RESPONSE TO EARLY GENERATION SELECTION FOR STRIPE RUST AND FUSARIUM HEAD BLIGHT RESISTANCE IN WINTER WHEAT BREEDING POPULATIONS

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ABSTRACT

RESPONSE TO EARLY GENERATION SELECTION FOR STRIPE RUST AND FUSARIUM HEAD BLIGHT RESISTANCE IN WINTER WHEAT BREEDING POPULATIONS

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Early generation selection for resistance to wheat stripe rust Puccinia striiformis f.sp. Tritici and Fusarium head blight (FHB) in segregating populations can increase the frequency of resistance among derived lines. This study demonstrates an increase in the frequency of resistance and shifts in associated genomic regions among progeny derived populations undergoing selection. Populations segregating for resistance to stripe rust and FHB were split into an experimental group undergoing selection for resistance and a control without selection. To apply selection for resistance in the selected populations, susceptible genotypes were culled and resistant F2 and F3 plants were retained each generation. All genotypes in control populations were advanced without selection. Both selected and control populations were inoculated at the F4 generation to compare the frequency of resistant individuals. The frequency of resistance was also compared between selected and control-derived recombinant inbred lines (RILs) derived at the F4 generation. Experimental populations undergoing selection showed significantly higher resistance levelscompared to control populations. A significantly higher frequency of resistant genotypes were identified among RILs derived from populations undergoing selection. Signatures of selection for stripe rust were identified on eight unique chromosome regions and twelve for FHB. Selection for resistance during generational advancement results in an increased frequency of stripe rust and FHB resistance among inbred lines derived from segregating populations. The frequency of genomic regions associated with resistance is increased in parallel with phenotypic selection.

To my supportive and loving parents who I owe where I am.
To my always competitive siblings that pushed me to work my hardest.
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To my dogs Athena and Regi for always being by my side.

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CHAPTER 1: Review of Literature and Rationale

Part 1: Wheat

The widespread production and nutritional value of wheat is an essential food source to 36% of the world population (Anwaar et al. 2020). Wheat is the largest acreage crop in the world with 780 million tons produced in the year 2018 (Zhang et al. 2019). In countries where wheat is cultivated it accounts for 21% food calories and 20% protein (Muleta et al. 2020). Wheat is also a good source of vitamins and nutrients including iron, and phosphorus. With the world population growing the demand for high quality food such as wheat will increase. It has been estimated that a 60% increase in wheat production will be needed to meet demand by 2050 (Hu et al. 2020). The increase in demand will likely be met by increasing yields and the durability and agronomic traits by improved resistance to various biotic and abiotic challenges. Wheat along with oats and barley diverged from the grasses around 20 million years ago in Pre-Pottery Neolithic near East Fertile Crescent (Wani et al. 2014). Wheat species are classified into three types based on ploidy level: diploid, tetraploid, and hexaploid. Hexaploid wheat (genome AABBDD) is the type that is cultivated and used for milling purposes throughout the world. It has six sets of chromosomes totaling 42 chromosomes. Hexaploid wheat resulted from the hybridization between tetraploid wheat and diploid *Ae. tauschii* some 10,000 years ago (Dvorak et al. 1998). Since its cultivation the main objectives of breeding are for yield improvement, nutritional quality and stress tolerance (Wani et al. 2014). Farmers initially used natural and human selection as breeding methods, only advancing the highest yielding and best-looking varieties. This advanced to modern hybridization with crosses between genetically variable varieties. Since wheat flowers are complete and self-pollinate hybrid production is challenging. The use of chemical hybridizing agents, male sterility, hand emasculating, and plant

growth regulators to affect pollen development are currently used in hybrid production (Wani et al. 2014). The green revolution occurred in 1960-1980, jumpstarting wheat production and research. Large success was observed from the adaptation of high yielding varieties, increase in fertilizers, pesticides, and irrigation (Davies 2003). Dr. Norman E. Borlaug through CYMMIT, supplied high yielding, disease-resistant, semidwarf wheat varieties which allowed for substantial increases in yield outputs (Kiranjit 2011).

Part 2: Stripe Rust

Summary of the Problem

Stripe rust of wheat is caused by the rust fungus *Puccinia striiformis* Westend and is further classified by host specialization leading to the full name *Puccinia striiformis f. sp. Tritici* Westend. Stripe rust is one of the most serious pathogens affecting wheat with the ability to spread rapidly over vast areas under favorable conditions (Chen 2020). *P. striiformis* is believed to have originated from Transcaucasia where it then dispersed throughout the world reaching North America in the 1910s (Hassebrauk, 1965).

Impact Worldwide

Globally 5.47 million tons of wheat are lost annually as a result of stripe rust, at a cost of almost $1B USD in more than 60 countries (Getnet et al. 2020). Annually, stripe rust yield losses are 13% but with epidemics and susceptible lines being grown losses increase up to 100% (Ulukan 2020).

The frequency and severity of *Pst* epidemics have increased worldwide. Stepwise mutations have increased the prevalence of *Pst* races with virulence to widely developed resistance genes (Khan et al. 2020). Climate change is also a key factor allowing pathogens to

spread to new territories less prepared to handle them (Khazan et al. 2020). Increased tolerance to high temperatures have expanded the range in which *Pst* can thrive and has increased population sizes enabling higher rates of mutation.

Epidemics are occurring more frequently and with a greater effect. One of many substantial epidemics occurred in Ethiopia in 2018 that produced wheat yield losses up to 96% depending on the cultivar susceptibility (Getnet et al. 2020). In the U.S. in 2001, an epidemic caused losses of over 1M tons and additional epidemics impacted production in 6 of the following years leading to 2015. Losses over the years have increased from over 4.5 mil tons in 2015, significantly higher than the epidemic of 2001 (Chen 2020). The amount spent on fungicides has increased drastically with the global annual cost over $1B US dollars (Chen 2020). In countries with limited access to fungicides, the losses in wheat during epidemics is substantially higher.

Impact in Michigan

In Michigan wheat is produced on more than 500,000 acres with a farm gate value of $200M and contributing $3B across milling and food industries. The impact of stripe rust has been limited in Michigan, likely since spores of *P. striiformis* do not frequently overwinter. However, spores can be introduced from warmer regions by wind currents. In 2016, a stripe rust epidemic negatively impacted most wheat fields in Michigan, with up to 50% loss in some fields (Pennington 2016). This epidemic highlighted the potential impact of the disease and increased the research being conducted to control it in Michigan.

Stripe Rust Overview

Stripe rust can be identified on wheat plants as yellowish orange urediniospores between leaf veins on the adaxial surface forming stripes. On seedlings the uredinia are not confined by leaf veins but occur over the entire adaxial leaf surface.

Life Cycle and Spread

Stripe rust is a heteroecious macrocyclic fungus that requires a host to survive and reproduce (Chen 2020). Reproduction is observed as both sexual and asexual, with asexual occurring worldwide while asexual is limited to the Himalayan region (Figure 1). Asexual reproduction is observed as urediniospores which on average lead to 15 re-inoculation cycles a growing season (Schwessinger et al. 2016). Sexual reproduction requires an alternative host such as the barberry and produces both more spores and more genetic diversity than asexual reproduction (Schwessinger et al. 2016). Initial infection occurs by wind or human assisted dispersal of spores over large regions. Spores have been known to disperse by wind thousands of kilometers (Zadoks 1961).

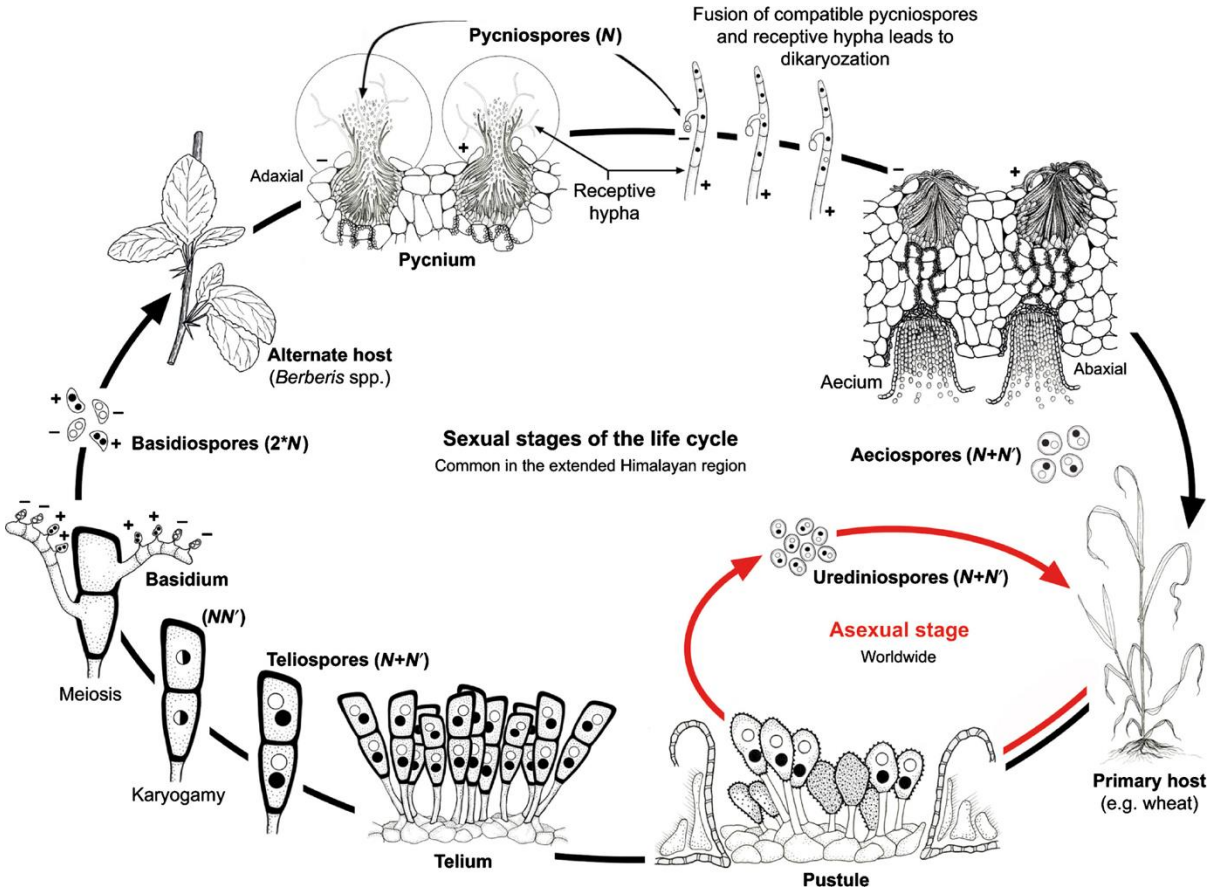

Figure 1. *Life cycle of Puccinia striformis f. sp. tritici.* Original illustration from Jacolyn A. Morrison at the USDA-ARS Cereal Disease Laboratory, St Paul, MN, USA (Schwessinger et al. 2016)..

P. striiformis life cycle starts with urediniospores infecting and reproducing asexually on the primary host wheat. Spores can germinate as soon as four hours after contact with the plant. Symptoms start to show one-week post infection and sporulation two weeks post infection (Khan et al. 2020). Asexual reproduction of urediniospores continues if environmental conditions remain ideal. As temperatures rise later in the growing season teliospores are produced which germinate readily forming basidiospores (Chen 2020). Basidiospores infect a second host which is often believed to be young barberry leaves which then

produces pycniospores and aeciospores. The aeciospores then infect primary hosts starting the cycle again (Schwessinger et al. 2016).

The year- round survival of *P. striiformis* is enabled in warm regions that provide optimal growth parameters year-round. In places where ideal pathogen growing conditions are not year-round, urediniospores survive by spreading from spring to winter wheat or with the help of volunteer wheat and wild grasses (Chen 2014). During late growth stages and high temperatures, stripe rust produces black telia that act as survival structures for season survival. Warmer winters also are shown to increase survival rates of the rust spores (Chen 2020).

Growth Parameters

Stripe rust is very sensitive and requires specific growth parameters in order to survive and reproduce. Of the three main rusts it is the most sensitive to its environment in terms of germination of urediniospores (Sharp 1967). The severity and frequency of stripe rust outbreaks varies heavily depending on environment, ecology, inoculum concentration, and host genotype (Ulukan 2020). Location also plays a role in disease severity with areas such as Europe, China, Australia, and India all prone to high severity (Khan et al. 2013).

The optimal environmental conditions for stripe rust growth and survival relies on temperature, moisture, and light exposure. Urediniospores of stripe rust require a minimum of three hours of dew on their hosts leaves in order to germinate and penetrate host tissues (Chen and Kang 2017). For proper germination the temperature is required to be 2-15°C while after germination the optimal growth temperature is 15-25°C (Sharp. 1965). Stripe rust is also dependent on humidity with greater than 50% required for sporulation (Chen 2014). These parameters highlight on this pathogen best growth being in late spring to early summer when

the conditions are easily meet. Stripe rust has also been shown to be more sensitive to air pollutants compared to other rusts and grow better with limited light exposure (Chen 2014). Even when environmental conditions are not optimal for stripe rust growth, cropping systems can influence its ability to grow and reproduce. This can be attributed to topography, cultivars used, spring and winter wheat used simultaneously, irrigation and other management practices (Jarraudi et al. 2020).

Damage Due to Stripe Rust

Infection results in yield losses due to reduced kernel weight, grain filling and number of grains per spike (Al-Maaroof et al. 2019). Yield losses are predicted to increase 0.39% for every 1% increase in stripe rust severity (King et al. 2007). A direct correlation has been shown between disease level and grain weight (Afzal et al. 2008). Stripe rust is shown to hinder the assimilation and movement of nutrients throughout the plant due to the loss of leaf area due to damage (Chunyu et al. 2019). Kernels of pathogen infected lines have been shown to be shrunken and reduced in size due to the decrease in translocation of nutrients and water to the pills and ovary of the flowers during development (Al-Maaroof et al. 2019). Spikes have been shown to have deficiencies in water, nutrient, and hormone balances (Al-Maaroof et al. 2019). This causes a loss of grain quality with a significant decrease in the protein and gluten content of wheat flour (Al-Maaroof et al. 2019).

When wheat is attacked by the stripe rust pathogen there is a disruption and reaction in the hosts vital functions such as cell division, photosynthesis, and respiration (Ulukan 2020). Leaf stress in susceptible lines is shown to significantly increase in severity from 31% at anthesis to 67% at 15 days after anthesis (Chunyu et al. 2019). The photosynthetic process is hindered due to the increase in chlorotic and necrotic regions on the flag leaf. The loss of

photosynthetic activity at critical growth stage between flowering and maturity are shown to have lower grain filling compared to controls by almost 20% (Chunyu et al. 2019). Infection by stripe rust also increases respiration, leading the plant to lose substantial CO_2 in photosynthesis (Al-Maaroof et al. 2019).

Resistance to Stripe Rust

Resistance to stripe rust can range from high to moderate in seedlings and adult plants. Resistance can be pathotype specific or non-specific and can be seen in the seedling stage for all stage resistance or limited to adult plant stage resistance. Seedling resistance can be race specific with a major gene which results in less durability compared to adult plant resistance which can be race non-specific with minor effect genes (Lie et al. 2018). Although, adult plant resistance can be race specific. The majority of resistance to stripe rust in Eastern soft winter wheat in the US is based on adult plant resistance. Resistance follows a distribution of phenotypes ranging from no visible infection, and small hypersensitive flecks, to uredinia surrounded by necrosis or chlorosis. Levels of resistance are ranked on a scale from 0-9 with the McNeal scoring system. A score of zero shows no visible uredia and is immune while a score of nine has abundant sporulation. Individuals with resistance are shown to have higher yields and less inputs used such as chemicals and labor (Abro et al. 2017). In order to combat the losses due to stripe rust, discovering and cultivating resistant lines is crucial.

Genes

Different genetic backgrounds result in different levels of resistance to pathogens. Certain genes have been associated with resistance to stripe rust over the years with many losing effectiveness due to pathogens ability to evolve into new races (Saharan et al. 2020). This makes

for the increasing need to identify new and effective resistance genes and incorporate them into different wheat varieties.

Currently there are over 80 official genes and over 300 quantitative trait loci (QTL) for stripe rust resistance (Chen and Kang 2017). *Yr17* is located on chromosome 2AS and is correlated with an infection type of 1 with only small necrotic flecks visible. *Yr15* is located on chromosome 1BS and encodes a protein with predicted kinase and pseudo kinase domains leading to infection scores of 0 (Wang et al. 2020). *Yr-18* is located on chromosome 7D with resistance seen with up to 20% flag leaf area affected by the pathogen. *Yr-18* encodes putative ATP binding cassette transporters providing it with an increase in resistance (Wang et al. 2020). There are often linkage relationships seen with stripe rust resistance genes as they can belong to the same chromosome as other resistance genes (Chen 2014). This is seen with *Yr17* and the stem rust resistance gene *Sr38* which are both located on chromosome 2AS.

Further research is still needed in genome analysis to find and locate these resistance genes. Wheat landrace PI388060 has shown resistance to all stripe rust races tested on it with seedling scores of 0-4 for rust infection compared to susceptible scores of 8-9 (Khalid et al. 2020). This resistance is a result of one major gene discovered through crossing analysis but has yet to be identified opening up the possibility of a new resistance gene (Khalid et al. 2020). The continued study of resistant wheat cultivars is an effective approach to finding new resistance genes. The study conducted by Muleta provided genome wide mapping of stripe rust resistance in 441 winter wheat accessions of varying resistance (Muleta et al. 2020). Muleta was able to identify 19 genomic regions associated with stripe rust resistance, 15 of which were previously identified (Muleta et al. 2020). The remaining four regions had not yet been identified and

was thought to be highly stable and effective (Muleta et al. 2020). This shows the hidden potential many wheat cultivars might poses we just haven't found yet.

Pyramiding stripe rust genes increases resistance to multiple rust races and increases durability (Hu et al. 2020). This technique was done with Tian Hu's research with two quantitative trait loci (QTL) of stripe rust resistance, QYr.nafu-2BL and QYr.nafu-3BS found in wheat variety P9897 (Hu et al. 2020). These two QTLs were introgressed into three Chinese wheat cultivars that were high yielding but had lost their stripe rust resistance due to the rust race YR34, overcoming *Yr26* (Hu et al. 2020). These varieties had relied heavily on one trait making resistance evolution much more likely than if it possessed multiple resistance genes. Once introgressed with multiple traits, Hu's study showed a decrease in infection type and disease severity with the three Chinese lines (Hu et al. 2020). The lines originally produced infection types of 6 and 7 while the P9897 x Chinese crosses with both QTLs produced infection types of 0-2 and P9897 x Chinese crosses with one QTL falling somewhere in between (Hu et al. 2020). This shows that one QTL provides some resistance but multiple provide greater resistance and will also be more durable over time. A rust race will have a much harder time evolving resistance to multiple genes/QTL than it would with one.

Part 3: Fusarium Head Blight

Summary of the Problem

Fusarium head blight (FHB) is caused by a fungal pathogen effecting the growth and development of wheat. This disease was first identified in the early 1900's with multiple outbreaks farmers struggled to control (Mesterhazy 2006). At least 17 species of Fusarium have been implicated in causing FHB in wheat and other small grains with *F. graminearum*

and F. *culmorum* (Sakr 2020) being the most common. *F. graminearum* is the most studied species and used in research. FHB is a threat to farmers causing significant reductions in grain yield and quality, effecting wheat and other crops such as maize and barley (Windels et al. 2000). FHB also results in mycotoxin contamination causing significant economic impacts due to the rejection of the grain from food and brewing industries (Bertuzzi et al. 2020). From the toxicological and economic view, FHB is considered the most dangerous disease of wheat diseases (Mielniczuk et al. 2020).

Impact Worldwide

FHB is a threat to wheat production across all growing regions. China, the leading producer of wheat, had nine epidemics between 1991 and 2007 with yield losses greater than 20%. In the United States losses due to FHB epidemics from 1993 to 2001 totaled more than $2 billion with Canadian losses of more than $520 million (Xia et al. 2020). The 1993 epidemic reduced yields by 40-50% in the spring wheat producing states of North Dakota and Minnesota (Windels et al. 2000). Mycotoxins produced by Fusarium also play a huge factor. In the epidemic of 1996, 60% of the winter wheat acres harvested in Ontario had toxic mycotoxin levels higher than 5mg/kg leading to rejection of grain at elevators (Xia et al. 2020). More breeding efforts need to be made to increase FHB resistance, to prevent future epidemics and global losses.

Fusarium Head Blight Overview

FHB is initially seen as a tan/ bleached discoloration at the base of an individual spikelet of the wheat head. As the disease develops discoloration spreads to bleach the entire spikelet and head of the wheat. During high humidity there is also growth observed as pink sporodochia

along with mycelium layers (Mielniczuk et al. 2020). Infection is typically confined to heads with occasional brown streaking along the stem.

Life Cycle and Spread

The general disease lifecycle of *F. graminearum* is illustrated in Figure 2. Its lifecycle initiates with airborne spores landing on flowering spikelet's and infecting them. Plants are most susceptible at flowering with symptoms developing 14 to 21 days after infection. The fungus infects individual florets through the flower and then can spread throughout the spike through the rachis into developing caryopses (Birr et al. 2020).

F. graminearum has both telomorph and anamorph stages in its life cycle. The anamorph *F. graminearum* reproduces and infects wheat primarily by asexually produced conidia. Conidia are formed on crop residue or the surface of infected plants in masses that leads to rain splash dispersal (Deacon 2006). Infection can initially hit seedlings and transfer up the stem as the plant grows. The teleomorph of *F. graminearum* (*Gibberella zeae*) begins with the formation of hyphae with binucleate cells (Trail 2009). These develop into fruiting body initials which then develop into flask shaped perithecia (Trails and Common, 2000). Perithecia are filled with ascospores, which are the products of meiosis that are discharged into the air for dispersal. The perithecia are the overwintering structures that survive on host plant residues such as maize and wheat. No-tillage farming therefore allows a greater amount of *Fusarium* to survive to the next cropping season (Windels 2000).

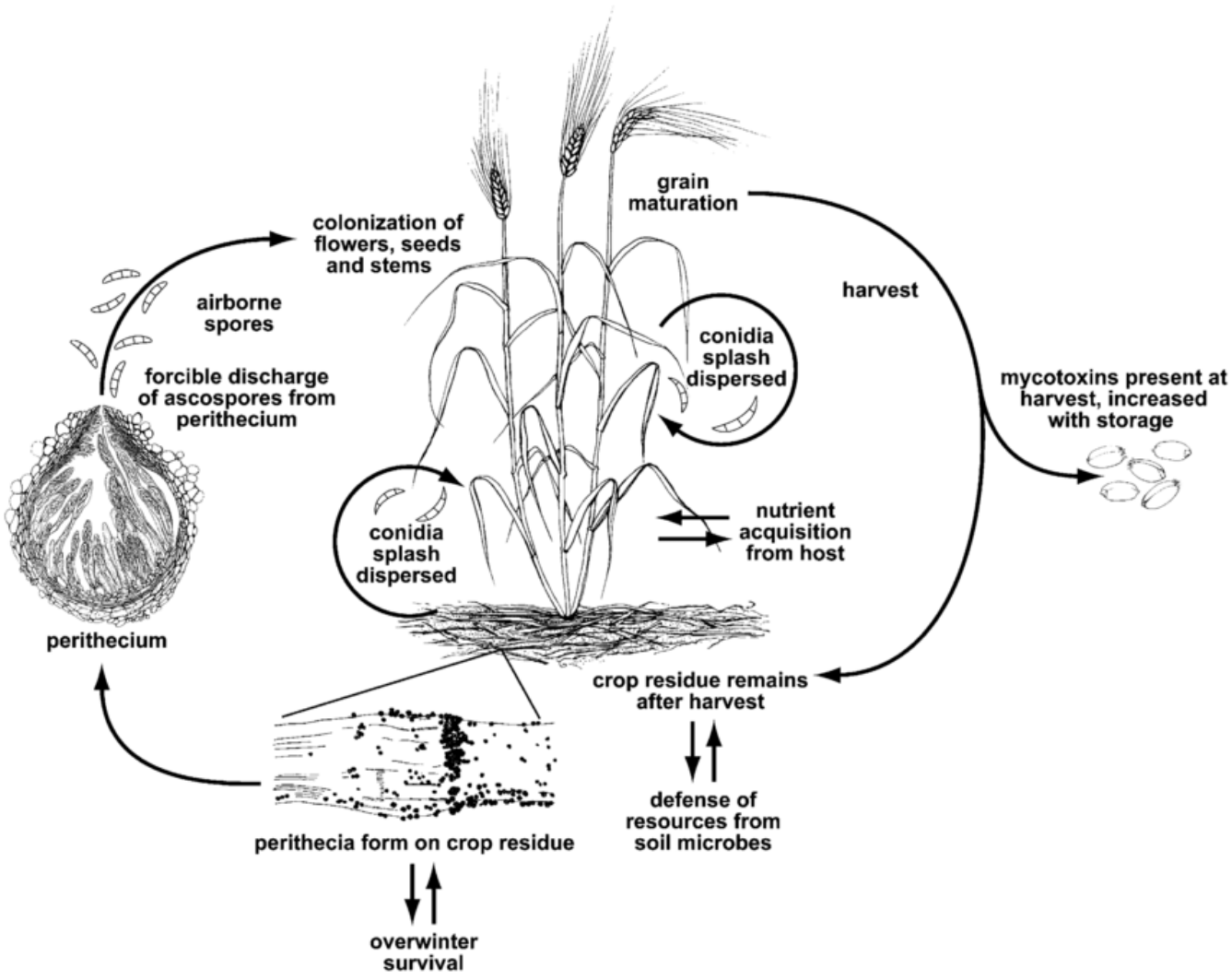

Figure 2. *Lifecycle of F. graminearum (Trail 2009).*

Growth Parameters

FHB is most destructive in regions that are warm and humid (Zhu et al. 2020). *F. graminearum* is typically found in regions with annual temperatures above 15°C or in more temperate climates during growing seasons (Mielniczuk et al. 2020). A study was conducted comparing the amount of pathogen present using their DNA amounts which also determined which species performed best under differing environmental variables. Positive

correlations were found with precipitation and humidity and quantities of *F. graminearum* DNA during flowering of wheat (Birr et al. 2020). Not all species of Fusarium are the same with *F. avenaceum* growing optimally at average annual air temperature 5-15°C and F. *poae* colonizing heads in much drier air (Mielniczuk et al. 2020).

Annual fluctuations in temperature and humidity lead to variation in the frequency and severity of FHB epidemics. A study was conducted by Bertuzzi analyzing the different FHB species and occurrence amounts over a five-year period. Their results showed the overall dominance of *F. graminearum*, peaking in 2013 with occurrence at 92.4% over other species (Bertuzzi et al. 2020). The less dominant species shows yearly shifts in frequency with less dominant occurring more frequently when environmental conditions were not favorable for dominant species (Bertuzzi et al. 2020). The highest Fusarium totals have been shown in the year 2013 and 2017 at 64.9% and 67.6%, respectively (Birr et al. 2020). This shows how yearly fluctuations of infection are observed due to optimal conditions for the promotion of infection not always present. Peak infection years for *F. graminearum* will have temperatures around 21°C and relative humidity around 85% for at least 48 hours (Manstretta et al. 2015).

Damage Due to Fusarium Head Blight

Even a mild *Fusarium* infection can result in reduced yield, with shriveled and chalky grain. Infection results in damage to the starch granules and causes changes in starch composition (Packa et al. 2012). Infection to the stem can also lead to the wheat head senescing early due to the lack of water and nutrient transportation (Birr et al. 2020). Besides the loss associated with the grain, one of the main concerns when looking at Fusarium infection is the production of mycotoxins such as deoxynivalenol (DON). DON in animal feeds results in lower weight gain due to the loss of appetite and vomiting. Other symptoms have been observed such

as necrosis of tissues such as bone marrow and lymphoid, and inflammation of intestinal epithelium (Mielniczuk et al. 2020). These symptoms are observed at levels of 1-3 ppm with the worst symptoms being shown >10ppm (Wolf et al. 2003). Animals are less sensitive to DON with the ability of swine for example able to consume up to 5 ppm as compared to humans requiring less than 1 ppm in food products.

Resistance to Fusarium Head Blight

Resistance to FHB in wheat is classified into five types with type I and II being the most observed (Hales et al. 2020). Type I is shown to have resistance to initial infection while type II is the resistance to the spread of infection within the spike. Type III is resistance to kernel infection, type IV is tolerance of infection, and type V is resistance to the accumulation of DON. Resistance is genetic and evidenced in discrete phenotypic characteristics and the expression of resistance genes.

Resistance Sources

Naturally wheat varieties with awns are shown to be more susceptible as compared to awnless varieties (Mesterhazy 2006). This is believed to be due to the larger head area picking up more airborne conidia and dew which enhances Fusarium development. Taller wheat is also commonly associated with an increased resistance to infection. This is believed to be due to Fusarium splashing up to infect its host, shorter wheat varieties being closer to the ground and splash zone are therefore more often infected than taller plants whose heads are farther away.

Many large effect QTL have been identified for FHB resistance (Zhu et al. 2020) with *Fhb1* having the largest effect. *Fhb1* is located on chromosome arm 3BS and is considered the most common and widely studied QTL for FHB resistance. *Fhb1* is associated

with Type I-IV resistance. Type II resistance of *Fhb1* is the most studied and has been shown to reduce the type II response by up to 50% compared to related lines without *Fhb1* (Eldoliefy et al. 2020). *Fhb1* provides Fusarium resistance through various possible ways. One study found that resistance arises from the loss-of-function of the histidine rich calcium binding protein (Su et al. 2019). While other research states resistance is due to a gain-of-function as a result of a different start codon upstream of the original (Li et al. 2019). Other data support the gain-of-function hypothesis, possibility due to multiple genes being involved in the *Fhb1* resistance. *Fhb1* effectiveness can increase when combined with other resistance factors.

The most FHB resistant spring wheat in the U.S is the variety, Glenn, that was released in 2005 additional resistance from the Chinese landrace Sumai3. This variety lacks the *Fhb1* gene, acquiring FHB resistance through other ways. Glenn achieves its resistance through the pyramiding of 12 minor effect QTL (Eldoliefy et al. 2020). This variety was also discovered to have a new major QTL 5BL which was associated with resistance, showing the constant evolution in resistance and breeding. These findings opened up the possibility of other resistance factors being discovered and used in breeding.

Other resistance factors that have been found includes the susceptibility factor that is located on the short arm of chromosome 4D. The loss of this region has been shown to increase resistance to FHB but not lower DON levels (Hales et al. 2020). The 4D region associated with resistance was narrowed to a 31.7 Mbp interval containing almost 300 high confidence genes (Hales et al. 2020).

Another form of resistance arises from manipulation of plant defense responses such as the production of reactive oxygen species (ROS). One such study compared three wheat lines

and found that lines with lower catalase activity (CAT) were shown to slowdown fungal growth, giving the plant more time to produce a defense response (Spanic et al. 2020). The Guaiacol peroxidase activity (GPOD) and Ascorbate peroxidase activity (APX) were shown to be produced in higher amounts in more resistant lines, showing their role in increasing plant defense (Spanic et al. 2020). A variety of other resistance factors have been discovered or are yet to be, making the continued research in this area essential to breeding more resistant lines in the future.

Part 4: Identifying Shifts in Allele Frequencies

The use of new plant breeding technologies has allowed for more advanced plant breeding methods. With tissue collection, wheat can be analyzed and compared to other populations or past generations in order to discover shifts in allele frequencies. Analyzing shifts in allele frequencies can verify how selection is shifting a population and help identify regions with resistance.

Genotyping technologies enable the identification and sequencing of genomic regions associated with traits like disease resistance and for detecting shifts in allele frequencies. Genotyping by sequencing (GBS) identifies single nucleotide polymorphisms (SNP) across the genome. Low cost and high throughput make this technology ideal for implementation on a large scale in plant breeding programs. GBS was utilized in a study on red clover (*Trifolium pratense L.*) survival in various field experiments by comparing survivor vs original populations allele frequencies. SNP data was obtained by GBS of individuals vs pooled DNA to provide analysis of these two techniques along with the main goal of looking for shifts in allele frequencies. From the main study it was found that survivor populations diverged from the original

population in different directions with 27 SNP's having a shift in allele frequency ranging from 0.09 to 0.22 for a $P < 0.1$ (Ergon et al. 2019). It was also found that pooled DNA resulted in a higher accuracy as compared to individuals only when less than 50 reads or more than 600 reads from the sequencing pool (Ergon et al. 2019). These results show that individuals under selection undergo shifts in allele frequencies and using pooled DNA for GBS is sufficient for determining these shifts.

Performing phenotypic selection on wheat has also been shown to cause shifts in allele frequencies. A study was performed on wheat root angle that after two rounds of selection shifted mean root angle by as much as ten degrees when parent lines were phenotypically distinct (Richard et al. 2018). Correlating with the phenotypic change was the change in allele frequency at several genomic regions. Even lines with no phenotypic change had multiple regions under selection identified on various chromosomes. This is believed to be due to multiple genes each with lesser effects as compared to the other lines. Overall, 13 genomic regions "hotspots" associated with seminal root angle were identified with six of these appearing to be newly identified QTL (Richard et al. 2018). This was all achieved with two rounds of selection, showing the potential to shift allele frequencies in wheat lines using phenotypic selection.

Part 5: Chemical Control of Plant Pathogens

Fungicides are used throughout the world to control a variety of fungal diseases such as Fusarium, septoria, and rusts. If used properly they can be an effective strategy to control stripe rust and Fusarium head blight. For developing countries, the cost and availability of fungicides results in many small farms going without. As a result, huge losses have

been observed in stripe rust epidemic years for countries such as Uzbekistan that lost 35% yield in 1998 while similar countries with fungicide used only lost around 2%. With improper and overuse, fungicides have the increased potential to select for resistant pathogens. Fungicides are also not ideal due to the increase in negative health effects to growers. Exposure can occur by absorbance through the skin and lungs, or contamination in the water supply causing severe effects such as headaches, dizziness, convulsions, epilepsy, stroke, respiratory disorders, leukemia, heart attacks, cancer, brain and liver tumors, and death (Oluwole et al. 2009). Fungicides are also associated with effecting the nearby biodiversity which is seen by decrease numbers of frogs, insects, aquatic organisms and birds (Oluwole et al. 2009). Although fungicides are commonly used, they are not the best approach when dealing with stripe rust control due to its variety of problems.

Breeding

Breeders cross wheat lines in order to increase positive agronomic traits, and to increase resistance to pathogens and disease. Identification of an ideal trait in one wheat line can be crossed with another line producing hybrids. Newly created hybrids must be tested and analyzed in order to determine if they are superior to their parent lines. Plant breeding allows for genetic divergence into the creation of new cultivars. Breeding is the most cost effective and environmentally approved approach to controlling pathogens (Hu et al. 2020). The ability to have multiple growing seasons with the use of greenhouses also provides the ability to grow out new lines faster and more productively than field use alone.

Breeding does come with some challenges that have yet to be overcome. The initial challenge is finding resistance genes and having the ability to transfer them. Resistance

cannot always be found in the same species and therefore do not breed well together. This is seen with many resistance genes being found in other closely related species to wheat such as other grasses or in wild wheat relatives such as *Aegilops tauschii* (Lee et al. 2020). Breeding with these species is a hard process due to problems such as embryonic failure. Once resistance genes are identified and the use of genetic linkage good resistance traits to be linked with poor agronomic traits. Although there are many challenges for plant breeding, research is constantly being devoted to overcoming them.

CHAPTER 2: Response to Early Generation Selection for Stripe Rust Resistance in Winter Wheat Breeding Populations

Introduction

Stripe rust of wheat is caused by the rust fungus *Puccinia striiformis* f.sp. *tritici.* Stripe rust is one of the most serious pathogens affecting wheat with the ability to spread rapidly over vast areas under favorable conditions (Chen 2020). Globally 5.47 million tons of wheat are lost annually as a result of stripe rust, at a cost of nearly $1B USD (Getnet et al. 2020). Yield losses are due to reduced kernel weight, number of grains per spike and photosynthetic capacity (Al-Maaroof et al. 2019). Increased aggressiveness increases the scope and severity of epidemics worldwide (Milus et al. 2009). Larger population size has increased the frequency of *P. striiformis* f. sp. *tritici* races virulent to widely deployed resistance genes, due to stepwise mutations (Khan et al. 2020). With the increased ability to survive, mutate and reproduce under a broader range of temperatures, epidemics are occurring more frequently and with a greater effect.

Resistance to stripe rust can be expressed across all growth stages in wheat or at the adult plant stage. Resistance can be race-specific or race non-specific and can be observed at the seedling stage through adult plant stages as all stage resistance. All stage resistance is generally race specific conferred by a major gene which is less durable compared to adult plant resistance which is race non-specific with minor effect genes (Liu et al., 2018).

The resistance response to stripe rust can be characterized as no visible interaction, small hypersensitive flecks, or uredinia surrounded by chlorosis or necrosis (Chen 2014). Infection type is ranked on a scale from 0-9 (McNeal et al., 1971). A rating of zero shows no visible uredia

and is immune while a score of 9 has abundant sporulation. and an absence of any resistance response.

Resistant varieties is the most cost effective and environmentally approved approach to controlling pathogens (Hu et al. 2020). Wheat varieties with resistance to stripe rust demonstrate higher yields under stripe rust disease pressure (Abro et al. 2017). Currently there are over 80 official genes and over 300 quantitative trait loci (QTL) for stripe rust resistance (Chen 2020). Using DNA markers, breeding programs can use this knowledge in selecting for stripe rust resistance lines. Other forms of selection are typically done in field settings with visual selections for resistance. Field trials are dependent on environmental effects often leading to ineffective selection. Field trials also provide one grow out a year resulting in much longer timeframes for shifts in resistance to be observed.

Early generation selection aims to improve breeding efficiency by culling undesirable genotypes in early generations allowing the advancement of desirable genotypes (Clement et al. 2014). Performing early generation selection has been done on a variety of crops and traits of interest. In a study focused on root angle, after two rounds of selection the mean root angle was shifted by as much as ten degrees (Richard et al. 2018). A shift in allele frequency was observed at several genomic regions that correlated with the shift in the population mean. Even lines with no phenotypic change had multiple regions under selection across multiple chromosomes. Overall, 13 genomic regions “hotspots” associated with seminal root angle were identified with six of these appearing to be newly identified QTL (Richard et al. 2018).

In the absence of selection, at a single disease resistance locus, an approximately equal proportion of resistance and susceptibility alleles are expected among inbred individuals. As directional selection is applied for major gene disease resistance in early inbreeding generations,

a shift in allele frequencies is expected, every generation. Selection for resistance and against susceptibility removes the homozygous susceptible individuals. A corresponding increase in frequency is expected for resistance and susceptibility alleles are expected to decrease.

The objective of this study was to perform early generation selection in wheat breeding populations segregating for stripe rust resistance to test whether artificial selection increasing the frequency of stripe rust resistance in segregating populations and recombinant inbred lines (RILs) derived from populations undergoing selection. This study is done in a controlled greenhouse setting allowing infection and selection to be consistent across populations and generations. The ability to control temperature in the greenhouse allows for two generations of selection each year. With selection for stripe rust, lines can be analyzed for shifts in resistance and allele frequencies.

Materials and Methods

Plant Materials

The 12 populations used in selection experiments were derived from crosses among 15 soft winter wheat parents in the 2019 crossing cycle of the Michigan State University wheat breeding program (Table 1). Parents include experimental soft red and soft white winter wheat genotypes from breeding programs at Cornell, Michigan State University, University of Arkansas, University of Georgia, University of Missouri, University of Tennessee, The Ohio State University, and Virginia Polytechnic Institute and State University. The F_1 plants from each cross were self-pollinated to generate segregating F_2 populations used to initiate selection for stripe rust resistance.

Table 1. *Pedigrees of populations used in stripe rust selection experiments*.

Line	Pedigree
MSU19000017	AR06037-17-2/MI14R1140
MSU19000034	E2041/VA09W-188WS
MSU19000052[a]	GA 081298-16LE1/MI14W0064
MSU19000069	E5011B/VA09W-75
MSU19000070	E5011B/VA09W-188WS
MSU19000108	MI14R0011/TN1704
MSU19000141	MI14W0064/VA09W-75
MSU19000177	MI15W0193/E5011B
MSU19000195 [a]	MO080104/MI16W0355
MSU19000222 [a]	OH12-195-22/MI14R1140
MSU19000240	VA09MAS1-12-5-1-3/E5011B
MSU19000262	VA09W-188WS/NY05158-833

[a] Recombinant inbred lines (RILs) derived from these populations at the F_4 generation from selected and control groups.

Experimental Design

Each F_2 population was split into two streams, a control stream and stream undergoing selection for stripe rust resistance. At the F_2, F_3, and F_4 generations, populations were planted in 15.2cm pots, with two untreated control pots and two pots undergoing selection inoculated with stripe rust. Populations were grown at 250 seeds per pot in the F_2 generation and 150 seeds per

pot in the F_3 and F_4 generations. At each generation, all heads were bulk harvested and threshed together.

In populations undergoing selection, after stripe rust inoculation and disease development, heads were removed from susceptible plants. An infection type greater than four (McNeal et al., 1971) is considered susceptible in this study. The number of plants removed and remaining in each pot was recorded at the F_2, F_3, and F_4 generations. The proportion of resistant individuals in each population in each generation was calculated by dividing the number of resistant heads remaining after selection by the total number of heads prior to selection.

A set of 200 RILs were randomly derived from five populations at the F_4 generation, 20 RILs from the control stream and 20 RILs from selected stream. All F_4 plants were inoculated with stripe rust and rated. The entire experimental structure can be visualized in Figure 3.

Stripe Rust Inoculation

P. striiformis inoculations were done using race Pst-37 collected in 2019 at Mason, MI propagated on the spring wheat variety 'Morocco'. Inoculations were performed when the flag leaf was fully emerged on all plants in an individual pot. The amount of 60-70mg stripe rust urediniospores were added to 15ml of Soltrol 170 (Chevron Phillips, Borger, TX) and applied to leaves using an airbrush. Inoculated plants were placed in a dew chamber for 16 hours at 16 °C. Plants were returned to the greenhouse for disease development.

Stripe Rust Rating and Culling of Susceptible Genotypes

Stripe rust evaluations of parents, segregating populations and RILs were performed 14-16 days after inoculation using a 0-9 scale (McNeal et al., 1971). In segregating populations undergoing selection, plants with scores greater than 4 on the 0 to 9 scale were considered susceptible.

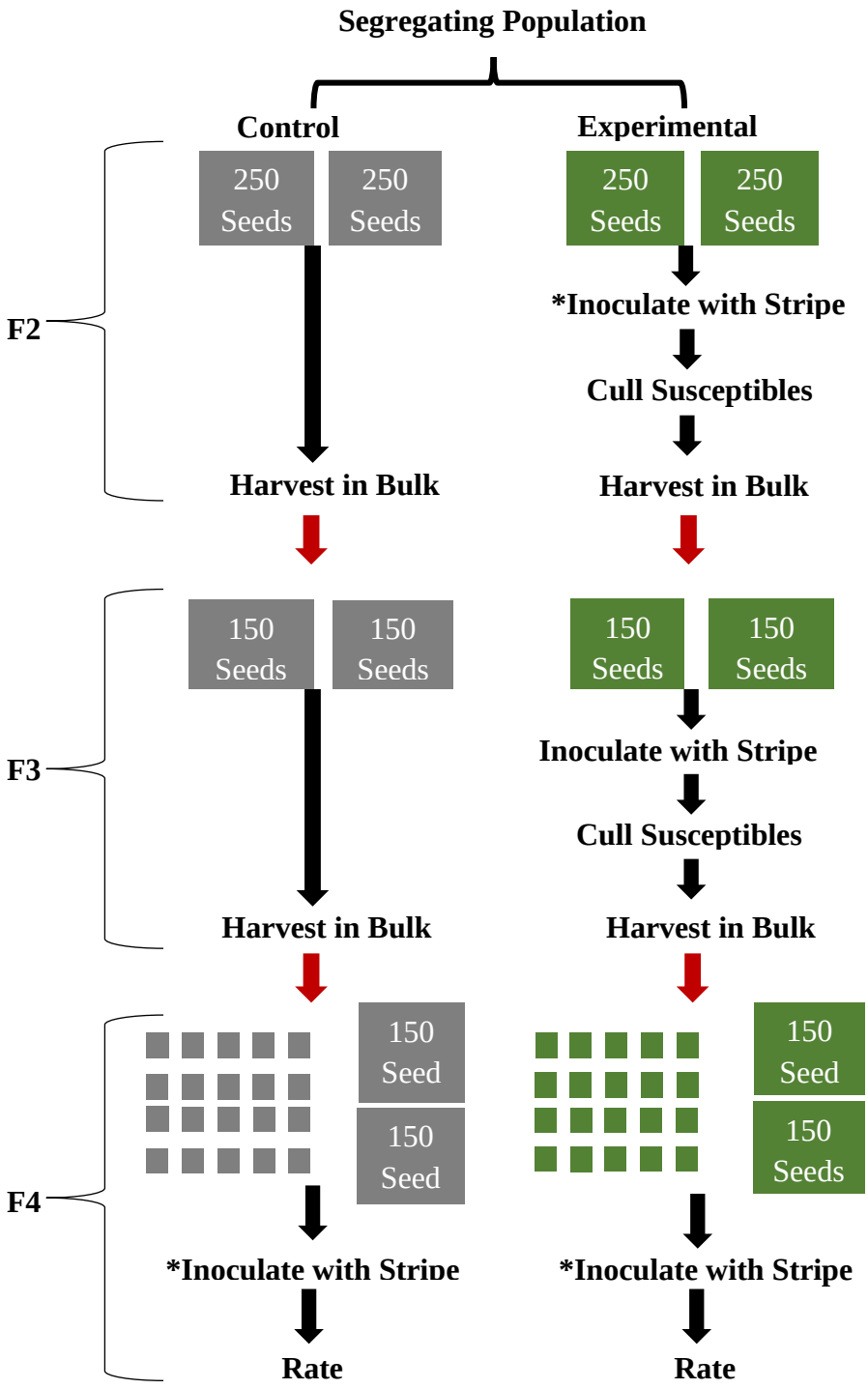

Figure 3. *Experimental Design*. Large boxes represent populations. Small boxes represent recombinant inbred lines derived at the F4 generation. The * indicates the generation where the number of stripe rust resistance genes was predicted by chi square analysis.

Plant Growth Conditions

Seedlings of all segregating populations and parental genotypes were vernalized at 4°C for 8 weeks in 15.24 cm x 15.24 cm ClearSeal® Hinged Lid Plastic Container (Dart Container Corporation, Mason, MI) under 8-h light/16-h dark photoperiods with light supplied with LEDs, and watered as needed with 5mg/ml solution of Thiram (T24201, Sigma Aldrich, St. Louis, MO). After 8 weeks segregating populations were transplanted to 15.24 cm plastic pots in the greenhouse with saturated Sure Mix potting media (Michigan Grower Products Inc., Galesburg, MI). To decrease plant height in segregating populations, chlormequat trimethylammonium chloride (Cycocel®, OHP Inc., Bluffton, SC), was applied at a rate of 40mL per gallon was made. A second application of chlormequat trimethylammonium chloride was made when 25% of plants had a visible internode. A 22-hour photoperiod was used to accelerate development. Greenhouse temperature ranged from 21°C to 27°C. Watering was performed as needed and continued until 75% of all plants in a pot reached physiological maturity (Feekes 11). Water soluble fertilizer (20-20-20 NPK) was applied two weeks after transplant and once weekly until plants reached Feekes 11 at a rate of 188g/ 3.8L H20 at 1:50 dilution.

RILs were vernalized in 288 cell trays containing saturated Sure Mix potting media (Michigan Grower Products Inc., Galesburg, MI) with a single seed in each well RILs at 4°C for 8 weeks. Individual seedlings were transplanted into 7.62 cm plastic pots. Watering was performed as needed and continued until 75% of all plants in a pot reached physiological maturity (Feekes 11). Water soluble fertilizer (20-20-20 NPK) was applied 2 weeks after transplant and once weekly until plants reached Feekes 11 at a rate of 188g/ 3.8L H20 at 1:50 dilution.

DNA Isolation and Genotyping

Parents and 80 RILs from populations MSU19000195 and MSU19000222 were genotyped using genotyping-by-sequencing (GBS). Tissue was collected RILs and parental DNA was isolated according to Wiersma et al. (2016). GBS libraries were prepared according to Poland et al. (2012) using *PstI* and *MspI* enzymes scaled to a 24uL volume in 384-well format. Libraries were sequenced on an Illumina Next Seq 500 instrument to generate 92bp single end reads. An average of 2.3 million reads were generated for each parent and RIL. Single nucleotide polymorphisms (SNPs) were called using the TASSEL 5 GBS pipeline (Glaubitz et al., 2014) using a kmer length of 64bp and minimum kmer count of 5. Reads were aligned to the RefSeq v1.0 wheat reference genome assembly (International Wheat Genome Sequencing Consortium et al., 2018) using the Burrows-Wheeler Aligner (Li and Durbin, 2009). SNPs were filtered for minimum and maximum allele frequencies of 0.4 and 0.6, respectively for selected and control RILs within populations.

Predicting the Number of Stripe Rust Resistance Genes

Chi square analysis was used to predict the number of stripe rust resistance genes segregating in each population. In the F_2, populations are expected to segregate 3 to 1, 15 to 1 and 63 to 1 ratios, resistant to susceptible for one, two and three genes, respectively. The initial gene number predictions made in the F_2 were tested again by inoculating the F_4 control populations with stripe rust. F_4 control populations that were selfed are expected to segregate approximately in 1.3 to 1, 4.2 to 1 and 11 to 1 ratios, resistant to susceptible for one, two and three genes, respectively.

A chi-square test was performed in the populations undergoing selection at the F_4. The observed ratio of resistant to susceptible was compared to an expected ratio of a selfing with selection and removal of the homozygous susceptible genotypes at the F_2 and F_3 generation. In the F_4, populations undergoing selection the ratio of resistant to susceptible genotypes are expected to be 15 to 1, 19 to 1 and 31 to 1 for one, two and three genes, respectively ratio.

Comparisons between Control and Selected Populations

The proportion of resistant individuals was compared between F_4 control and selected populations. The proportion of resistant individuals for each replicate of selected populations was calculated as the number of resistant plants remaining divided by the total number of plants.

Comparisons between Recombinant Inbred Lines Derived From Control and Selected Populations

The Mann Whitney U test was used to compare the mean infection type among RILs derived from control or selected populations and compare RILs from control and selected groups within populations. Mann Whitney U tests were performed in Excel. Boxplots of comparisons were developed in RStudio version 4.0.2.

Detecting Signatures of Selection

Differentiation between 20 experimental and 20 control RILs was estimated in two populations by computing Fst values at individual SNP positions across the genome. Fst values for individual SNPs were calculated according to Weir and Cockerham (1984) using vcftools (Danecek et al., 2011). In MSU19000195 (MO080104/MI16W0355), a total of 2,535 SNPs were tested and in MI19000222, (OH12-195-22/MI14R1140) 4,370 SNPs were tested. A Fst significance threshold for each population was determined using Rosner's Outlier Test implemented in R (R Core Team, 2019) using the rosner function of the EnvStats package (Millard and Kowarik, 2020).

Results

Stripe Rust Response in Parental Genotypes

Populations were initially selected from the MSU wheat breeding program based on preliminary stripe rust resistance data on the parental genotypes. Parents of each population were inoculated with stripe rust race Pst-37 and given a score for infection type (Table 2). Most parents show resistance with infection types ranging from 0 to 4. Four out of the fifteen parents scored greater than an infection type of 4 which for this study were classified as susceptible.

Table 2. *Stripe rust infection types of parents at 16 days after inoculation*.

Parent	Stripe Rust Infection Type
AR06037-17-2	1.5
Table 2 (cont'd)	
E2041	6.5

GA 081298-16LE1	0
E5011B	7.5
MI14R0011	1.5
MI14R1140	4
MI14W0064	6
MI15W0193	1.5
MI16W0355	4.5
MO080104	0.5
NY05158-833	2.5
OH12-195-22	0.5
TN1704	1.5
VA09MAS1-12-5-1-3	1.5
VA09W-75	2
VA09W-188WS	2

Determining the Number of Stripe Rust Resistance Genes Segregating

The number of genes segregating in each population was estimated at the F_2 and F_4 generations (Table 3). Populations were shown to have one to three genes segregating for stripe rust resistance. All F_2 populations were not significantly different from expected for the predicted gene number. This trend is also observed in the F_4 with only three populations showing significant differences from expected based on the number of genes predicted in the F_2.

Table 3. *Chi square analysis of F_2 selected and F_4 control populations to predict number of segregating genes present in the population.*

Population	Pedigree	Genes	F_2 P-value	F_4 P-value
MSU19000017	AR06037-17-2/MI14R1140	3	0.493	0.287
MSU19000034	E2041/Venus	1	0.528	0.002*
MSU19000052	GA 081298-16LE1/MI14W0064	2	0.378	0.657
MSU19000069	Jupiter/VA09W-75	1	0.455	0.112
MSU19000070	Jupiter/Venus	2	0.694	0.222
MSU19000108	MI14R0011/TN1704	2	0.756	2.6E-4*
MSU19000141	MI14W0064/VA09W-75	1	0.278	0.557
MSU19000177	MI15W0193/Jupiter	2	0.249	0.474
MSU19000195	MO080104/MI16W0355	1	0.381	6.4E-6*
MSU19000222	OH12-195-22/MI14R1140	2	0.822	0.533
MSU19000240	VA09MAS1-12-5-1-3/Jupiter	2	0.385	0.056
MSU19000262	Venus/NY05158-833	3	0.146	0.405

* Significant difference at the .05 probability level.

F_4 Selected Populations

Chi square analysis of F_4 selected populations demonstrated the frequency of resistant genotypes was not significantly different from expected following two rounds of selection against susceptible genotypes (Table 4). F_4 selected populations showed an increase in resistance compared to control populations (Table 5). Control F_4 populations that had no selection ranged from 60-97% resistant individuals. F_4 populations undergoing two prior rounds of selection at the F_2 and F_3 generations had proportions of resistant individuals ranging from 80-

100%. Within each population there was an observed increase in proportion of resistant individuals in all but one of the 12 populations. This decrease in resistance could be due to the fact that the population already had a high level of resistance with three genes, so selection did not play role in increasing the already high frequency of resistant genotypes.

Table 4. *Chi square analysis of F_4 selected populations in comparison to expected ratios after selection.*

Population	Genes	P-value
MSU19000017	3	0.474
MSU19000034	1	0.497
MSU19000052	2	0.241
MSU19000069	1	0.064
MSU19000070	2	0.506
MSU19000108	2	0.446
MSU19000141	1	0.182
MSU19000177	2	0.208
MSU19000195	1	0.550
MSU19000222	2	0.772
MSU19000240	2	0.825
MSU19000262	3	0.104

Table 5. *Number of predicted genes segregating and frequency of stripe rust resistant F_4 individuals within selected and control populations*. Values presented for control and selected groups are the proportion of resistant individuals.

Population	Genes	Control	Selected
MSU19000017	3	97	100
MSU19000034	1	80	90
MSU19000052	2	79	98
MSU19000069	1	71	80
MSU19000070	2	83	97
MSU19000108	2	60	94
MSU19000141	1	62	89
MSU19000177	2	83	92
MSU19000195	1	84	98
MSU19000222	2	81	94
MSU19000240	2	67	95
MSU19000262	3	93	88

F_4 Recombinant Inbred Lines

The average infection type of control and selected RILs were compared for each population. A significant decreases in infection type was observed for RILs derived from all but one selected populations (Table 6, Figure 4). The largest decrease in infection types was 2.9 for MSU190000141. Population MSU19000052 had demonstrated a decrease in infection type for selected RILs at 0.9 but this decrease was not significant. Among all populations, an average decrease infection type decrease of 1.85 was observed. Among all populations the decrease in infection type was highly significant (p=1.67E-12).

Table 6. *Mean infection types of recombinant inbred lines (RILs) derived from control and selected populations and the difference between control and selected means*. P-values are from the Mann-Whitney U test for differences in mean infection types of RILs derived from selected and control populations.

Population	Control	Selected	Decrease	P-value
MSU19000052	4.25	3.35	0.90	0.064
MSU19000141	5.95	3.05	2.90	1.270E-05*
MSU19000195	5.90	4.20	1.70	0.001*
MSU19000222	4.25	2.45	1.80	9.871E-05*
MSU19000240	3.75	1.80	1.95	2.629E-4*
All Populations	4.82	2.97	1.85	1.670E-12*

* Significant difference at the .05 probability level.

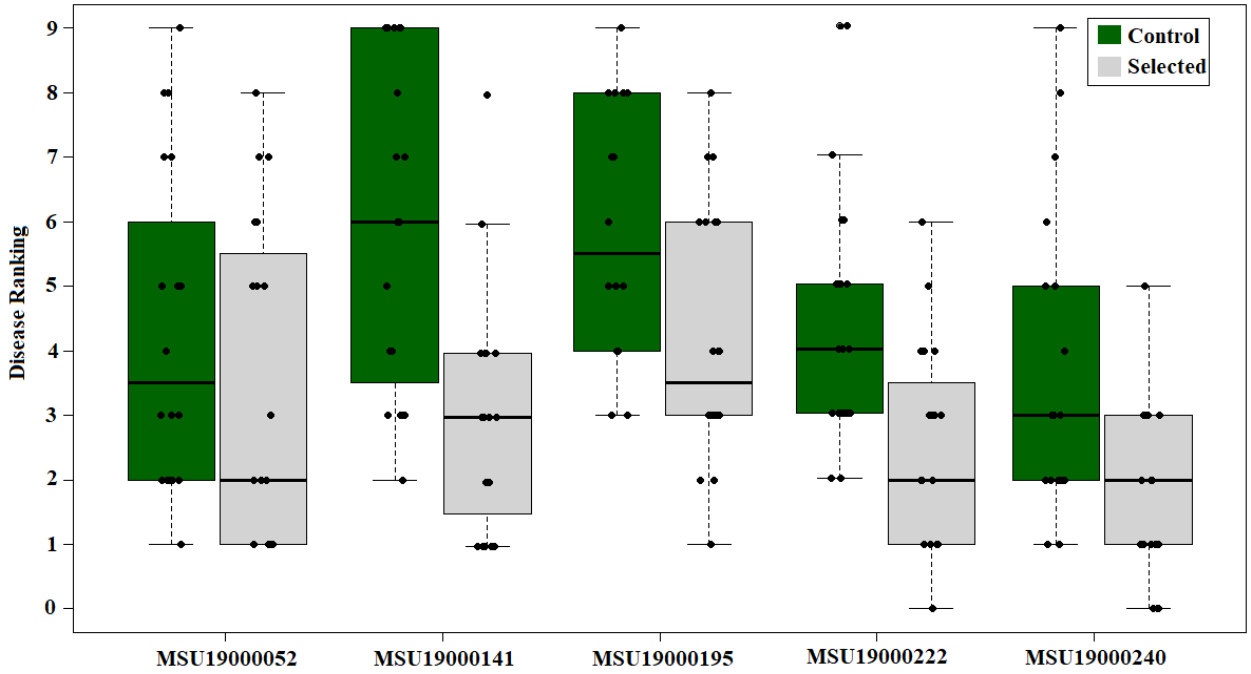

Figure 4. *Infection types of recombinant inbred lines derived from selected and control populations.*

Signatures of Selection for Stripe Rust Resistance

Allele frequency shifts were detected in two populations. In MSU19000195 RILs, a total of 2,535 SNPs were tested for selection. Four regions on chromosomes 1AS, 4AS, 4AL, 5AL and 6BS were differentiated between control and selected RILs. In MSU19000222 RILs a total of 5,442 SNPs were tested for selection. Four chromosome regions on 3AL, 5DS, 7BL and 7DS were differentiated between control and selected RILs.

Table 7. *Genomic regions differentiated between recombinant inbred lines derived from selected and control populations*.

Population	SNPs	Significant SNPs	Chr.*	Mb**
MSU19000195	2,535	10	1AS	25.3
			4AS	86.3
			4AL	153.8
			5AL	115.2
			6BS	153.4
MSU19000222	5,442	5	3AL	213.2
			5DS	11.0
			7BL	246.7
			7DS	99.8

* Chromosome location of significant SNP

** Position of the most significant SNP in Mbp

Discussion

In this study, early generation selection for resistance to stripe rust was shown to increase the frequency of resistance from the F_2 to F_4 generation. F_4 populations undergoing selection in the F2 and F3 generations demonstrated a significant increase in the proportion of resistant

individuals when compared to the control. Due to the lack of replicates significance could not be calculated between populations but a clear increase in resistance is observed. Future studies can be done with higher replication in order to provide proof of significant increases in resistance. The Chi square test was an alternative to the significance test since F_4 populations should follow an expected ratio for susceptible and resistant heads in a population. The Chi square analysis showed that the resistant verse susceptible F_4 heads are not significantly different than what we would expect them to be, providing an alternative test to show the study did perform as hypothesized.

RILs showed significant differences between the control and selected in all but one population, MSU1900052. Due to the phenotype being more intermediate, it was a challenge to distinguish susceptible verse resistant heads for this population. Infection types of four or less are considered resistant as is shown in all averages of selected RILs. Heads scoring over four had been removed from previous populations decreasing the number of susceptible genotypes present in the F_4 RILs. If more generations with selection were performed this value would continue to decrease with the removal of almost all homozygous recessive individuals from the population. The control RILs did not have selection performed which is reflected in average infection type of almost six for certain populations. A score of six is still considered moderately resistant but this study used high standards in selection in order to observe faster and stronger selection pressure. Scores higher than five have moderate sporulation and necrosis which decreases the yield and quality of wheat. Removing these individuals from the population and only keeping scores of four or less provides for high selection pressures but still maintains diversity without strong fixation of traits.

Shifts in allele frequencies were observed between selected and control RILs showing selection moving a population towards higher resistance when under selection. Allele comparison between selected and control found significant differences for multiple SNPs. Some SNPs that were significantly different are associated with chromosomes known for stripe rust resistance. *Yr18* is be located on 7D which was found to be significantly different in MSU19000222. *Yr17,* located on 2A, is the resistance gene most commonly used in breeding programs (Milus. 2015). Although we had selected out parents with known *Yr17* resistance genes, these were not detected as significant differences in allele comparisons.

These findings show that adding early generation selection does increase frequency of stripe rust resistance among inbred wheat lines derived from segregating populations. Use of this research will allow for the rapid advancement of resistance in breeding programs. In correlation with other research, rapid advancement of resistance will produce higher yielding wheat to meet the increasing demand (Ellis. 2014). The ability to perform multiple rounds of selection in a greenhouse speed breeding type of approach (Minibulks) makes this study unique to the Michigan State University wheat breeding program. After selection for resistance lines will be yield tested with top performers being released as new varieties. Future studies can expand upon this research by performing selection not only for resistance but also selection for susceptibility. Selecting susceptible lines and selecting resistant lines from the same starting population can show a stronger divergence and shift in allele frequencies.

CHAPTER 3: Response to Early Generation Selection for Fusarium Head Blight Resistance in Winter Wheat Breeding Populations

Introduction

Fusarium head blight (FHB) of wheat in the United States is caused primarily by the ascomycete fungus *Fusarium graminearum* Schwabe. FHB is a threat to farmers causing significant reductions in grain yield and quality, effecting wheat and other crops such as corn and barley (Hales et al. 2020). Even a mild FHB infection can result in reduced yield, with shriveled and chalky grain that accumulate high levels of mycotoxins including deoxynivalenol (DON). Infection results in damage to the starch granules and causes changes of the starch composition (Packa et al. 2012). Infection to the stem can also lead to the wheat head to senesce early due to the lack of water and nutrient transportation (Birr et al. 2020). FHB is most destructive in regions that are warm and humid (Zhu et al. 2020). *F. graminearum* is typically found in regions with annual temperatures above 15 °C or in more temperate climates during growing seasons (Mielniczuk et al. 2020). In the United States losses due to FHB epidemics from the year 1993-2001 totaled more than $2B (Xia et al. 2020).

Developing resistant varieties is the most cost effective and environmentally conscious approach to controlling FHB (Hu et al. 2020). Resistance to FHB in wheat is classified into five types affecting the initial infection and spread of the disease in the wheat spike and grain (Hales et al. 2020). Breeding for resistance to FHB involves selection type II resistance to the spread of infection within the spike (Chen et al., 2019). Many large effect QTL have been identified for FHB resistance (Zhu et al. 2020) with *Fhb1* having the largest effect. *Fhb1* is located on chromosome arm 3BS and is considered the most common and widely studied QTL for FHB

resistance. Type II resistance conferred by *Fhb1* has been shown to reduce infection by up to 50% compared to related lines without *Fhb1* (Eldoliefy et al. 2020). In wheat breeding programs, *Fhb1* can be selected based on the resistance phenotype and marker assisted selection.

Visual selection for FHB resistance in a wheat breeding context is typically done in a disease nursery setting to evaluate the level of resistance under heavy FHB disease pressure. Field trials are limited by the ability to perform only one grow out a year resulting in much longer timeframes for shifts in resistance to be observed. Field trials are also dependent on the environment due to the interaction it has on disease development. Bokore et al. (2017) evaluated spring wheat varieties and found that environment played a role in the expression of *Fhb1* resistance with higher levels of resistance at specific locations. Each location had variable and unique weather conditions, and therefore disease development, leading to difficult and inaccurate field selections. Progress on breeding for resistance to FHB depends on the ability to accurately and efficiently evaluate resistance.

Early generation selection aims to improve breeding efficiency by removing genotypes carrying undesirable traits in early generations while increasing the frequency of individuals carrying desired traits (Clement et al. 2014). In wheat and rye, early generation selection for visual FHB resistance and lower DON was successful in removing susceptible genotypes early in a breeding program (Miedaner et al., 2003). As early as the F_3 generation, transgressive segregants could be identified with reduced DON and higher FHB resistance.

Selective pressures on plants will lead to shifts in allele frequencies. A study performed in *Arabidopsis* demonstrated allele frequency shifts in response to high temperatures and low precipitation (Exposito-Alonso et al.,2019). Under these harsh growing conditions selection eliminated 63% of genotypes and 5% having significant changes in allele frequency. Applying

selection during generation advancement in a breeding context should also cause shifts in the frequency of alleles associated with phenotypes under selection.

The objectives of this study were to determine the impact of early generation selection for FHB resistance. The impact of selection on type I and type II resistance was evaluated in both segregating populations and inbred lines derived from populations undergoing selection. Shifts in allele frequencies in response to selection were detected.

Materials and Methods

Plant Materials

The 26 populations used in selection experiments were derived from crosses among 32 soft winter wheat parents in the 2019 crossing cycle of the Michigan State University wheat breeding program (Table 8). Populations were selected based on the expectation of segregation for resistance to FHB and the presence of *Fhb1*. Parents include experimental soft red and soft white winter wheat genotypes from breeding programs at Cornell, KWS Cereals (Champaign, IL), Limagrain Cereals (West Lafayette, IN), Michigan State University, University of Arkansas, University of Illinois, University of Kentucky, University of Missouri, The Ohio State University, and Virginia Polytechnic Institute and State University. The F_1 plants from each cross were self-pollinated to generate segregating F_2 populations used to initiate selection for FHB resistance.

Table 8. *Populations evaluated in the current study*

Line	Pedigree
MSU19000053	IL12-17257/MI16R0677
MSU19000076 [b]	KWS095/MI16R0936
MSU19000078 [b]	KWS095/VA09MAS1-12-5-1-3
MSU19000110	MI14R0082/MO151126
MSU19000111 [a]	MI14R0082/X08-1181-61-15-5
MSU19000112 [ab]	MI14R0267/KWS095
MSU19000113	MI14R0267/LES15-5443
MSU19000114	MI14R0267/MI16R0936
MSU19000115	MI14R0267/MO151126
MSU19000119 [ab]	MI14R0330/KY09C-1245-100-1-3
MSU19000162	MI14W1039/VA16W-149
MSU19000167 [b]	MI15R0388/IL13-20616
MSU19000185	MI16R1172/KY07C-1145-94-12-5
MSU19000186	MI16R1172/MI14R0267
MSU19000187	MI16R1172/VA09MAS1-12-5-1-3
MSU19000188 [b]	MI16W0209/MI14W0190
MSU19000196 [a]	MO151126/MI16R1172
MSU19000197 [b]	MO151126/OH11-118-18
MSU19000206 [c]	NY09087-15-69-1124/IL13-20616
MSU19000208 [c]	NY09087-15-69-1124/MI14R0330
MSU19000217 [b]	OH11-118-18/VA09MAS1-12-5-1-3

Table 8 (cont'd)

MSU19000255	VA16W-149/AR07133C-19-4
MSU19000259 [b]	VA16W-149/MI14W0190
MSU19000260 [a]	VA16W-149/MI15R0388
MSU19000263	X08-1181-61-15-5/IL14-11911
MSU19000264[b]	X08-1181-61-15-5/MI14W0190

[a] Recombinant inbred lines (RILs) derived at the F_4 generation from selected and control groups.

[b] Populations segregating for *Fhb1*

[c] Populations fixed for *Fhb1*

Experimental Design

Each F_2 population was split into two streams, a control stream and stream undergoing selection for stripe rust resistance. At the F_2, F_3, and F_4 generations, populations were planted in in 15.2cm pots, with two untreated control pots and two pots undergoing selection inoculated with stripe rust. Populations were grown at 250 seeds per pot in the F_2 generation and 150 seeds per pot in the F_3 and F_4 generations. At each generation, all heads were bulk harvested and threshed together.

In populations undergoing selection, after FHB inoculation and disease development, susceptible heads were removed. The number of plants removed and remaining in each pot was recorded at the F_2, F_3, and F_4 generations. The proportion of resistant individuals in each population in each generation was calculated by dividing the number of resistant heads remaining after selection by the total number of heads prior to selection.

A set of 200 RILs were randomly derived from five populations at the F_4 generation, 20 RILs from the control stream and 20 RILs from selected stream. All F_4 plants were inoculated with FHB and rated for disease severity. The entire experimental structure can be visualized in Figure 5.

Parents of all populations were also grown in 15.2cm pots at 150 seeds per pot and evaluated for FHB incidence and severity. Parents with FHB severity less than 50% were classified as resistant and greater than 50% severity as susceptible.

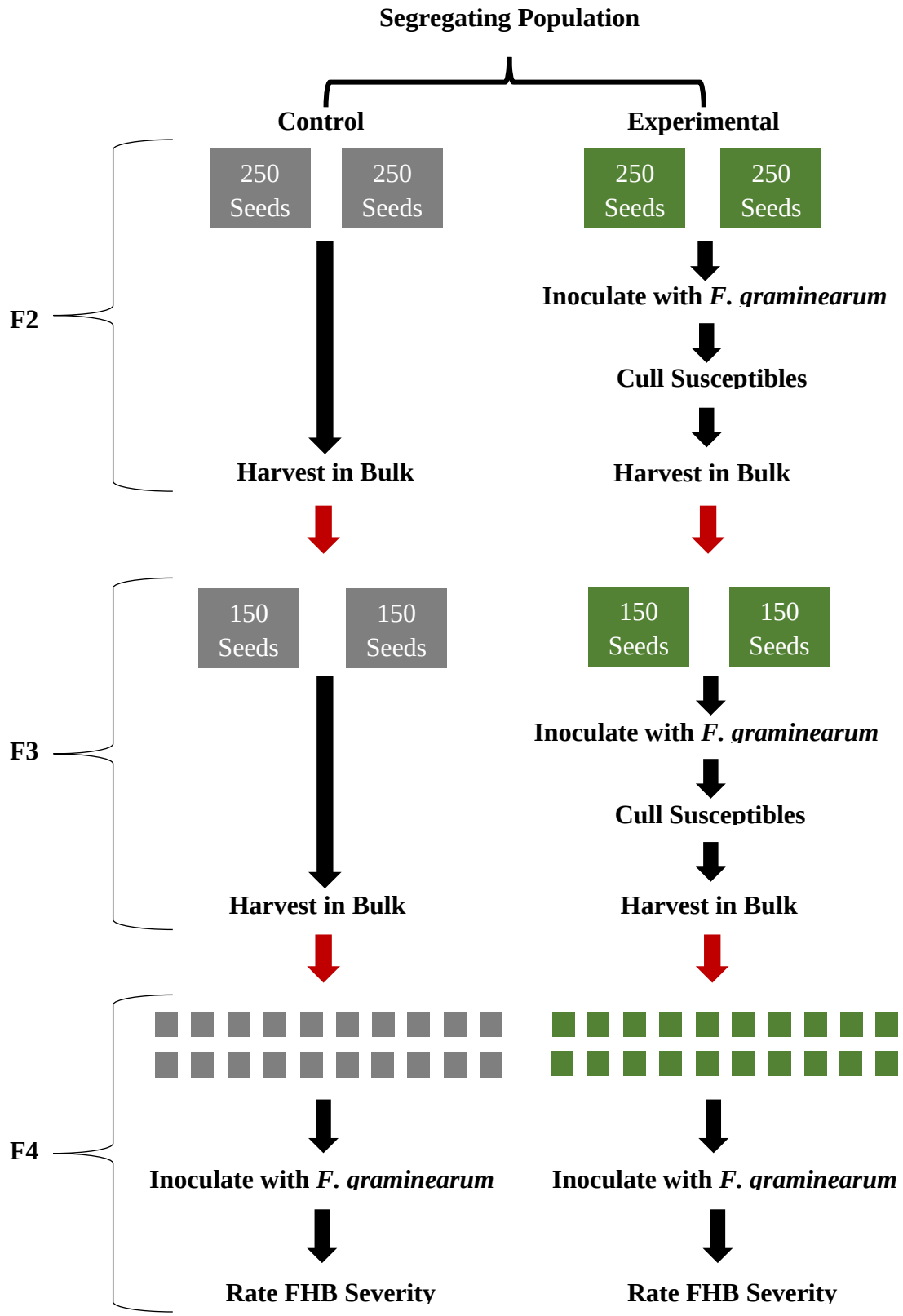

Figure 5. *Experimental Design*

Fusarium Conidia Preparation

F. graminearum conidia were cultivated by inoculating 100ml of Carboxymethyl Cellulose broth with 10μL of conidia of the *Ph-1* isolate obtained from the Trail lab at Michigan State University. Broth was incubated at 25˚ C for three to five days while agitated at 225 rpm. Flasks were then filtered with miracloth to remove mycelium and centrifuged at 4000 rpm for ten minutes. The pellet formed at the bottom of the tubes was the conidia. Liquid above the pellet was discarded and the remaining pellet was diluted in ddH2O to a concentration of $1x10^5$. A Graco TC Pro cordless handheld airless sprayer (Graco Inc., Minneapolis, MN) was used to apply 50ml of conidia per population.

Evaluation of FHB Resistance

Incidence and severity were recorded for 24 parental genotypes. Severity (type II resistance) was rated at 14- and 21-days post inoculation as the percentage of spikelets in a single head showing FHB infection. Incidence (type I resistance) was rated as the percentage of heads within a pot showing FHB infection.

Inoculation of Segregating Populations

Inoculation of F_2 and F_3 populations with *Fusarium* conidia was initiated when 50% of the heads in a pot reached anthesis. After inoculations 45.7 cm x 45.7 cm x 101.6 cm plastic bags (Uline, Pleasant Prarie, WI) propped up by modified tomato cages were used to cover the pots in order to maintain a humid environment (Figure 6). Bags remained on the pots for 24 hours after inoculation and were removed for 24 hours followed by a subsequent inoculation.

Each experimental population was inoculated 3 times following this pattern to ensure inoculation of all heads flowering at different times.

FHB resistance in segregating populations was assessed 21 days after the final inoculation. Heads with 50% or greater severity culled from populations. The percentage of resistant individuals in each population in each generation was calculated by dividing the number of heads remaining after culling by the total number of heads prior to culling.

Figure 6. *Post Inoculation Experiment Set Up.*

Inoculation of Recombinant Inbred Lines

RILs were inoculated with *Fusarium* conidia at anthesis. After inoculation, heads were covered in a 5.1cm x 5.1cm 12.7cm plastic bag (Uline, Pleasant Prarie, WI) for 3 days. Severity was recorded at 14- and 21-days post inoculation as the percent of spikelets in a single head that showed infection.

DNA Isolation and Genotyping

Parents and 200 RILs from five populations were genotyped using genotyping-by-sequencing (GBS). Tissue was collected RILs and parental DNA was isolated according to Wiersma et al. (2016). GBS libraries were prepared according to Poland et al. (2012) using *PstI* and *MspI* enzymes scaled to a 24uL volume in 384-well format. Libraries were sequenced on an Illumina Next Seq 500 instrument to generate 92bp single end reads. An average of 2.3 million reads were generated for each parent and RIL. Single nucleotide polymorphisms (SNPs) were called using the TASSEL 5 GBS pipeline (Glaubitz et al., 2014) using a kmer length of 64bp and minimum kmer count of 5. Reads were aligned to the RefSeq v1.0 wheat reference genome assembly (International Wheat Genome Sequencing Consortium et al., 2018) using the Burrows-Wheeler Aligner (Li and Durbin, 2009). SNPs were filtered for minimum and maximum allele frequencies of 0.4 and 0.6, respectively for selected and control RILs within populations.

The 80 RILs from populations MSU19000112 and MSU19000119 were evaluated for the presence of *Fhb1*.

Comparisons between Control and Selected Populations

The proportion of FHB-resistant individuals in F_2 and F_3 populations was compared using a standard t-test. The proportion of resistant individuals for each replicate of populations undergoing selection for FHB resistance was calculated as the number of resistant plants remaining after selection divided by the total number of plants.

Comparisons between Recombinant Inbred Lines Derived from Control and Selected Populations

Comparisons were made between RILs derived from control or selected populations using a type 1 t-test. Comparisons were made between RILs derived from control and selected groups within and among populations. T-tests were performed in Excel. Boxplots of comparisons were developed in RStudio version 4.0.2.

Detecting Signatures of Selection

Differentiation between 20 experimental and 20 control RILs was estimated in two populations by computing Fst values at individual SNP positions across the genome. Fst values for individual SNPs were calculated according to Weir and Cockerham (1984) using vcftools (Danecek et al., 2011). A Fst significance threshold for each population was determined using Rosner's Outlier Test implemented in R (R Core Team, 2019) using the rosner function of the EnvStats package (Millard and Kowarik, 2020).

Results

FHB Response of Parental Genotypes

Parents of each population were inoculated with *Fusarium* and rated for severity and incidence (Table 9). Based on severity scores at 21 days after inoculation parents were classified as susceptible with scores >50% and six parents resistant with scores ≤30%. All other parents fell into the slightly to moderately resistant category.

Table 9. *Fusarium head blight parents of selected populations. Incidence and severity scores at 14 and 21 days after inoculation.* Resistance score, susceptible (S), moderately resistant (MR), resistant (R).

Parent	Incidence	Severity (14 days)	Severity (21 days)	Resistance
AR07133C-19-4	33	53	95	S
IL12-17257	85	57	80	S
IL13-20616 [a]	88	39	45	MR
IL14-11911	13	13	19	R
KWS095 [a]	70	27	45	MR
KY07C-1145-94-12-5	60	51	79	S
KY09C-1245-100-1-3	45	36	75	S
LES15-5443	65	50	73	S
MI14R0082	10	10	12	R
MI14R0267	100	53	90	S
MI14R0330 [a]	68	33	60	S

Table 9 (cont'd)

MI14W0190 [a]	43	26	40	MR
MI14W1039	65	70	100	S
MI15R0388	15	38	83	S
MI16R0677	53	25	60	S
MI16R0936	43	50	83	S
MI16R1172	65	43	60	S
MI16W0209	78	47	73	S
MO151126	35	13	15	R
NY09087-15-69-1124 [a]	8.5	13	13	R
OH11-118-18 [a]	13	19	19	R
VA09MAS1-12-5-1-3	70	28	92	S
VA16W-149	45	43	74	S
X08-1181-61-15-5	18	11	30	R

[a] Parents with *Fhb1*

FHB Selection in F_2 and F_3 Generations

For each population the percent resistance was calculated at the F_2 and F_3 generation (Table 10). An increase in resistance was observed in 18 of the 26 populations with the largest increase being 17%. While increases in resistance were observable from the F_2 to the F_3, the increase was statistically significant in only one population, MSU19000053 (IL12-17257/MI16R0677). Six populations showed a decrease in resistance while two populations stayed at 100% for both F_2 and F_3 generations.

Table 10. *Comparison of populations from the F2 to F3 generation.* T-test comparison for significance.

Population	Percent Resistant F2	Percent Resistance F3	Percent Change in Resistance	Pvalue
MSU19000053	86	54	-32	0.04*
MSU19000076	87	82	-5	0.22
MSU19000078	83	84	1	0.25
MSU19000110	91	98	7	0.06
MSU19000111	86	99	13	0.10
MSU19000112	75	92	17	0.17
MSU19000113	81	83	2	0.26
MSU19000114	83	83	0	0.47
MSU19000115	93	86	-7	0.06
MSU19000119	77	90	13	0.06
MSU19000162	84	89	5	0.10
MSU19000167	86	88	2	0.24
MSU19000185	97	98	1	0.03*
MSU19000186	84	66	-18	0.16
MSU19000187	80	82	2	0.46
MSU19000188	82	83	1	0.17
MSU19000196	82	85	3	0.29
MSU19000197	96	95	-1	0.17

Table 10 (cont'd)

MSU19000206 [c]	100	100	0	-
MSU19000208 [c]	100	100	0	-
MSU19000217	83	87	4	0.39
MSU19000255	82	86	4	0.31
MSU19000259	87	92	5	0.17
MSU19000260	82	93	11	0.28
MSU19000263	89	100	11	0.12
MSU19000264	93	90	-3	3.9E-03*

* Significant difference at the .05 probability level.

[c] Populations fixed for *Fhb1*

FHB Response of F_4-derived RILs

FHB severity was rated in 20 RILs derived from control and selected groups for each of the five populations at 14 and 21 days after inoculation (Table 11, Figure 7 and 8). Severity rating decreased in all RILs for 14 and 21 days after inoculation with decreases of up to almost 20% severity at 14 days after inoculation and up to 29% severity at 21 days after inoculation. More significant differences were observed at 21 days after inoculation compared to 14 days when looking at individual populations. Across all populations there was a significant difference at both 14 and 21 days after inoculations showing the overall effects of selection.

Table 11. *T-test for differences in mean FHB severity among RILs derived from selected and control populations at 14 and 21 days after inoculation.*

	14 Days			21 Days		
Line	**Control**	**Selected**	**P-value**	**Control**	**Selected**	**P-value**
MSU19000111	34.7	33	0.43	56.25	48.2	0.22
MSU19000112	40.75	29.16	0.08	74.35	47.21	0.01*
MSU19000119	49.7	28.8	1.69E-3*	79.2	50.15	3.30E-4*
MSU19000196	23.9	18.8	0.23	47.35	34.6	0.09
MSU19000260	43.45	37.1	0.26	62.95	49.6	0.09
Across all populations	38.5	29.37	0.008*	64.02	45.94	1.64E-05*

* Significant difference at the .05 probability level.

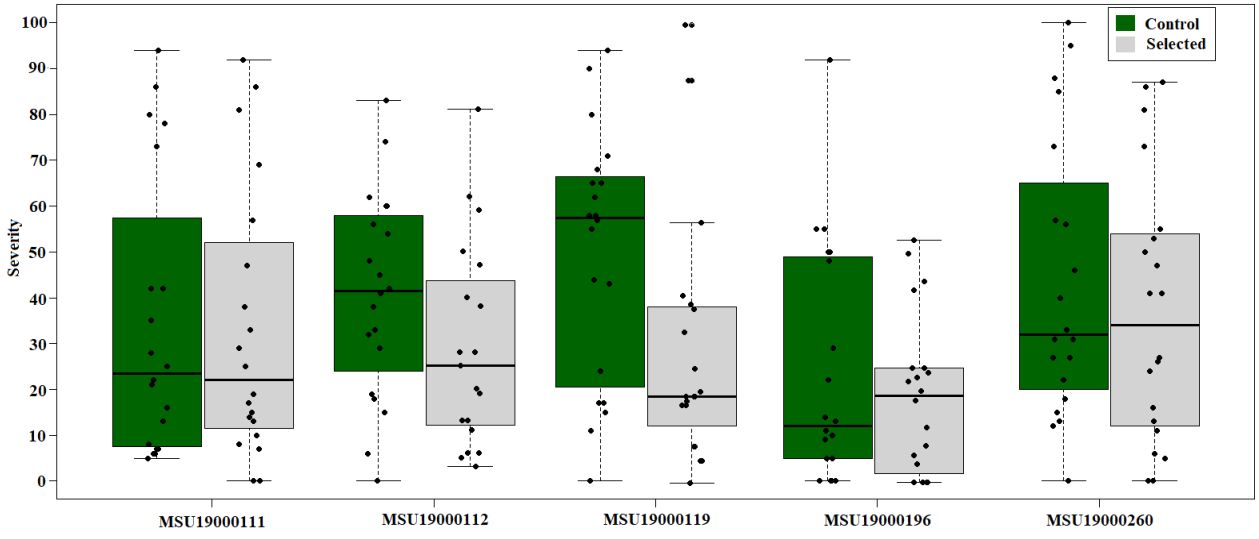

Figure 7. *Differences in mean FHB severity among RILs derived from selected and control populations 14 days after inoculation.*

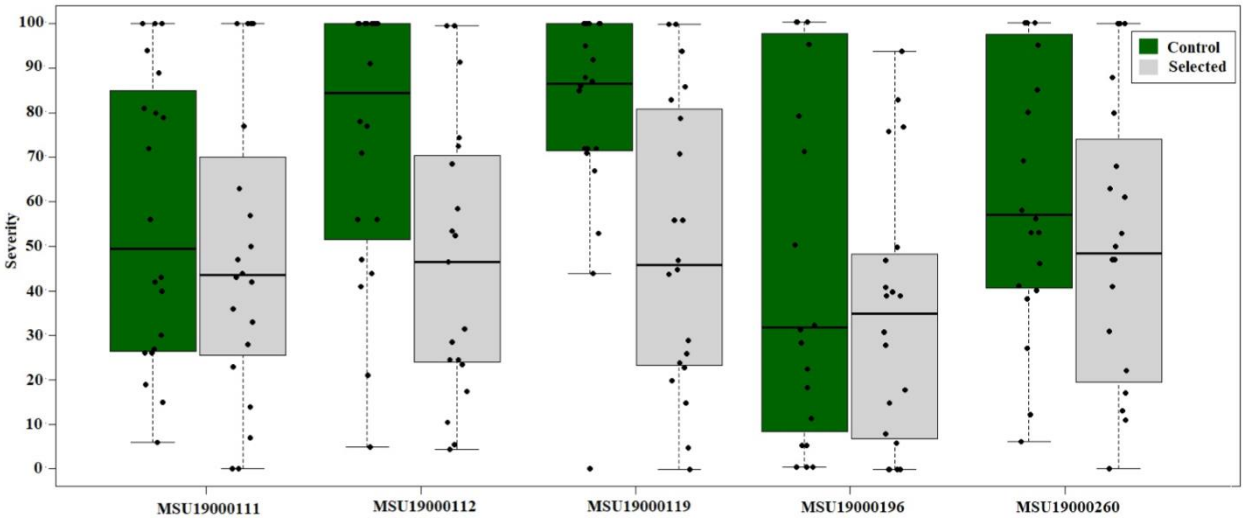

Figure 8. *Differences in mean FHB severity among RILs derived from selected and control populations 21 days after inoculation.*

Signatures of Selection for FHB Resistance

Between 842 and 5,657 SNPs were tested for selection in five populations. Allele frequency shifts were detected on 12 chromosomes. A total of 70 SNPs were found to be under selection across populations (Table 12). Selection on chromosome 1BS was detected in two populations, MSU19000111 and MSU19000119. Other regions were specific to each population. Although *Fhb1* was found at higher frequency among RILs derived from selected populations, selection for SNPs on 3BS was not detected using Fst.

Table 12. *Genomic regions differentiated between recombinant inbred lines derived from selected and control populations*.

Population	SNPs	Significant SNPs	Chr*	Mb**
MSU19000111	4,565	9	1BS	52.9
			6DS	82.5
			7AL	163.0
MSU19000112	2,624	4	2AS	90.8
			4BL	186.5
			5AL	227.0
MSU19000119	842	28	1BS	5.0
			2AL	286.1
			2BL	322.1
			5BL	125.5
			6BS	11.3
			7DS	112.4

Table 12 (cont'd)

MSU19000196	2,339	12	3BS	111.8
			3BS	213.7
			6DS	414.5
			6DL	14.0
MSU19000260	5,657	17	6AL	157.8
			6BL	253.4
			7AS	158.7

* Chromosome location of significant SNP

** Position of the most significant SNP in million base pairs

Discussion

Early generation selection for disease such as fusarium head blight was shown to increase a population's resistance from the F_2 to F_3 generation. Although there was an increase in resistance, significance was only observed on one of the populations. One generation of selection does not provide enough of a selection force to observe significant differences. With more generations significant differences would have likely been observed. Some populations showed a decrease in resistance from the F2 to F3 generation. This could be due to escapes or the chance that the F2 population was inoculated during a low humidity span or unideal temperature causing

stress to the conidia, reducing growth. Two populations were also shown to stay at 100% resistance for the F2 and F3 generation. These populations were ones where both parents had *Fhb1* so the offspring were expected to show 100% resistance. This resistance will remain over the generations which correlates to what we observed for these populations.

RILs, which had two rounds of selection, showed more significant differences. All populations showed a decrease in severity of the selected compared to the control. This shows that selection is working and moving towards higher resistance. Greater differences were observed at 21 days after infection compared to 14 days after infection. This is due to type 2 resistance, which does not protect against initial infection. So, like susceptible populations, type 2 resistance may appear to be closer in severity scores at 14 days. Unlike susceptible populations type 2 resistant populations will not have an increase in severity since the infection does not spread unlike the susceptible populations. Therefore 21 days after infection has a greater difference in severity numbers as to be expected by this reasoning. Although severity was less for selected lines and changes from susceptible to moderately susceptible were observed there was no significance differences across individual populations. This could be due to the need for more selective generations or increasing our standards for selection. Instead of removing minibulk heads if they showed >50% infection, increasing this number to be around 70-75% may produce a more significant shift in resistance over generations.

The phenotypic selection applied in this study is sufficient to identify resistant genotypes in segregating populations. In two populations fixed for *Fhb1*, all F_2 to F_3 individuals were identified as resistant. While these populations were 100% resistant others were as low as 54% resistant identifying as more susceptible genotypes and distinguishing susceptible verse resistant.

RILs that segregated for *Fhb1* showed significant shifts in resistance between selected and control as compared to RILs that are not segregating for *Fhb1*.

Shifts in allele frequencies were observed between selected and control RILs showing selection moving a population towards higher resistance when under selection. Allele comparison between selected and control found significant differences for multiple SNPs. Some SNPs that were significantly different are associated with chromosomes known for fusarium head blight resistance. FHB resistance is associated with chromosome locations 3B and 5B (Ghavami et al. 2011) which were located on MSU19000119 and MSU19000196. Located on 3BS is the *Fhb1* gene which is most commonly used for FHB resistance (Li et al. 2019). Although multiple lines should have shown significant allele differences in the 3BS region due to know *FHb1* in the selected parents this was not shown with the Fst results. Later tests did show that *FHB1* was found in higher amounts in the selected verse control individuals.

These findings show that adding early generation selection does increase frequency of fusarium head blight resistance among inbred wheat lines derived from segregating populations. Use of this research will allow for the rapid advancement of resistance in breeding programs. In correlation with other research, rapid advancement of resistance will produce higher yielding wheat to meet the increasing demand (Ellis. 2014). The ability to perform multiple rounds of selection in a greenhouse speed breeding type of approach (Minibulks) makes this study unique to the Michigan State University wheat breeding program. After selection for resistance lines will be yield tested with top performers being released as new varieties. Future studies can expand upon this research by performing selection not only for resistance but also selection for susceptibility. Selecting susceptible lines and selecting resistant lines from the same starting population can show a stronger divergence and shift in allele frequencies.

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