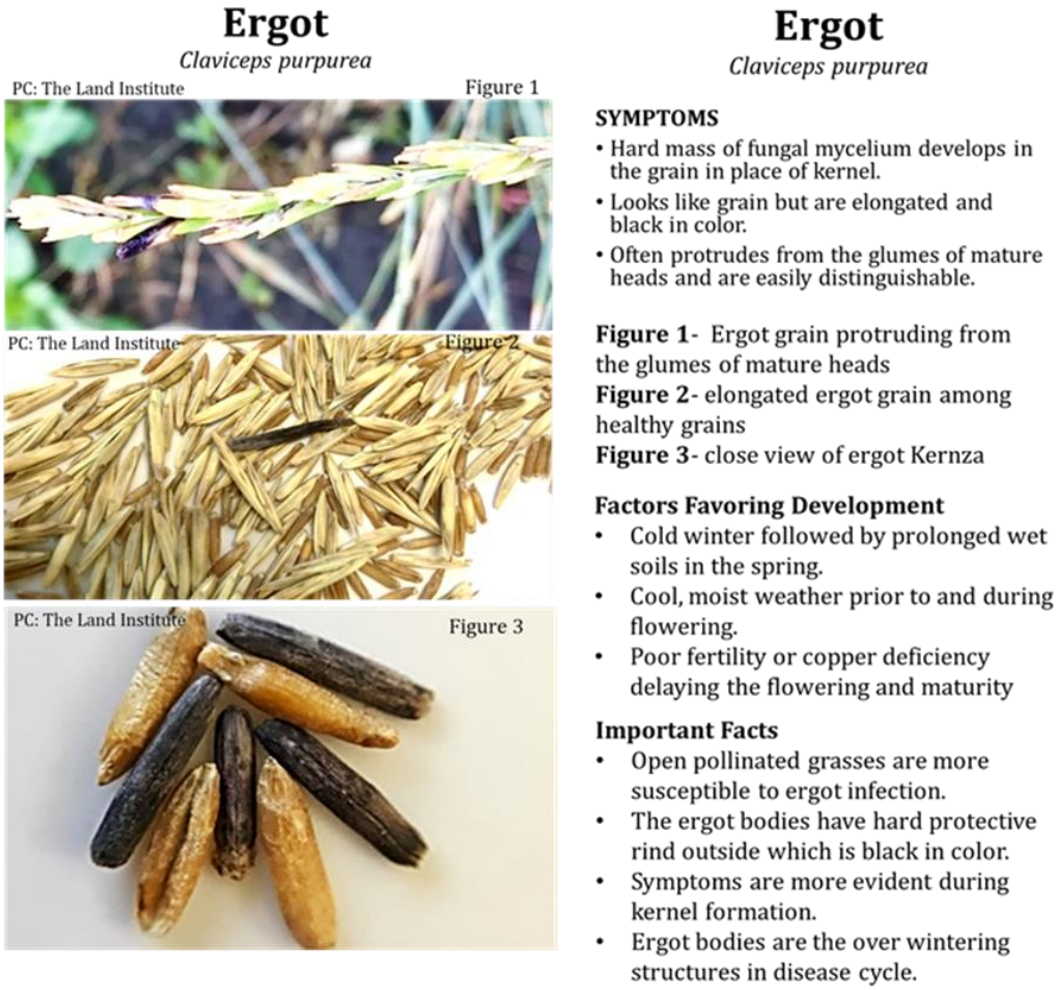


Figure 3.3. Bacterial leaf streak of IWG.

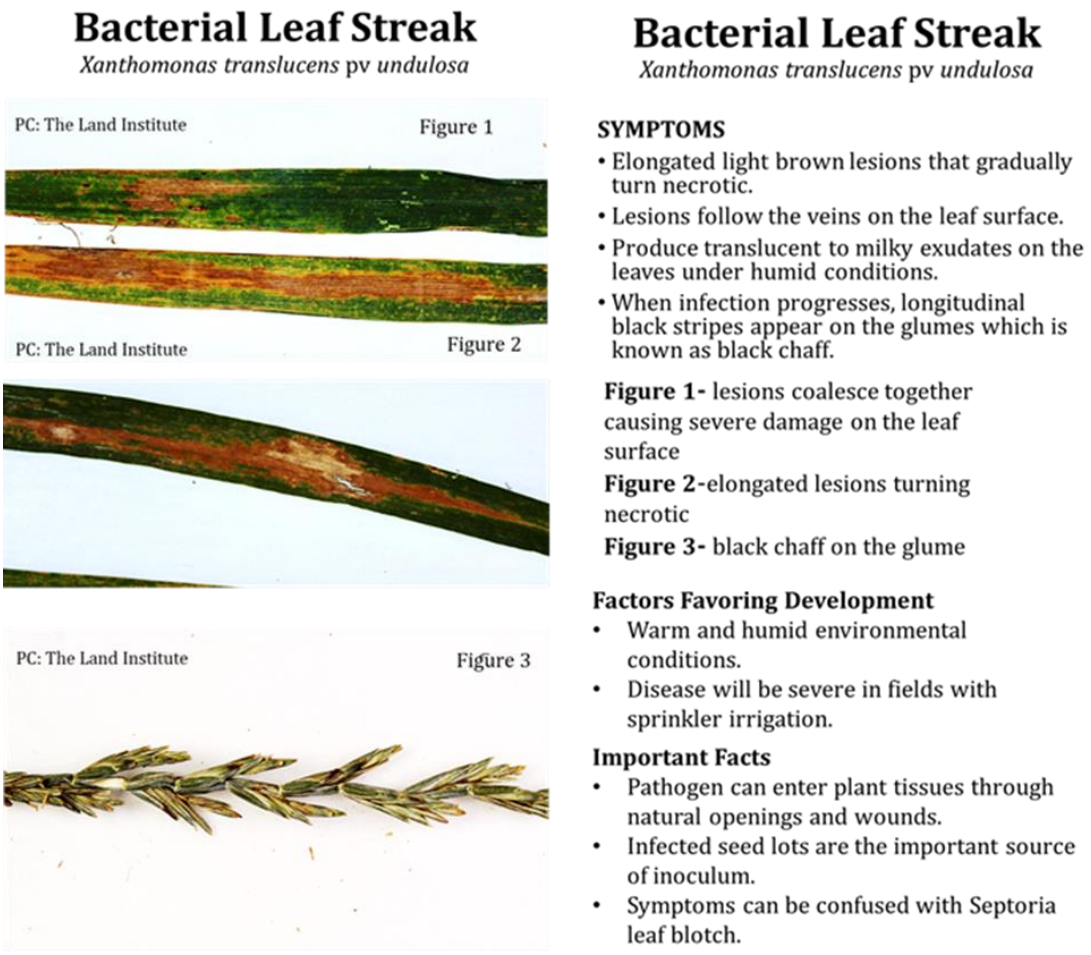


Figure 3.4. Anthracnose of IWG.

Anthracnose

Colletotrichum cereale

PC: The Land Institute Figure 1

Anthracnose

Colletotrichum cereale

SYMPTOMS

- Elongated, chlorotic leaf spots with small, black, spore-bearing structures in the center.
- Water-soaked lesions which later become bleached and necrotic.
- Leaves die from their tips down.

Figure 1- chlorotic leaf patch on the crop stand

Factors Favoring Development

- High humidity and shade.
- Excess water on the leaf canopy for a longer period of time.
- Poor soil drainage.

Important Facts

- Fungus survives on the soil surface and in soil as mycelium and conidia on host residues.
- Fungal conidia can be spread by wind, water or human activities.
- Symptoms often appear as a patch.

Figure 3.5. Net blotch of IWG.

Net Blotch

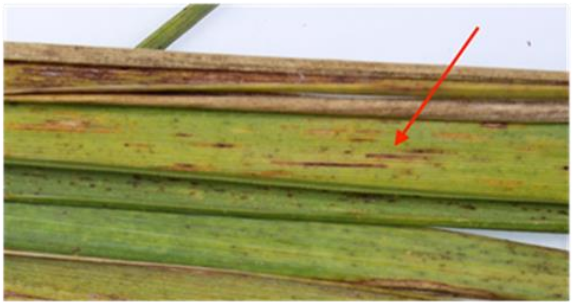
Pyrenophora teres f. *teres*

PC: The Land Institute

Figure 1

PC: The Land Institute

Figure 2



Net Blotch

Pyrenophora teres f. *teres*

Symptoms

- Tiny dark brown spots on the leaves
- Lesions are large and cigar shaped
- As the lesion expand, they become oval or square and coalesce to form a net-like pattern on the leaf.

Figure 1- lesions coalesce to become cigar shaped and net like pattern

Figure 2- coalescing lesion on the chlorotic leaf

Factors Favoring Development

- 6 hours of wetness at a temperatures between 50°F and 77 °F.
- High humidity and mild temperatures in spring and summer
- Thick crop

Important Facts

- Fungus survives saprophytically between cropping season and is present as mycelium on host crop residues
- Pathogen get dispersed by wind and water.
- Primary inoculum comes from the infested stubble in which pathogen can survive up to 3 years.

Figure 3.6. Ramularia leaf spot of IWG.

Ramularia leaf spot

Ramularia collo-sygni

PC: The Land Institute Figure 1

PC: The Land Institute Figure 2

Ramularia leaf spot

Ramularia collo-sygni

Symptoms

- Small brown, round spots are seen on the upper leaves at the flowering stage.
- Spots enlarge and develop into rectangular, reddish-brown necrotic spots.
- Spots are surrounded by the chlorotic halo and are restricted by the leaf veins.

Figure 1-distinct brown round spot with chlorotic halo

Figure 2- rectangular mature lesion, with chlorotic halo visible and brownish center

Factors Favoring Development

- Moist weather and leaf surface wetness for a longer period of time.
- Spring rainfall
- Infested materials

Important Facts

- Loss of green area in infected leaves which leads to yield loss
- Symptoms appear late in the growing season after ear emergence.
- Leaf becomes chlorotic starting from the tip and leaf margins.

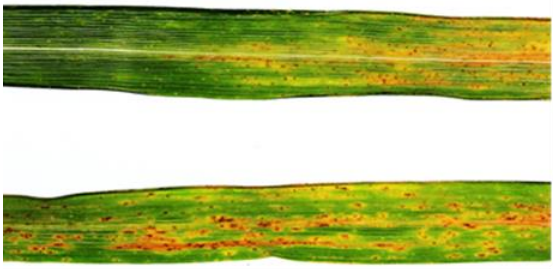
Figure 3.7. Septoria leaf blotch of IWG.

Septoria Leaf blotch

Zymoseptoria tritici

PC: The Land Institute Figure 1

PC: The Land Institute Figure 2



Septoria Leaf blotch

Zymoseptoria tritici

Symptoms

- Foliar lesions (broadly elliptical) with yellowish halo and contains small dark-brown to black specks (pycnidia).
- Lesions are mostly elongated with rougher edges
- A curved cirrhus extrudes from the ostiole of the pycnidium, when relative humidity is high and leaf surface is not actually wet.

Figure 1- lesions with pycnidia in them
Figure 2- presence of chlorotic halo on the lesions

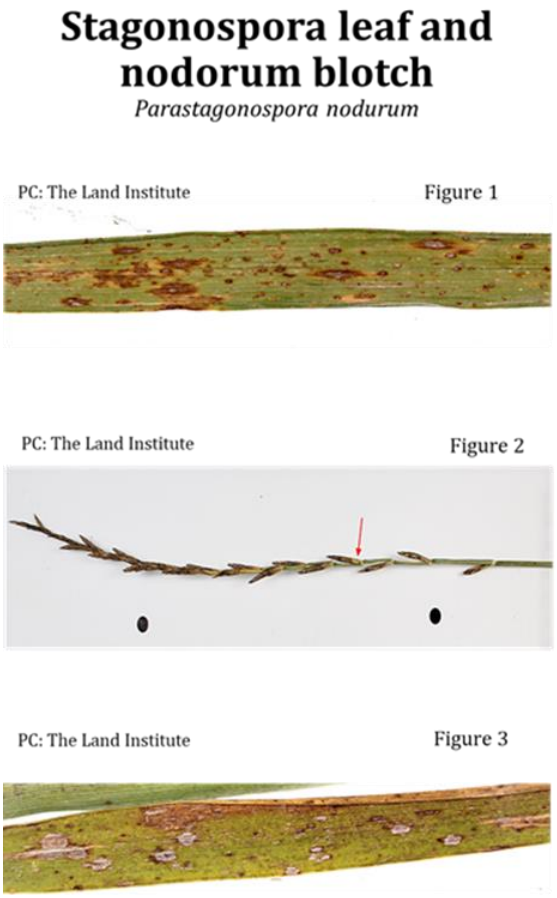
Factors Favoring Development

- Cool, moist weather for at least 48 hours.
- Temperature between 50°F to 68°F.
- High relative humidity and moist leaves.

Important Facts

- Symptoms is more prominent in the early growing season during stem elongation to flag leaf emergence.
- More common on lower leaves.
- Fungus overwinters as mycelium or pycnidia on infected plants, seeds or plant debris.

Figure 3.8. Stagonospora leaf and nodorum blotch of IWG.



Stagonospora leaf and nodorum blotch

Parastagonospora nodurum

Symptoms

- Presence of dark-brown flecks or spots with prominent yellow halo.
- As lesions enlarge, they become dark brown and centers turn grayish-white in color causing leaf blotch.
- Light brown pycnidia may develop but are not easy to see with the naked eye like in Septoria blotch.
- Purple brown or grayish streaks will be often seen on the heads which is known as glume blotch.

Figure 1- dark brown lesion with grayish-white center
Figure 2- infection on glumes
Figure 2- grayish- white center in focus

Factors Favoring Development

- High humidity and extended period of leaf wetness.
- Warm temperature between 68°F to 81°F .

Important Facts

- Symptoms are predominantly seen on the upper leaves.
- Fungus infects seed and is seed-borne.

Figure 3.9. Tan spot of IWG.

Tan spot

Pyrenophora tritici-repentis

PC: The Land Institute

Figure 1

PC: The Land Institute

Figure 2

Tan spot

Pyrenophora tritici-repentis

Symptoms

- Lesions appear as tan to brown flecks which later expand to form irregular oval or lens shaped lesions with a yellow or chlorotic halo and a dark fleck in the center.
- Oval to diamond shaped spots on the leaf.
- Absence of dark fruiting bodies.
- Black pseudothecia will be visible in the stubble that feels like coarse sandpaper to the touch.

Figure 1- oval or diamond shaped lesions with chlorotic halo
Figure 2- dark fleck visible in the center of the lesion

Factors Favoring Development

- Rainy, misty and foggy weather lasting for 24 hours.
- Free moisture on the leaf surface.

Important Facts

- Lesions are observed in more mature leaves during tillering and jointing.
- When spots are abundant, leaves may yellow giving the field a yellow overcast.
- Fungus survives in crop residue and native prairie grasses.

Figure 3.10. Spot blotch of IWG.

Spot Blotch

Bipolaris sorokiniana

PC: The Land Institute Figure 1

PC: The Land Institute Figure 2

Spot Blotch

Bipolaris sorokiniana

Symptoms

- Small spots at the infection point and grows to oblong light brown lesions with brown margins
- Leaf spots will continue to elongate and coalesce covering the entire leaf during severe infection.

Figure 1- distinct black specks visible on the leaf
Figure 2- dirty blotch spread all over the leaf

Factors Favoring Development

- Warm temperature of 16-24°c and high relative humidity.
- High residual soil moisture.
- Pathogens transmits through infected seeds, stubble and soil, and secondary infection takes place through air.

Important Facts

- It can infect leaves, stem, roots, rachis and seeds.
- It produces toxin helminthosporol and sorokinianin.
- No chlorotic margin like tan spot.

Figure 3.11. Brome mosaic virus in IWG.

Brome Mosaic Virus

Brome Mosaic Virus

Symptoms

- Light yellow to white longitudinal streaks on the upper surface of leaves.
- Leaves are more broad and different than the normal leaves
- Stunted growth, chlorotic and necrotic patches.

Figure 1: Individual leaves infected with BMV
Figure 2: Virus symptoms on the crop stand

Factors Favoring Development

- Mechanical transmission in the field.
- Transmission may occur by flea beetle, cereal leaf beetle, bird cherry-oat aphid, Russian wheat aphid.

Important Facts

- BMV can infect a wide range of hosts.
- It is not known to cause disease in humans or animals.
- It can be controlled through sanitation, avoiding using infected material for propagation and use of resistant cultivars.

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