ABSTRACT

BUTOTO, ERIC. Genomic and Phenotypic Selection to Improve Resistance to Fusarium ear rot and Fumonisin Contamination in Maize. (Under the direction of Drs. James B. Holland and Jeffrey C. Dunne).

The fungus *Fusarium verticillioides* causes Fusarium ear rot (FER) disease and contaminates maize grain with the mycotoxin fumonisin (FUM). Consumption of FUM contaminated maize is associated with illnesses in humans and animals. This thesis explores phenotypic and genomic selection to improve resistance to FER and FUM contamination in the ReFus (Resistance for Fusarium ear rot) maize population developed from several cycles of recurrent selection.

Breeding for resistance to FER and FUM contamination is difficult and expensive; thus, in chapter two, I explored three possible 'shortcuts' to breed for improve resistance to FER and FUM: relying on natural instead of artificial inoculation of *F. verticillioides*, selecting inbred lines *per se* instead of topcross hybrids, and using grain test weight (TW) (also known as bulk density) as an indirect selection criterion. The entry-mean heritabilities for FER and FUM were considerably reduced under natural conditions compared to artificial inoculation, demonstrating the need to artificially inoculate maize ears with *F. verticillioides*. Selection among early generation inbred lines *per se* was an efficient method to improve resistance to their topcross hybrids. The high phenotypic correlation between FER or FUM of the inbred per se and TW of the topcross hybrids indicated that TW could be utilized as an indirect selection criterion to improve resistance to FER and FUM.

In chapter three, I empirically compared genomic and phenotypic selection ability to improve resistance to FER and FUM. Five rounds of genomic selection (GS) were achieved compared to two rounds of phenotypic selection (PS) from the common ReFus base population

and time frame. A random subsample of each GS and PS cycle was evaluated in three North Carolina environments in 2020. Both GS and PS are effective methods to reduce FER and FUM in maize. GS reduced FER and FUM more than PS in terms of gains per year (one calendar year, 2017). However, both methods performed similarly in terms of gains per cycle (from base population to GS C4 or PS C4). The final cycles of PS and GS performed similarly. We also observed a greater decrease in genetic variation in GS than PS. Balancing rapid genetic gain and loss of genetic variation will be important in a recurrent GS scheme; the use of an optimal contribution selection method can help maintain genetic variation during a long-term selection program.

Genomic and Phenotypic Selection to Improve Resistance to Fusarium ear rot and Fumonisin Contamination in Maize

by
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DEDICATION

This thesis is dedicated to Nowa, Nyakizungu, the family of Byicaza, Rushimisha, and Rugabisha, for their love and support.

BIOGRAPHY

Eric Butoto was born in Vyura, Democratic Republic of Congo (DRC), an agricultural community. He lived in Uvira, DRC, Burundi, and Rwanda before settling in the United States as a refugee in 2007. In high school, Eric loved biology but was unsure what he wanted to pursue in college. He decided to take a summer job at Pioneer Hi-bred (now Corteva Agriscience) as a summer maize pollinator, although he had lived in Iowa (the U.S. capital of maize) for a long time, it was the first time he had seen the research side of maize breeding. He was captivated by the sheer size and scientific expertise involved in creating the maize grown throughout Iowa. This experience inspired him to pursue a bachelor's degree in Agronomy at Iowa State University, where he immersed himself in plant breeding and genetics. His experience at Iowa State University and internships in the industry led him to continue his education and pursue a master's degree at North Carolina State University, working in the maize breeding and genetic program under the direction of Dr. Jim Holland.

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Chapter 1: Literature Review

Fusarium verticillioides (Saccardo) Nirenberg (Formerly *F. moniliforme* Sheldon, teleomorph *Gibberella moniliformis* Wineland) is a maize (*Zea mays L.)* pathogen and causal agent for Fusarium ear rot (FER), stalk and seedling rot and produces fumonisin (FUM), a mycotoxin (Blacutt, Gold, Voss, Gao, & Glenn, 2018; Munkvold, 2003b). FER and FUM reduce yield and grain quality, costing millions in production (Wu, 2006). Estimating yield loss due to *F. verticillioides* is extremely difficult due to the various factors contributing to yield; nonetheless, *Fusarium* species are estimated to cause yield loss between 10 to 30 percent (Logrieco et al., 2002).

F. verticillioides is ubiquitous in maize growing regions; however, FER and FUM severity increase in warmer and drier environments during the grain-filling periods (Shelby et al., 1994; Munkvold, 2003a; Cao et al., 2014). FER and FUM contamination could pose an even greater challenge in crop improvement with rising global temperature than currently (Gagkaeva and Yli-Mattila, 2020). *F. verticillioides* infected kernels can have symptoms such as asymptomatic, severe rotting, and "starbursting" manifested as a white or pinkish streak on the kernel due to the colonization of the pericarp by the fungus (Duncan and Howard, 2010; Morales et al., 2018).

Fumonisin

F. verticillioides is notorious for producing mycotoxins, including FUM, fusaric acid, and fusarins (Nelson et al., 1993). Fumonisins (FB_1, FB_2, FB_3) being the most important in cancer-promoting of the group (Marasas, 2001a; Bacon et al., 2008). High levels of FUM content are associated with various cancers and illnesses in humans and animals that consume contaminated grain. In animals, increased levels of FUM are associated with leukoencephalomalacia in horses,

pulmonary edema in swine, liver cancer in mice and rats (Marasas et al., 1988; Haschek et al., 2001; Marasas, 2001a). In humans, increased levels of FUM are associated with esophageal and liver cancer, neural tube defects, and growth retardation in children (Ueno et al., 1997; Marasas, 2001a; Missmer et al., 2006; Kimanya et al., 2010). Fumonisin disrupts sphingolipid metabolisms by inhibiting the enzyme sphinganine N-acyl-transferase, thus causing the accumulation of sphingoid bases. This accumulation of sphingoid bases is suspected of inducing the maladies associated with FUM contamination in humans and animals (Desjardins, Munkvold, Plattner, & Proctor, 2002; Nelson et al., 1993).

In 2002, FUM was classified as possibly carcinogenic by the International Agency for Research on Cancer (International Agency for Research on Cancer, 2002). As a result, international entities and individual countries have established a limit of FUM content in maize products for humans and animals. In the United States, the Food and Drug Administration established a recommended level of FUM (FB_1, FB_2, FB_3) to no more than 4 parts per million (ppm) in maize products intended for human consumption (Center for Food Safety and Applied Nutrition, 2001). The European Union established a maximum level of FUM (FB_1, FB_2) to 4 ppm in maize meant for further processing and 1 ppm for maize grain used for direct human consumption (EU, 2006).

Disease Cycle

Fusarium species (section Liseola) have many distinct biological species, referred to as mating populations (Leslie and Klein, 1996). *F. verticillioides* has two mating populations, A and F. Strains in mating population A produce abundant FUM and are the main strains found in maize. Strains in population F produce little to no FUM (Leslie, Plattner, Desjardins, & Klittich, 1992; Munkvold & Desjardins, 1997b). *F. verticillioides* is heterothallic, requiring two contrast

idiomophorms alleles (different sequences but mapped to the same position on homologous chromosomes), *MAT-1* and *MAT-2,* for successful sexual reproduction (Leslie and Klein, 1996). In nature, inoculum spores commonly found in maize fields are asexual, either macro or microconidia (Munkvold, 2003b). Sexual reproduction spores, ascospores, of *F. verticillioides* are not commonly found in nature (Leslie and Kleint, 1996; Cumagun, 2008). The main reservoir of inoculum is plant residue: *F. verticillioides* produce thick hyphae capable of overwintering in crop residue and serve as inoculum for the next crop. Soil can harbor *F. verticillioides*, when seeds are planted in infected soils, resulting in seeds rotting or seedling blight (Blacutt et al., 2018; Munkvold, 2003b). Also, seeds themselves can serve as carriers of inoculum; when infected kernels are planted, *F. verticillioides* can infect the whole plant systemically, moving to developing roots and stalk tissues and eventually infecting developing kernels (Munkvold, 2003b).

The primary mode of infection is through the silks. *F. verticillioides* microconidia are dispersed by the wind, rain, or insects, infecting silks and colonizing developing kernels (Munkvold, 2003b; Ooka, 1977). Insects such as European corn borers (*Ostrinia nubilalis*) and sap beetles (*Nitidulidea*) move microconidia from leaves and displace them to the developing kernels (Alma et al., 2005). Also, injury caused by insects creates an opening for the fungus to infect the stalk and developing kernels (Munkvold, 2003a; Blacutt et al., 2018).

Disease Management

Environmental conditions highly influence *F. verticillioide*s; temperature, water availability, and humidity greatly influence spore production (Rossi et al., 2009; Cao et al., 2014). The optimum temperature for FUM production is approximately 20 to 30° C (Santiago et al., 2015). Drought stress, before or during the grain filling period, can increase FER severity and

FUM content (Miller, 2001; Munkvold, 2003b; Shelby et al., 1994). Higher insect pressure is associated with greater FER and FUM content due to their role in dispersing the fungus and providing an opening for the fungus to enter the plant (Alma et al., 2005).

Agronomic practices can be implemented to reduce FER and FUM contamination. Perhaps the most important and economically feasible practice is planting maize hybrids with greater resistance. Early planting and harvesting and proper irrigation to alleviate drought stress can help reduce FER and FUM contamination (Munkvold, 2003a). Insecticide treatment and removing some crop residue are all practices that can help reduce FER and FUM contamination (Santiago et al., 2015). Post-harvest, artificial air drying, and proper storage conditions should be utilized when possible to reduce fungus growth that could further contaminate stored grain (Bush, Carson, Cubeta, Hagler, & Payne, 2004; Cao et al., 2014; Munkvold, 2003a).

The use of transgenic maize hybrids expressing *cry* genes (e.g., *cryIA(b) or cry9c*) from bacterium *Bacillus thuringiensis* (*Bt*) has been shown to reduce FER and FUM content; because the *cry* genes express crystalline proteins in maize plants tissue that kills insects that feeds on it, reducing insect infestation, which increases FER and FUM content (Munkvold et al., 1999). Another potential transgenic solution is directly disrupting FUM biosynthetic pathway genes or deploying enzymes that detoxify FUM before accumulating in plants (Duvick, 2001). However, the FUM biosynthetic pathway and enzymes contributing to FUM are not extensively understood (Duvick, 2001; Igawa et al., 2007). At the moment, the most economically and efficient method to reduce FER and FUM contamination is selecting for naturally occurring genetic resistance in maize germplasm.

Breeding for resistance to FER and FUM contamination

Artificial vs. natural inoculation of Fusarium verticillioides

Some breeders rely on natural inoculations because they assume that natural inoculation is sufficient to improve resistance to FER and FUM (Mesterházy et al., 2012). However, due to the strong environmental influence on *F. verticillioides*, disease severity under natural conditions can vary significantly from one year to another, even in disease-conducive environments (Eller, 2009; Mesterházy et al., 2012). Artificial inoculation increases disease severity and decreases plot variability, ensuring that reliable data is collected to compare genotypes. Bolduan et al. (2009) reported entry-mean basis heritability of 0.32 for FER under natural conditions compared to 0.65 when artificially inoculated with *F. verticillioides*. Low heritability and larger environmental variance for FER under natural conditions can hinder selection for improved resistance.

Genotypic and phenotypic correlation between FER and FUM contamination

Robertson et al. (2006) reported a genotypic correlation of 0.87 and 0.96 between FER and FUM in the two maize populations. The phenotypic correlation between FER and FUM ranges from 0.4 to 0.74 in various populations (Robertson et al., 2006; Bolduan et al., 2009; Horne et al., 2016). Löffler et al. (2011) reported a phenotypic correlation of 0.68 and 0.79 between inbred lines *per se* and their topcross hybrids for FER and FUM, respectively, suggesting that selection for resistance for FER and FUM contamination should be initiated during the inbreeding process before evaluating topcross hybrids (Hung and Holland, 2012).

Heritability of FER and FUM

Variation for resistance to FER and FUM contamination exists in maize germplasm (Clements et al., 2004). However, resistance to FER and FUM is complex and controlled by

multiple genes with minor effects (Scott and King, 1984; Robertson-Hoyt et al., 2006; Zila et al., 2013). To date, no genotype has been discovered with complete immunity. Alleles for resistance to FER and FUM appear to be dominant, as demonstrated by Clements et al. (2004).

Robertson et al. (2006) reported low heritability on a single plot and single plant basis when artificially inoculated with *F. verticillioides*. In contrast, the entry-mean heritability with sufficient replications and environments was moderate to high for FER and FUM contamination in various populations (Robertson et al., 2006; Bolduan et al., 2009; Löffler et al., 2011; Horne et al., 2016). These results suggest that individual plant selection for FER resistance is not likely to be effective, whereas selection among lines based on their mean disease resistance values averaged across many plants and multiple environments is expected to be effective.

Correlated traits to FER and FUM

Physical traits of maize ears and kernels, including husk coverage, husk tightness, and pericarp thickness, have been correlated with FER and FUM contamination resistance. Husk coverage and tightness provide a layer of barrier from insects, reducing FER and FUM content (Warfield and Davis, 1996; Butrón et al., 2006; Cao et al., 2014). A thick pericarp can act as an extra protective barrier to reduce the penetration of *F. verticillioides* (Hoenisch and Davis, 1994; Sampietro et al., 2009). However, some studies have found little to no correlation between pericarp thickness or husk coverage and resistance to FER and FUM or genetic variation for those traits (Ivić et al., 2008; Links et al., 2020). Strong husk tightness or coverage could potentially reduce ear drying in the field, contributing to greater FER and FUM contamination (Enerson and Hunter, 1980).

For breeding purposes, indirectly selecting a correlated (preferably less costly or laborious) trait to reduce FUM contamination is appealing. Horne et al. (2016) demonstrated

empirically that FUM contamination was significantly reduced by selecting for resistance to FER, a highly correlated trait both genetically and phenotypically. Grain test weight (TW), also known as bulk density, has a moderate to high negative genotypic and phenotypic correlation with FER and FUM contamination (Morales et al., 2018); which suggests that TW (alone or in addition to FER) could potentially be used as a selection criterion in the inbred generation. However, further experimental studies are needed to understand the effects of strictly selecting TW to improve resistance to FER and FUM content.

Genetic architecture of FER and FUM

Quantitative trait loci mapping

The first quantitative trait loci (QTL) mapping study of FER was by Pérez-Brito et al. (2001), who found QTL explaining 11 to 44 percent of the phenotypic variance of FER in two $F_{2:3}$ populations. Robertson-Hoyt et al. (2006) utilized two populations, GEFR (crosses from a resistant line GE440 and susceptible line FR1064) and NCB (crosses from NC300 and B104), to conduct a QTL study for FER and FUM content. They identified numerous QTL with minor to moderate effects on FER and FUM content, of which three QTL for FER and FUM content were mapped in similar positions in the two populations. However, there were limited similarities between the QTL identified in their study and Pérez-Brito et al. (2001). Ding et al. (2008) used a recombinant inbred lines (RILs) population to identify a QTL on chromosome 3 (bin 3.04) that explains 13 to 22% of the FER variance in that population. The main conclusion of these studies is that resistance to FER and FUM contamination is highly polygenic, with multiple genes contributing to the resistance.

Genome-wide association study

As genetic markers became more readily available, extensive genome-wide association studies (GWAS) for FER and FUM content were feasible. Zila et al. (2013) utilized 47,445 single nucleotide polymorphism (SNP) markers scored on 267 inbred lines, which were evaluated in North Carolina and Spain to conduct a GWAS for FER; they identified three significant SNPs associated with minor effects on FER. Zila et al. (2014) conducted a large GWAS on 1,687 diverse inbred lines from the USDA maize gene bank with 200,978 SNPs. The authors identified seven significant SNPs. However, all the significant SNPs had a negligible effect on FER. Notably, all alleles associated with resistance were at high frequency in tropical maize than temperate, suggesting that tropical maize could serve as a source for more resistance in our current temperate cultivars. Chen et al. (2016) conducted a GWAS in 818 tropical maize inbred lines using 43,424 SNPs and identified 45 significant SNPs and 15 haplotypes; however, these also had a small effect on the FER variance. These studies confirmed that resistance to FER and FUM contamination is highly polygenic.

Genomic selection

Initially, marker-assisted selection was incorporated into breeding programs as an add-on to phenotypic selection for crop improvement. Marker-assisted selection works well when a trait is controlled by a significant major gene or by a significant effect QTL. However, most important agronomic traits (e.g., yield or resistance to FER and FUM) are influenced by many loci with small effects (Bernardo and Yu, 2007; Heslot et al., 2015; Bernardo, 2016). As a result, MAS has not generally been successful in the improvement of quantitative traits. Genomic selection (GS) is a possibly better way to leverage genetic marker data to improve quantitative traits, as it does not rely on a large effect QTL for accurate breeding value predictions. Genomic

selection uses individuals with genotypic and phenotypic data (training set) to build a statistical model that predicts genomic estimated breeding values (GEBVs) of untested individuals with only genomic data (selection set) (Meuwissen et al., 2001). The accuracy of GS depends on training population size, the heritability of the trait of interest, and the density of markers, and the use of a statistical model that is appropriate for a trait genetic architecture (Goddard and Hayes, 2007; Heffner et al., 2009; Heslot et al., 2012).

Training population

The size and composition of a training population directly affect the accuracy of the GS model. As the training population size increases, the accuracy increases up to a certain point (Lorenzana and Bernardo, 2009). For instance, Berro et al. (2019) demonstrated that the predictive ability of grain yield in wheat increased up to 600 individuals, then plateaued after that. At least as important as the sample size is the relationship between the training and selection sets, and there are different methods used to optimize the training set for a particular selection set. Rincent et al. (2012) utilized the average coefficient of determination (CDmean) and prediction error variance (PEVmean) to select individual genotypes for a training population in two maize diversity panels; dent and flint. The CDmean is defined as the squared correlation between the true and predicted contrast of genetic values. CDmean maximizes the expected reliabilities of the contrast between each selected candidate and the population mean. In the study, CDmean was superior to PEVmean for optimizing the training population.

Isidro et al. (2015) assessed five different training set optimization algorithms: CDmean, PEVmean, stratified sampling, stratified CDmean, and random sampling. The authors concluded that the best method depended on the trait architecture and the population structure of the training population. In a population with a substantial population structure, stratified sampling is

preferable to the other methods. However, with a low population structure, CDmean is superior. Rincent, Charcosset & Moreau (2017) addressed the population structure issue by demonstrating that the CD method could be adapted to various population structures.

Marker density

Increasing the density of markers increases the prediction accuracy as more markers are likely to be in LD with causative variants (Solberg et al., 2008). Like with the training population size, there are diminishing returns as the marker number increases. For example, Norman et al. (2018) showed that prediction accuracy for four traits (glaucousness, relative humidity, thousand kernel weight, and grain yield) in wheat increased from 100 to 17,181 markers, with the highest prediction accuracy at 17,181. However, at 5,000 markers, the prediction accuracy plateaued with an incremental increase from 5,000 to 17,181 markers (Norman et al., 2018). The decision on marker density depends on the rate of LD decay in the population, which is influenced by multiple factors, including species, mating system, size of the population, and recombination rate (Heffner et al., 2009). Linkage disequilibrium estimated as the square correlation (r^2) between marker incidence coefficients of pairs of markers can be utilized to measure the marker density relative to decay of LD decay (Calus and Veerkamp, 2007). In a biparental population, where the training set members are full-sibs of the selection set, a relatively small number of markers can achieve adequate accuracy as the two sets share extensive haplotype blocks (Bernardo and Yu, 2007; Lorenzana and Bernardo, 2009).

Heritability of a trait of interest

The heritability of a trait is another important factor that directly influences the prediction accuracy of GS. For traits with low heritability, larger training sets are required to adequately predict breeding values (Hayes and Goddard, 2010). Prediction accuracy increases with higher

heritability when other factors are held constant. Zhao et al. (2013) reported a GS accuracy of 0.6 for grain yield (h^2 = 0.5) and 0.8 for grain moisture (h^2 = 0.7).

Prediction accuracy of genomic selection

Genomic selection prediction accuracy is reported in a few different ways; it can be reported as the correlation between the GEBVs and true (observed) phenotypic values divided by the square root of heritability (Lorenzana and Bernardo, 2009). It can also be reported as a mean square error of predictions (MSE) (Lorenz et al., 2011).

Genomic selection models

There are various statistical models used for GS. These current statistical models are essential in overcoming the "large *p,* small *n*" issue. The *p* refers to the number of markers compared to the population size, *n*. Large *p* and small *n* issue make it difficult to predict marker effect on a phenotype; one issue that arises is overfitting due to multicollinearity between markers (Heslot et al., 2012). Genomic selection models address this by using shrinkage methods, variable selection, or dimension reduction.

Genomic best linear unbiased predictor (GBLUP) incorporates genomic information in the form of relationship matrices with mixed models to predict breeding values of individuals. First introduced by Henderson (1975), BLUP incorporated additive pedigree relationship matrix (**A**) based on pedigree information to predict breeding values of animals. However, as sequencing technology became more readily, markers were used to create a matrix of pairwise realized genomic relationships (**G**). The **G** matrix indicates how much of the genome is shared based on identity by descent state between individuals, which is more accurate and reflective of the relationships among individuals than pedigree-based measures (VanRaden, 2008; Hayes et al., 2009). In GBLUP, **A** is substituted with a **G** matrix to compute the GEBVs for all individuals

(training and selection set). Thus, GBLUP models are computationally efficient and can incorporate multiple traits in the model (Jia and Jannink, 2012).

Ridge regression best linear unbiased prediction (RR-BLUP), also known as random regression best linear unbiased prediction, is a shrinkage method that can also be used for GS. In RR-BLUP, marker effects are treated as random and their estimates are shrunk towards zero; a penalty parameter λ determines the magnitude of shrinkage, the sum of squared regression weight (Whittaker et al., 2000; Piepho, 2009; Crossa et al., 2017). This penalty parameter λ avoids overfitting in the model. RRBLUP and GBLUP are essentially the same when both models scale the marker effects equivalently (Habier et al., 2007; Jannink et al., 2010).

Bayesian approaches to GS assume that marker variance comes from a prior distribution, which allows the marker variances to vary across the genome, which is more reflective of some genetic architectures, unlike GBLUP and RRBLUP, assuming that each marker variance is minimal (Meuwissen et al., 2001). Machine learning models such as random forests (Ogutu et al., 2011), support vector machine (Ogutu et al., 2011), and extreme gradient boosting (Holland et al., 2020) can be incorporated into GS. Machine learning models are increasingly being explored and used in GS; however, GBLUP and RR-BLUP remain the most popularly used models.

Genomic vs. phenotypic selection

Is GS superior to PS? Phenotypic selection (PS) is expected to perform better than GS in terms of gains per cycle depending on the trait heritability and sufficient replication in multiple environments. However, GS significantly outperforms PS in term gains per unit time (i.e., year) as genotyping of the selection set can be conducted in winter nurseries or greenhouses, thus reducing generation time (Heffner et al., 2009, 2010; Lorenzana and Bernardo, 2009). Genomic

selection has a significant advantage over PS for perennials crops such as oil palm or any other long generation crop and for traits that are costly to phenotype (Wong and Bernardo, 2008; Lipka et al., 2014; Crossa et al., 2017; Crain et al., 2020). Using simulation data, Wong & Bernardo (2008) found that cost per unit gain was 26-57% lower using GS than PS using markers cost $1.50. In terms of cost benefits of GS and PS, Beyene et al. (2019) found that GS reduced cost by 32% compared to PS based on rough estimates of the cost of conducting a two-row yield plot trial and genotyping of lines. However, both of these studies did not factor in human labor, which could change the cost-benefit of GS and PS.

Many studies demonstrate the feasibility of GS compared to PS, mainly accessing the predictive ability of different GS models for various traits and crops. However, there are limited studies that empirically compare GS and PS based on selection. For instance, Combs & Bernardo (2013) compared GS to PS backcrossing in semidwarf maize for grain yield and other traits. They concluded that mean performance for the grain yield, plant height, and root and stalk lodging was better with GS than PS. Asoro et al. (2013) evaluated GS, MAS, and PS by achieving two rounds of selection for each method and found that GS was a superior method to increase the mean β-glucan over PS. After one round of GS and PS in two bi-parental populations, Vivek et al. (2017) concluded that genetic gain per year was higher for GS than PS when looking at grain yield.

Beyene et al. (2019) used GS and PS to select high-performing double haploid lines; these inbreds were topcrossed and evaluated for grain yield under well-watered conditions and managed drought stress. They reported that hybrids advanced through GS and PS performed similarly. Sallam & Smith (2016) reported similar gains for GS and PS for Fusarium head blight and deoxynivalenol in barley when advancing one round of GS and PS. Rutkoski et al. (2015)

compared GS and PS for resistance to stem rust in wheat. In two years, two generations of GS compared to one generation of PS were achieved. Gains per year were similar between GS and PS. They also observed significantly less genetic variance following GS than PS.

Long term genomic selection

In a long-term GS scheme (i.e., rapid recurrent selection conducted over many cycles), many factors can reduce the accuracy of GS prediction over time. First, by design, many GS schemes involve at least two selection cycles compared to one in PS in a similar time. In addition, higher accuracy of GS can lead to a selection of more closely related individuals in each generation, and as a result, inbreeding tends to be higher in GS (Rutkoski et al., 2015). Second, the decay of QTL- marker linkage disequilibrium due to recombination and because markers in LD with a QTL become fixed over generations, resulting in reduced accuracy in later selection cycles (Goddard, 2009; Müller et al., 2017). Lastly, some favorable QTL alleles can be lost during the intense selection of GS (Jannink, 2010).

One method to address inbreeding is utilizing optimal contribution selection (Meuwissen, 1997). Optimal contribution selection works by balancing the genetic merit of individuals and their relationship to others. Optimal contribution selection picks individuals with high GEBV while imposing a threshold for an acceptable inbreeding rate. Thus, it ensures that genetic variation persists over generations (Meuwissen, 1997; Woolliams et al., 2015).

An alternative method to ensure long-term GS gains is placing weight on rare favorable alleles. Placing weight on rare favorable alleles causes the GS model to initially increase genetic variance early on and allow gains to be maintained over a more extended period of selection (Goddard, 2009). In addition to placing weight on rare favorable alleles, it allows GS to maintain

more polymorphic markers than unweighted GS models, permitting QTL-marker LD to persist over a more extended period (Jannink, 2010; De Beukelaer et al., 2017).

Theoretical studies such as (Bernardo and Yu, 2007; Muir, 2007; Jannink, 2010; De Beukelaer et al., 2017; Gorjanc et al., 2018) utilize simulation to explore long-term GS. However, long-term empirical studies are limited at the moment. Although, more empirical studies are forthcoming as the usage of GS continues to increase in plants.

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Chapter 2: Effects of artificial inoculation on trait correlations with resistance to Fusarium ear rot and fumonisin contamination in maize

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ABSTRACT

Breeding for resistance to Fusarium ear rot (FER) and fumonisin (FUM) contamination in maize is labor-intensive, time-consuming, and expensive. The objective of this study is to explore three possible 'shortcuts' to improve the efficiency of breeding for resistance to FER and FUM: relying on natural instead of artificial inoculation of *F. verticillioides*, selecting inbred lines *per se* instead of topcross hybrids, and using grain test weight (TW) as an indirect selection criterion. We selected the 27 most resistant and 26 most susceptible $S_{0:1}$ lines from the third cycle of a broad-based recurrent selection population and topcrossed them to a common inbred line. The resulting topcross hybrids were evaluated in three North Carolina environments under artificial and natural inoculation of *F. verticillioides*. The entry-mean heritabilities for FER and FUM are considerably reduced under natural conditions compared to artificial inoculation; therefore, artificial inoculation should be practiced for efficient selection for resistance. We found a high correlation between FER and FUM content of $S_{0:1}$ lines *per se* and their topcross hybrids, suggesting that selection among early generation inbred lines *per se* is an efficient method to improve resistance to their topcross hybrids. Lastly, TW of inoculated $S_{0:1}$ lines *per se* was strongly and negatively correlated with FER and FUM of their topcross hybrids, suggesting that TW can be utilized as an indirect selection criterion to improve resistance to FER and FUM contamination.

Abbreviations: FER, Fusarium ear rot; FUM, fumonisin; TW, grain test weight; BLUEs, best linear unbiased estimators; ELISA, enzyme-linked immunosorbent assay

INTRODUCTION

Fusarium verticillioides Nirenberg (synonym *F. moniliforme* Sheldon, teleomorph *Gibberella moniliformis* Wineland), a common pathogen of maize worldwide, is responsible for Fusarium ear rot (FER). Fusarium ear rot is especially prevalent in warmer and drier conditions during the grain-filling period (Shelby et al., 1994; Miller, 2001). *F. verticillioides* can infect maize via silks, wounds in the stalks, and kernels damaged by insects; infected seeds can also lead to systemically infected adult plants (Blacutt et al., 2018). *F. verticillioides* reduce yield and grain quality, costing millions of dollars in production (Wu, 2006).

F. verticillioides infection can range from asymptomatic to complete rotting of maize kernels (Munkvold & Desjardins, 1997). *F. verticillioides* is notorious for producing mycotoxins, most importantly fumonisins (Bacon et al., 2008). Fumonisins (FB_1, FB_2, FB_3) have adverse health effects on humans and animals that consume contaminated maize, including leukoencephalomalacia in horses, pulmonary edema in swine, and liver cancer in rats (Gelderblom et al., 1988; Haschek et al., 2001; Marasas et al., 1988). In humans, consumption of maize grain in regions with higher levels of FUM contamination is associated with esophageal (Marasas, 2001a) and liver (Ueno et al., 1997) cancers, neural tube defects (Gelineau-van Waes et al., 2005; Missmer et al., 2006), and growth retardation in children (Kimanya et al., 2010; Shirima et al., 2013). As a result, the International Agency for Research on Cancer classified FUM as possibly carcinogenic to humans (International Agency for Research on Cancer, 2002), and the United States Food and Drug Administration recommends a limit of no more than 4 $\mu g\ g^{-1}$ of the toxin for a variety of maize grain products (Center for Food Safety and Applied Nutrition, 2001).

Agronomic practices such as early harvest, followed by forced drying, and proper storage help reduce FER and FUM contamination (Bush et al., 2004; Munkvold & Desjardins, 1997). Irrigation during grain filling alleviates drought stress, which can also reduce the susceptibility of maize plants to infection (Chotena, Makus, Simpson, & Anderegg, 1980). The use of transgenic maize hybrids expressing *cry* genes from bacterium *Bacillus thuringiensis* (*Bt*) has been shown to reduce FER and FUM because they reduce insect infestation (Munkvold et al., 1999). Insects such as European corn borers and sap beetles play an important role in fungal dispersal; they can move microconidia from leaves and displace them to the developing kernels. Also, injury caused by insects creates an opening for the fungus to infect the stalk and developing kernels (Munkvold, 2003a).

These management strategies are only partially effective and can be expensive for farmers to implement. Naturally occurring genetic resistance to FER and FUM contamination is an alternative strategy that may be easier and less expensive for farmers to use, but resistance to FER and FUM is complex and highly polygenic (Clements & White, 2004; Nankam & Pataky, 1996). Genetic variation for resistance to FER and FUM contamination exists, but no fully immune genotype has been discovered, and incorporating resistance alleles in hybrids has proven to be difficult (Clements et al., 2004; Munkvold, 2003; Zila et al., 2014). The heritability of FER and FUM under artificial inoculation is low on a plot-basis but moderate to high on an entry mean-basis with sufficient replication within and across environments (Robertson et al., 2006; Bolduan et al., 2009; Zila et al., 2014; Horne et al., 2016).

Breeding for resistance to FER and FUM contamination in maize is costly, laborious, and time-consuming. The most reliable method is to select among lines by crossing them to an appropriate tester and evaluating topcross hybrid ears directly for FER and FUM content under

artificially inoculated conditions, increasing disease severity and decreasing plot variability (Clements et al., 2003; Mesterházy et al., 2012). However, this requires an additional generation beyond the final generation of line development and selection to produce topcross hybrids.

Can we make this process more efficient by introducing 'shortcuts' to work around the bottlenecks of a generalized hybrid selection program? One shortcut would be to rely on natural infection by *F. verticillioides* in field trials and eliminate artificial inoculation. If traits measured under natural infection were predictive of resistance measured under artificial inoculation, breeders could shift resources away from artificial inoculation towards evaluating more genotypes under natural infection. This would save considerable labor currently needed for inoculation during a typically busy field season and remove the need to maintain viable and aggressive cultures of *F. verticillioides*, which requires some expertise in mycology. A second shortcut would involve shifting primary selection for resistance to the inbred generation *per se*, allowing a reduction of one generation and considerable land and labor required to produce topcross hybrids.

A final shortcut could be the use of indirect selection on traits that are easier or less time-consuming to measure than FER and FUM themselves. Without considering the economic and time trade-offs, indirect selection can be more effective than directly selecting on FER and FUM content if the product of the square root of the heritability of the measured trait and the genetic correlation between the measured and target traits is greater than the square root of the heritability of the target trait (Falconer and Mackay, 1996). If the time or expense required to measure are less for correlated traits than the target traits, indirect selection can be more economically efficient than direct selection, even when the expected response to indirect selection is lower. For example, Robertson et al. (2006) suggested that selection for resistance to

FER would indirectly improve FUM resistance because of the strong positive genetic correlation between these traits. Measuring FUM requires enzyme-linked immunosorbent assay (ELISA) or other chemical assays, which are significantly more costly to phenotype than FER, which can be scored visually. Horne et al. (2016) empirically demonstrated that FUM contamination was reduced significantly by indirectly selecting FER. Would it be possible to go further and replace the evaluation of FER with a trait that is easier to measure? Grain test weight (TW) is correlated with FER and could serve as an indirect selection criterion to reduce FER and FUM (Morales et al., 2018). Test weight would have the advantage of being quantitatively measured by machine (avoiding potential biases that could influence visual scoring of FER). It could also be performed quickly on a mechanically harvested grain sample, allowing it to be assayed routinely from yield trials.

The objective of this study is to explore possible ways to improve the economic and time-efficiency of selection to reduce FER and FUM contamination in maize: first, would gain from selection for resistance to FER and FUM contamination be reduced by relying on natural instead of artificial inoculation of *F. verticillioides* in environments that are conducive to the development of FER? Second, is the correlation between FER and FUM evaluated in inbreds *per se* and their topcross hybrids sufficiently strong to permit initial selection among inbreds to be used to dramatically reduce the number of topcross hybrids to be tested directly? Third, could indirect selection based on correlated traits be an efficient way to reduce FER and FUM contamination?

MATERIALS AND METHODS

ReFus population

The Resistance to Fusarium ear rot (ReFus) population was developed from 22 inbred founders using Design II factorial mating (Comstock and Robinson, 1948). Half of the lines were selected as potential donors of resistance alleles based on previous field evaluation for FER and FUM under artificial inoculation of *F. verticillioides,* and the other half was selected for overall agronomic performance. Two rounds of random intermating without selection took place, and those lines were self-fertilized to recover 206 Cycle 0 $S_{0:1}$. More details on the creation of the ReFus population can be found in Eller (2009) and Horne et al. (2016).

ReFus Cycle 0 $S_{0:1}$ lines were evaluated at Clayton and Lewiston-Woodville, NC, and 20 Cycle 0 $S_{0:1}$ lines with the highest index values (including FER, grain yield, and percentage lodging), were selected to form a balanced bulk from remnant $S_{0:1}$ and intermated twice to create Cycle 1 S_0. Next, those Cycle 1 S_0 lines were self-fertilized to produce Cycle 1 $S_{0:1}$. Then, following a similar selection scheme, two more selection rounds produced 489 ReFus Cycle 3 $S_{0:1}$.

Selection of the most resistant and susceptible lines from a common population

A population of 489 $S_{0:1}$ lines from the third cycle of a broad-based recurrent selection population (Horne et al., 2016) was evaluated under artificial inoculation with *F. verticillioides* in replicated trials in three locations in North Carolina (Clayton, Kinston, and Lewiston-Woodville) from 2014 to 2016 (Holland et al., 2020). A mixed linear model was constructed with FER as the response. The model included environment and experimental design effects and random line effects, with covariance structure proportional to a realized genomic relationship matrix based on 6,086 single nucleotide polymorphic markers (Holland et al., 2020). This model

was used to predict genomic estimated breeding values for FER for each line in the population. The 27 $S_{0:1}$ lines with the lowest predicted FER were selected to represent the "resistant $S_{0:1}$ group" and the 26 $S_{0:1}$ lines with the highest predicted FER were selected to represent the "susceptible $S_{0:1}$ group". Each of the selected lines was then crossed with a common relatively susceptible tester, FR1064. We refer to the resulting topcross hybrids as part of the "resistant topcross group" if their selected parental line was in the resistant $S_{0:1}$ group or part of the "susceptible topcross group" if their selected parental line was in the susceptible $S_{0:1}$ group. Seven check hybrids (31G66, DK697, DKC6208RIB, DKC6579, DKC6929, FR1064×FR615, and FR1064×NC358) were included as entries. One of the resistant $S_{0:1}$ group lines was later discovered to have seed contamination; therefore, this line and its topcross hybrid were excluded from subsequent analyses and results.

Experimental Designs

The 53 topcross hybrids plus the seven check hybrids were evaluated in two sets of experiments. A disease evaluation experiment was designed to measure the effects of hybrid and inoculation treatment on FER, FUM, and other traits. A second experiment evaluated the topcross yield and agronomic performance under natural conditions. Both experiments were designed as randomized 6 x 10 α-lattice designs with two replications at each of the three locations in North Carolina (Clayton, Kinston, and Lewiston-Woodville) in 2017. For the disease experiment, plots were single 4.86-m rows, including a 1-m alley. Interrow spacing was 0.97 m at Clayton, 0.76 at Kinston, and 0.91 m at Lewiston. Target planting densities were 43,200 plants ha^{-1} in Clayton, 55,000 plants ha^{-1} in Kinston, and 45,600 plants ha^{-1} in Lewiston. The same plot lengths, spacings, and planting densities were used for the yield evaluation trial, but experimental units were two-row plots.

Disease evaluation experiment methods

The disease evaluation experiment was designed as a split-plot overlayed on an incomplete block design. The whole plot factor was hybrid and the sub-plot factor was inoculation treatment, artificial inoculation of *F. verticillioides* or no inoculation. Half of the plants in each whole-plot were artificially inoculated with *F. verticillioides* and the other half were not. *F. verticillioides* has limited spread from inoculated non-inoculated plants within a season (Yates and Sparks, 2008).

The inoculum consisted of six North Carolina isolates of *F. verticillioides* (NC-4D3, NC-4B1, NC-1E3, NC-1E2, NC-3C1, NC-2A2) selected for high fumonisin production *in vitro* (Eller, 2009). Approximately seven days after 50% of the plants in the experiment were silking, 5 ml of a solution containing spores from all six isolates at a concentration of 2×10^6 spores mL^{-1} was injected into the silk channel in half the plants in the plot. After seven days, the same plants were inoculated with the same volume and concentration through the husk to simulate infection caused by insect damage. Inoculations were performed with a modified Solo (Newport News, VA) backpack sprayer with a syringe to hold and deliver conidial suspensions. One drop of Tween-20 was added to each liter of inoculum suspension to break the surface tension of the water.

In the disease experiment, ten inoculated and ten non-inoculated ears from each plot were harvested and dried using forced air. Each ear was scored for FER on a scale from 0-100% (increments of 5%) as the percentage of kernels displaying visible symptoms of infection. The ears were then shelled, the grain was bulked, and the total grain weight was recorded. Grain yield (g $plant^{-1}$) was calculated by dividing the total weight of each sub-plot by the number of ears in

that sub-plot. Test weight (kg m^{-3}) was measured with the Dickey-John GAC 2000 grain analysis computer (Auburn, IL) adjusted to a constant 15.5 g kg^{-1} grain moisture.

Samples of 500 g of grain from each sub-plot were ground to a fine powder using a Romer II Series Mill (Romer Labs, Union, MO). A 20-g sample of the ground powder was used to measure FUM concentration using an ELISA kit from Helica Biosystems Inc (Santa Ana, CA).

Yield evaluation experiment methods

A separate experiment was designed to evaluate topcross yields under natural conditions using two-row plots and machine harvesting for more accurate yield measurements. In the yield evaluation experiment, days to silking (DTS) (when at least 50% of the plants have at least 2 cm of visible silk) and days to anthesis (DTA) (when at least 50% of the plants had a least 50% of the anthers producing pollen) were recorded for each plot in Clayton only. At all locations, plant height was measured on four plants per plot as the distance from the soil to the flag leaf node, and ear height was measured as the distance from the soil to the primary ear-bearing node. Plots were mechanically harvested, and grain yield (Mg ha^{-1}) and grain moisture were measured on each plot. Grain yield was adjusted to 15.5 g kg^{-1} moisture.

Statistical analysis

A natural log transformation was applied to FER and FUM to reduce the relationship between predicted values and residual variance. Mixed linear models were used for the analysis of the data**.** The model used for the disease experiment was:

$$Y_{ijklm} = \mu + L_i + R(L)_{ij} + B(RL)_{ijk} + T_l + TL_{il} + H_m + HL_{im} + HT_{lm} + HTL_{ilm} + H(RL)_{ijm} + \varepsilon_{ijklm}$$

where Y_{ijklm} is the observed trait value on a sub-plot, μ is the overall mean, L_i is the random effect of location *i,* $R(L)_{ij}$ is the random effect of replication *j* in location *i,* $B(RL)_{ijk}$ is the random effect of incomplete block *k* nested within replication *j* and location *i,* T_l is the fixed effect of inoculation treatment *l,* TL_{il} is the random effect of the interaction between inoculation treatment *l* and location *i,* H_m is the fixed effect of hybrid *m,* HL_{im} is the random effect of the interaction between the hybrid *m* and location *i,* HT_{lm} is the fixed effect of the interaction between hybrid *m* and inoculation treatment *l,* HTL_{ilm} is the random effect of the interaction between hybrid *m,* inoculation treatment *l,* and location *i,* $H(RL)_{ijm}$ is the whole-plot error variance (random effect of the interaction between hybrid *m,* and replication *j* nested within location *i*), and ε_{ijklm} is the sub-plot error variance.

The model used for the yield evaluation study was:

$Y_{ijkm} = \mu + L_i + R(L)_{ij} + B(RL)_{ijk} + H_m + HL_{im} + \varepsilon_{ijkm}$, where the terms are the same as defined for the disease experiment model. Analyses were performed using ASReml-R version 4.1 (Butler et al., 2017).

Best linear unbiased estimators (BLUEs) were estimated for the hybrid and treatment main effects and their combinations in the disease experiment. Standard errors for comparing the differences among hybrid group means were computed as a function of the average standard errors of pairwise comparisons of individual hybrids and the group sample sizes. Phenotypic correlations estimated within and across inoculation treatment were based on BLUEs for hybrid by treatment combinations. For the yield evaluation experiment, BLUEs were estimated for hybrids and the same procedure was used to calculate standard errors comparing groups of hybrids.

A separate model was used for estimating heritabilities within a treatment. This model was identical to the previous model but excluded checks and fit hybrids as random effects. The heritability estimates are subject to upward bias since the topcross hybrids were sampled from the extremes of the $S_{0:1}$ line distribution for resistance. Therefore, the reported heritability estimates are useful to compare the relative response to selection on various traits but not for predicting the actual response to the prediction. Heritability was estimated with Cullis estimator (Cullis et al., 2006; Piepho and Möhring, 2007):

$$H_c = 1 - \frac{SED^2}{2 * V_g}$$

Where SED is the average standard error of pairwise differences among hybrid predictions within a treatment and V_g the genetic variance of hybrids within the treatment.

For estimating the correlation between the $S_{0:1}$ line values and their topcross hybrids, we used $S_{0:1}$ line mean values from Holland et al. (2020) for FER and FUM. In addition, TW of thirty-two $S_{0:1}$ lines was measured in the 2016 experiment described in Holland et al. (2020), permitting estimation of the correlation between TW of the $S_{0:1}$ lines *per se* and FER, FUM, and grain TW of their topcross hybrids.

RESULTS AND DISCUSSION

The effects of treatment on trait means and heritabilities in the disease evaluation experiment

The hybrid main effect was significant ($p < 0.05$) on all traits. The inoculation treatment main effect was significant ($p < 0.05$) only for hand-harvested grain yield, although nearly significant ($p < 0.10$) for FER, FUM, and TW (Table 2.1). Compared to natural conditions, artificial inoculation increased FER and FUM by 23.7% and 45.0%, respectively, on average. Compared to natural conditions, artificial inoculation decreased grain TW by 1.6% and grain yield decreased by 5.5% on average, similar to results reported by Nagy et al. (2006). On average, DK697 and the resistant topcross group had the lowest FER, and 31G66 and the resistant topcross group had the lowest FUM content under artificial inoculation. On average, FR1064×NC358 and the resistant topcross group had the lowest FER and FUM contents under natural conditions (Table 2.2).

The hybrid by inoculation treatment interaction was significant for FUM and TW, indicating that the relative performance of genotypes for these traits differs under artificial and natural inoculation (Table 2.1). Hybrid by treatment interaction was not significant for FER; however, congruent with Silva et al. (2007) and Eller et al. (2010), who found no significant difference in the ranking of genotypes with and without inoculation. The entry mean-basis heritabilities for FER and FUM were 0.57 and 0.67 under artificial inoculation but reduced close to zero under natural conditions (Table 2.1). This is congruent with, although more extreme than, the results of Bolduan et al. (2009), who found reduced heritability for FER under natural conditions (0.32, compared to 0.65 under artificial inoculation). Although we did not find a significant hybrid by treatment interaction for FER, the dramatic reduction in heritability under natural conditions suggests that the relative response to selection under natural conditions is

expected to be lower under natural conditions; therefore, artificial inoculation should be utilized when possible to differentiate relative resistance amongst genotypes. Under natural conditions, disease pressure will vary even in geographic regions favorable to Fusarium ear rot development; artificial inoculation improves the chances of obtaining reliable disease scores across years and locations regardless of the local disease pressure (Eller, 2009; Czembor et al., 2019).

Residual variances were stratified by location and inoculation treatment, with lower residual variance for FER observed in the inoculated treatment (Table A.1). Inoculation helps create a more uniform presence of the pathogen (Mesterházy et al., 2012), and this is reflected by the decrease in heterogeneity within plots in the inoculation treatment. In contrast, residual variances for FUM and TW were greater in the artificially inoculated treatment. The increased residual variance for FUM due to inoculation may be an effect of scale, as the mean values for FUM are increased under inoculation, and the inflation of residual variance is more than offset by the increase in genetic variance due to inoculation (Table A.1). The increased residual variance for TW cannot be an effect of scale, as its mean value was decreased under inoculation; however, this increase was offset mainly by a large increase in the genetic variance under inoculation. This suggests that while inoculation introduces additional residual variance for TW, it also reveals additional genetic variance that is likely due to differences in resistance to FER.

Performance of resistant vs. susceptible topcross groups

The $S_{0:1}$ lines used to create the topcross hybrids were artificially inoculated with *F. verticillioides* and evaluated as lines *per se* as described in Holland et al. (2020). The resistant $S_{0:1}$ group of lines *per se* had a mean of 9.4% FER compared to a mean of 45.4% of the susceptible $S_{0:1}$ group (Figure 2.1A). For FUM, the resistant $S_{0:1}$ group had a mean of 9.0 μg g^{-1} compared to 44.9 μg g^{-1} of the susceptible $S_{0:1}$ group as lines *per se* (Figure 2.1B). Under

artificial inoculation in the disease evaluation of topcross hybrids described in this study experiment, the resistant topcross group had a mean FER significantly ($p < 0.001$) lower than the mean of the susceptible topcross group (15.6%) (Figure 2.1C). The resistant topcross group also had a significantly ($p < 0.001$) lower mean FUM than the susceptible topcross group (26.8 μg g^{-1}) (Figure 2.1D).

In the disease experiment, there was a significant difference between the mean FER, FUM, and TW of the resistant and susceptible topcross group under artificial inoculation and natural conditions ($p < 0.05$, Table 2.2). When artificially inoculated, the resistant topcross group mean FER percentage was significantly ($p < 0.05$) lower than other topcross hybrids except for FR1064xNC358, DK697, and 31G66 (Table 2.2). Under natural conditions, the resistant topcross group mean FER percentage was significantly ($p < 0.05$) lower than the rest of the topcross hybrids except for FR1064xNC358 and DK697. When artificially inoculated, the mean FUM of the resistant topcross group content was significantly ($p < 0.05$) lower than the susceptible topcross group and DKC6929 hybrid. Under natural conditions, the resistant topcross group FUM mean was significantly ($p < 0.05$) lower than the susceptible group, DKC6929, DKC6579, and DKC6208RIB (Table 2.2).

The correlation between artificially inoculated $S_{0:1}$ lines *per se* mean values and their artificially inoculated topcross mean values for FER, FUM, and TW were significant ($p < 0.001$) and positive ($r = 0.68$ for FER; $r = 0.76$ for FUM; $r = 0.82$ for TW) (Figure 2.2). In agreement with Löffler et al. (2011) and Clements et al. (2004), who reported a similar correlation coefficient between inbred lines and their topcross hybrids for FER and FUM. This is expected as more resistant inbred lines *per se* tend to produce more resistant hybrids due to partial dominance of resistance (Clements et al., 2004). The correlations between artificially inoculated

$S_{0:1}$ line *per se* mean values and their topcross mean values for FER, FUM, and TW under natural conditions were also positive and significant ($p < 0.001$) but lower than the correlation coefficients when the topcrosss hybrids were artificially inoculated (Figure A.1). These high phenotypic correlations between inbred lines *per se* and their topcross hybrids when both are artificially inoculated suggest that breeders can effectively screen inbreds for FER and FUM resistance, reserving the development and evaluation of topcross hybrids at the later stages of the breeding program. Preliminary selection of more resistant lines before making topcross hybrids can save time and resources while increasing resistance to FER and FUM contamination.

For the yield evaluation experiment, the mean machine-harvested grain yields for the resistant and susceptible topcross group were not significantly ($p < 0.05$) different. This result is congruent with previous studies that demonstrated that selecting for resistance to FER in nonuniform lines does not significantly change yield (Eller, 2009; Horne et al., 2016). The mean yields of the resistant and susceptible topcross groups, FR1064×NC358, and FR1064×FR615 were similar and significantly ($p < 0.05$) lower than the rest of the commercial hybrids (Table 2.3). This is not surprising as the $S_{0:1}$ lines used to create these topcross hybrids are not uniform and have not been selected for other agronomic traits except for FER.

Phenotypic correlations of topcross hybrids within and across treatments

Trait correlations were estimated based on the BLUEs of topcross hybrids within and across treatments in the disease experiment. FER and FUM were positively correlated both with and without inoculation ($r = 0.70$, $p < 0.001$, when inoculated; $r = 0.74$, $p < 0.001$, when not inoculated) (Figure 2.3). When comparing hybrids evaluated in a common environment, hybrids with more symptomatic kernels contain more fumonisin content, despite the lower correlation between kernel rot and fumonisin content that is sometimes observed when comparing seed lots

(Desjardins et al., 1998; Robertson-Hoyt et al., 2007; Horne et al., 2016; Morales et al., 2018). Under artificial inoculation, both FER and FUM are significantly ($p < 0.001$) and negatively correlated with TW ($r = -0.73$, FER; $r = -0.59$, FUM) (Figure 2.3), in agreement with Morales et al. (2018). Under natural conditions, the correlation between FER and TW is reduced ($r = -0.62$, $p < 0.001$), but the estimated correlation between FUM and TW is the same as when artificially inoculated (Figure 2.3).

Trait correlations were reduced across treatments. The correlation between FER and FUM evaluated with and without inoculation was 0.60 and 0.46, respectively (Figure 2.3). Critically, the correlation between FUM under inoculation and FER without inoculation was only 0.20 (reduced from 0.70 when FER was evaluated under inoculation; Figure 2.3). This result suggests that if FUM measured under inoculation is indicative of the potential for mycotoxin contamination under conducive conditions, then selecting against FER as a correlated trait will only be very effective if FER is measured under inoculation.

Test weight correlation between $S_{0:1}$ lines and their topcross hybrids

Among the 53 $S_{0:1}$ lines selected to make topcross hybrids for this study, 32 had TW measured in the $S_{0:1}$ generation *per se* in the experiments conducted in 2016 (reported by Holland et al., 2020), permitting estimation of the correlation between TW of the $S_{0:1}$ lines *per se* and FER and FUM of their topcross hybrids. Test weight of $S_{0:1}$ lines was significantly ($p < 0.001$) and negatively correlated with FER and FUM of their topcross hybrids under artificial inoculation ($r = -0.73$, FER; $r = -0.66$, FUM) (Figure 2.4). Tissue degradation caused by *F. verticillioides* and other contributing factors such as insect damage could explain the lower TW found in more susceptible lines (Miller, 2001; Duncan and Howard, 2010). Surprisingly, these correlations are equal to or stronger than those between TW and FER or FUM; all measured on

the topcross hybrids in the same experiment under artificial inoculation (Figure 2.3). Extending the results reported by Morales et al. (2018), this strong negative correlation between line *per se* TW and FER or FUM of their hybrids indicates that selecting lines *per se* for higher TW under artificial inoculation could be an efficient and practical way to improved hybrids for their resistance to FER and FUM contamination. This indirect selection criterion could potentially replace some of the effort put into visual ratings of FER for selection.

CONCLUSIONS

The objective of this study was to address three potential 'shortcuts' that could be implemented in a hybrid maize breeding program for resistance to FER and FUM contamination. One shortcut would be to eliminate artificial inoculation from FER resistance screening trials. Our results suggest that even in an environment such as North Carolina, which tends to have among the highest natural infection levels by *F. verticillioides* and grain contamination by FUM content (Shelby et al., 1994), relying on natural infection is not a good strategy. This is demonstrated by the considerable reduction in the heritability for both FER and FUM under natural conditions compared to artificial inoculation and the low correlations between FUM measured under inoculation and FER and FUM measured without inoculation. Therefore, we suggest that the effort required to maintain fungal cultures and perform calibrated artificial inoculation remains necessary for evaluating resistance to this quantitative disease.

Our results suggest that initial germplasm screening can be effectively performed on lines *per se* without requiring topcross hybrid evaluations of resistance until the final stages of hybrid breeding, in agreement with Hung and Holland (2012). The significant difference for disease resistance between topcross hybrids grouped based on their resistance levels measured in $S_{0:1}$ lines along with the strong correlation between FER and FUM values in inbred *per se* and hybrid generations under inoculation support the delay of direct evaluation of hybrids for resistance to FER and FUM until the later advanced stages of hybrid selection when the number of selection candidates has been narrowed considerably.

Finally, grain TW measured on $S_{0:1}$ lines under artificial inoculation had strong and favorable correlations with FER and FUM content in the topcross hybrids. This result suggests that TW could be utilized as a useful indirect selection criterion to improve resistance to FER

and FUM contamination of both inbred lines and their topcross hybrids. Although measuring TW still requires the harvest of grain, it has the advantage of quantitively measured by a machine, reducing bias and lack of precision resulting from visual rating of ear disease. In our experience, TW of ten ears can also be measured faster than visual ear rot scores of the same number of ears. Finally, TW can be measured on bulk grain samples, making it more amenable to mechanized measurement, as TW can be measured on total plot grain with many combined harvesters. The correlation between TW and FER or FUM measured with a mechanical combine harvester could potentially be lower than reported in this study because lighter infected kernels tend to be mechanically removed from grain samples, whereas such kernels are maintained by hand harvest and shelling. Also, in the disease experiment and under natural conditions, TW had a significant ($p < 0.01$) and negative correlation with hand-harvested grain yield ($r = -0.43$; Figure 2.3), suggesting that indirect effects of selecting on TW need to be monitored carefully. Our correlations estimates are based on relatively small sample size, however, such that the predicted effects of strictly selecting grain TW on the genetic gain of yield is uncertain and should be further investigated in the future.

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TABLES

Table 2.1. *P-values* for the fixed effects of topcross hybrid, treatment, and their interaction for natural log-transformed Fusarium ear rot score [ln(FER)] and fumonisin content [ln(FUM)], hand-harvested grain yield, and test weight. Results are based on 59 hybrids evaluated under two inoculation treatments at three locations. Entry mean-basis heritability was estimated within each inoculation treatment, excluding check hybrids.

p-values				
Source	ln(FER)	ln(FUM)	Grain yield	Test weight
Hybrid	<0.001	<0.001	0.025	<0.001
Treatment	0.068	0.075	0.022	0.088
Hybrid×Treatment	0.311	0.030	0.904	<0.001
Entry mean-basis heritability estimates within a treatment				
Inoculated	0.57	0.67	0.05	0.88
Non-inoculated	0.00	0.00	0.15	0.89

Table 2.2. Means of the natural log of Fusarium ear rot [ln(FER)] and fumonisin contamination [ln(FUM)], hand-harvested grain yield, and grain test weight for resistant and susceptible groups and the checks in the disease study under inoculated (INOC) and not inoculated (NOT) treatments. Within a trait, means not followed by a common letter are significantly different at 0.05 probability level.

	ln(FER)		ln(FUM)		Grain yield			Test weight	
	ln (%)		ln (μg g^{-1})		(g $plant^{-1}$)			(kg m^{-3})	
GROUP	INOC	NOT	INOC	NOT	INOC	NOT	% Diff*	INOC	NOT
Resistant Hybrids	2.4^{a}	1.7^{a}	2.6^{a}	1.5^{a}	146^{a}	156^{a}	-6.8 %	795^{a}	802^{a}
Susceptible Hybrids	2.8^{b}	1.9^{b}	3.3^{b}	1.8^{b}	147^{a}	161^{a}	-9.5%	767^{b}	793^{b}
FR1064×NC358	2.6^{ab}	1.8^{ab}	3.1^{ab}	1.4^{ab}	142^{a}	156^{a}	-9.9%	805^{c}	817^{c}
FR1064×FR615	3.4^{c}	2.8^{c}	3.1^{ab}	2.2^{ab}	142^{a}	155^{a}	-9.2%	745^{de}	742^{de}
DKC6929	3.1^{bc}	2.6^{cd}	3.5^{b}	2.6^{c}	160^{ab}	173^{ab}	-8.1%	745^{ef}	754^{de}
DKC6579	3.3^{c}	3.0^{c}	3.0^{ab}	2.3^{bc}	174^{b}	188^{b}	-8.0%	725^{d}	735^{e}
DKC6208RIB	3.0^{bc}	2.8^{c}	3.2^{ab}	2.7^{c}	163^{ab}	167^{ab}	-2.5%	741^{de}	757^{dg}
DK697	2.5^{abd}	2.0^{abd}	2.8^{ab}	1.8^{abc}	155^{ab}	160^{a}	-3.2%	775^{b}	791^{abf}
31G66	2.7^{abc}	2.3^{bc}	2.4^{a}	2.3^{abc}	171^{b}	174^{ab}	-1.8%	765^{bf}	773^{fg}
Mean	2.9	2.3	3.0	2.1	156	165	-5.5%	762	774

* Diff (%): Percent reduction of hand-harvest yield due to artificial inoculation.

Table 2.3. Mean grain yield, grain moisture, days to anthesis (DTA), days to silking (DTS), anthesis and silking interval (ASI), and plant height (PH) and ear height (EH) of topcross hybrids from yield evaluation experiment conducted at three North Carolina locations under natural conditions and with machine harvest. Within a trait, means not followed by a common letter are significantly different at 0.05 probability level.

GROUP	Yield	Moisture	DTA	DTS	ASI	PH	EH
	(Mg ha^{-1})	(g kg^{-1})	(D)	(D)	(D)	(m)	(m)
Resistant Hybrids	8.7[a]	180[a]	66[a]	66[a]	1[a]	2.1[a]	1.0[a]
Susceptible Hybrids	8.6[a]	180[b]	67[bc]	67[b]	0.8[a]	2.1[b]	1.0[b]
FR1064×NC358	8.5[a]	190[c]	66[abd]	67[abcf]	0[a]	2.0[c]	0.9[cf]
FR1064×FR615	7.8[a]	160[d]	65[abc]	65[abcd]	0[a]	2.0[c]	0.9[cf]
DKC6929	10.2[bd]	180[ab]	64[abc]	64[c]	0[a]	2.0[c]	0.9[c]
DKC6579	10.5[bc]	170[e]	66[abcd]	67[abce]	1.5[a]	2.2[d]	1.1[abe]
DKC6208RIB	10.2[b]	170[e]	64[c]	64[d]	0[a]	1.9[e]	0.9[c]
DK697	11.7[c]	190[c]	68[de]	69[e]	1.5[a]	2.2[d]	1.1[e]
31G66	10.0[bd]	180[ab]	68[e]	69[ef]	0.5[a]	2.2[d]	1.0[abf]
Mean	9.6	178	66	66	0.6	2.1	1.0

FIGURES

Figure 2.1 Means of the natural log-transformed Fusarium ear rot [ln(FER)] and fumonisin [ln(FUM)] of resistant and susceptible $S_{0:1}$ group *per se* and their topcross hybrids when artificially inoculated with *F. verticillioides*.

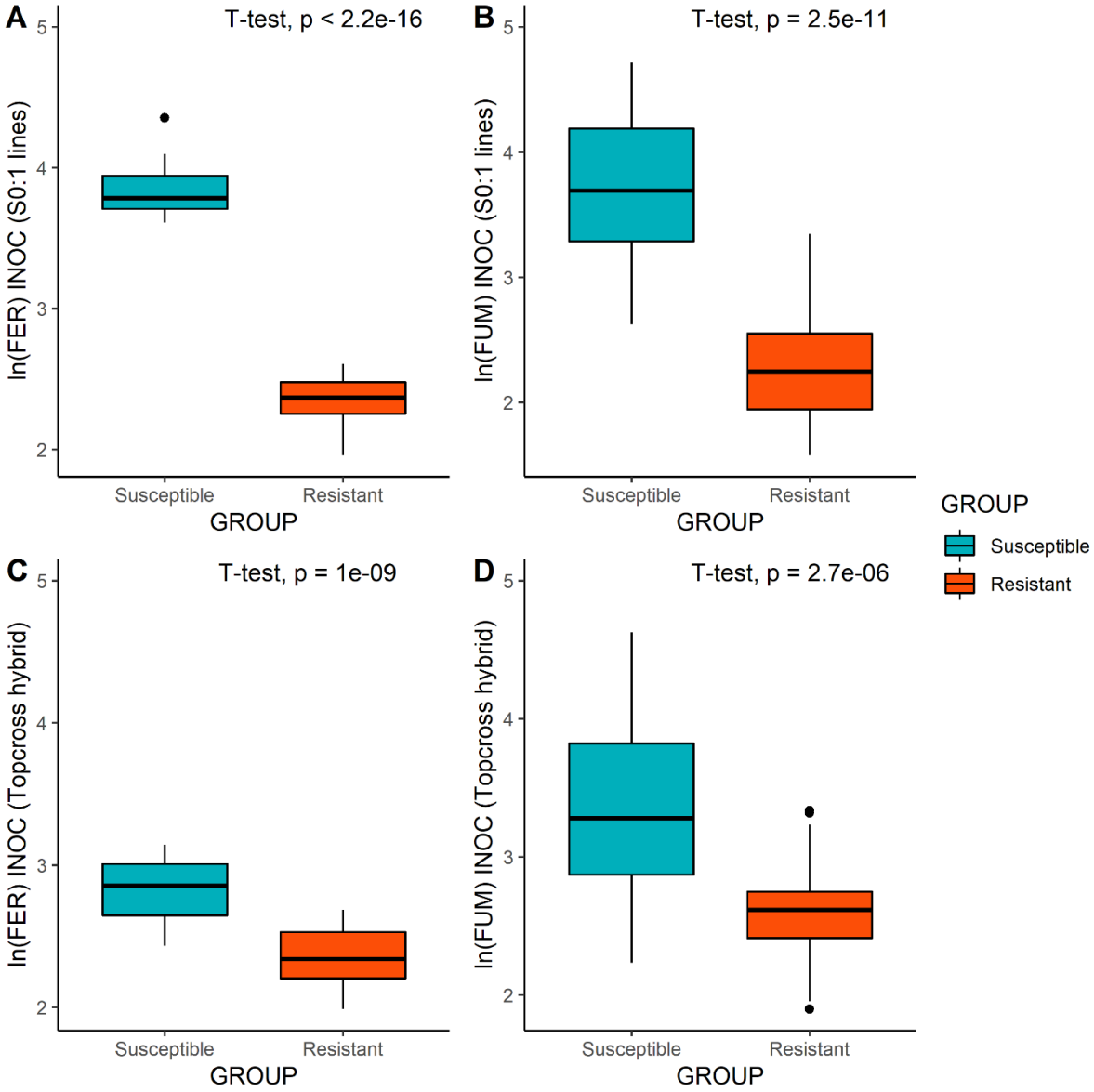


Figure 2.2. Estimated correlations between the resistant and susceptible $S_{0:1}$ line groups and their topcross hybrids for the natural log-transformed Fusarium ear rot [ln(FER)]) and fumonisin [ln(FUM)], and grain test weight (TW) artificial inoculation.

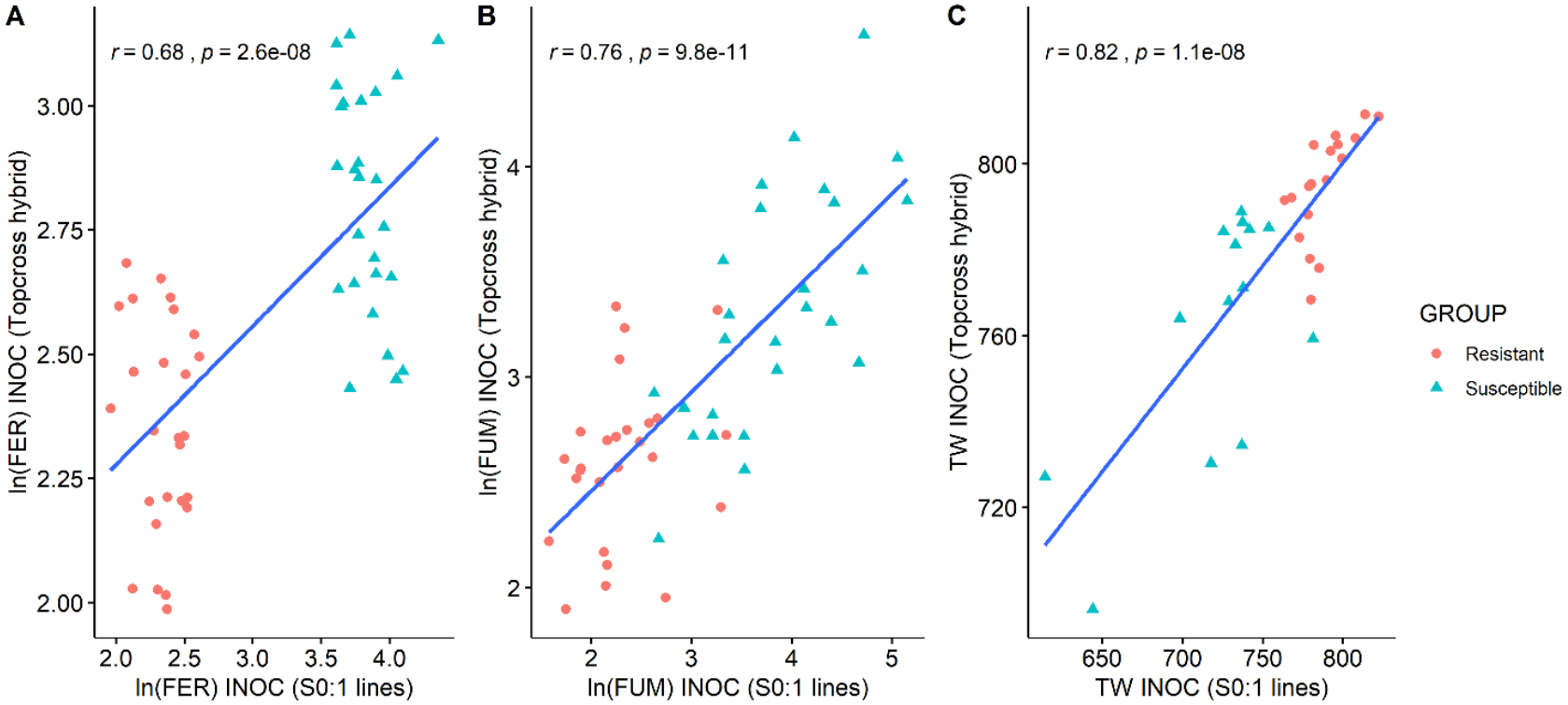


Figure 2.3. Matrix of correlations of topcross hybrid mean values of the natural log-transformed Fusarium ear rot [ln(FER)] and fumonisin [ln(FUM)] contamination, grain yield (YIELD), and grain test weight (TW) within and across treatments (artificially inoculation, INOC, and natural conditions, NOT). Noncolored correlation coefficients are not significant at $p < 0.01$.

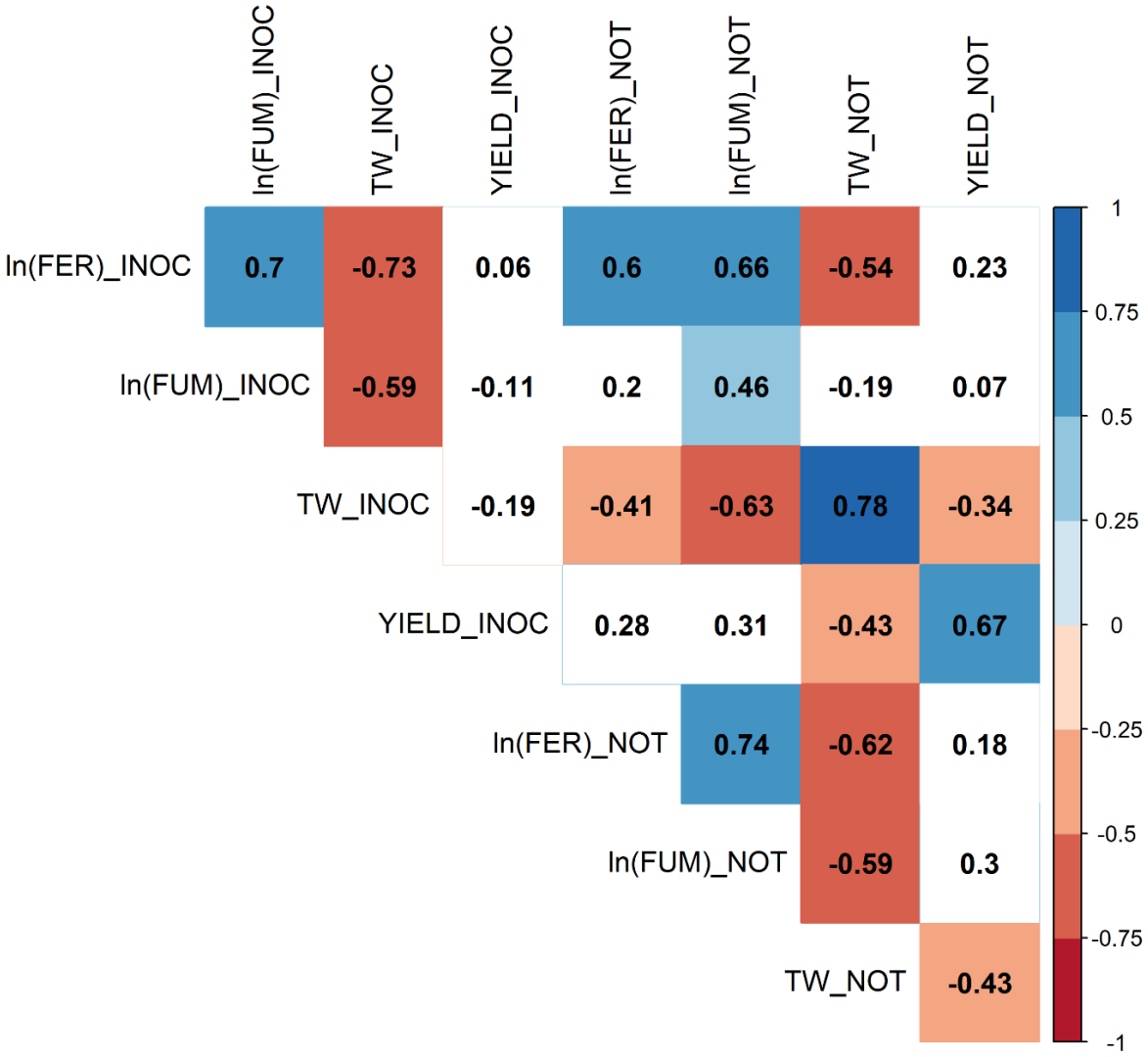
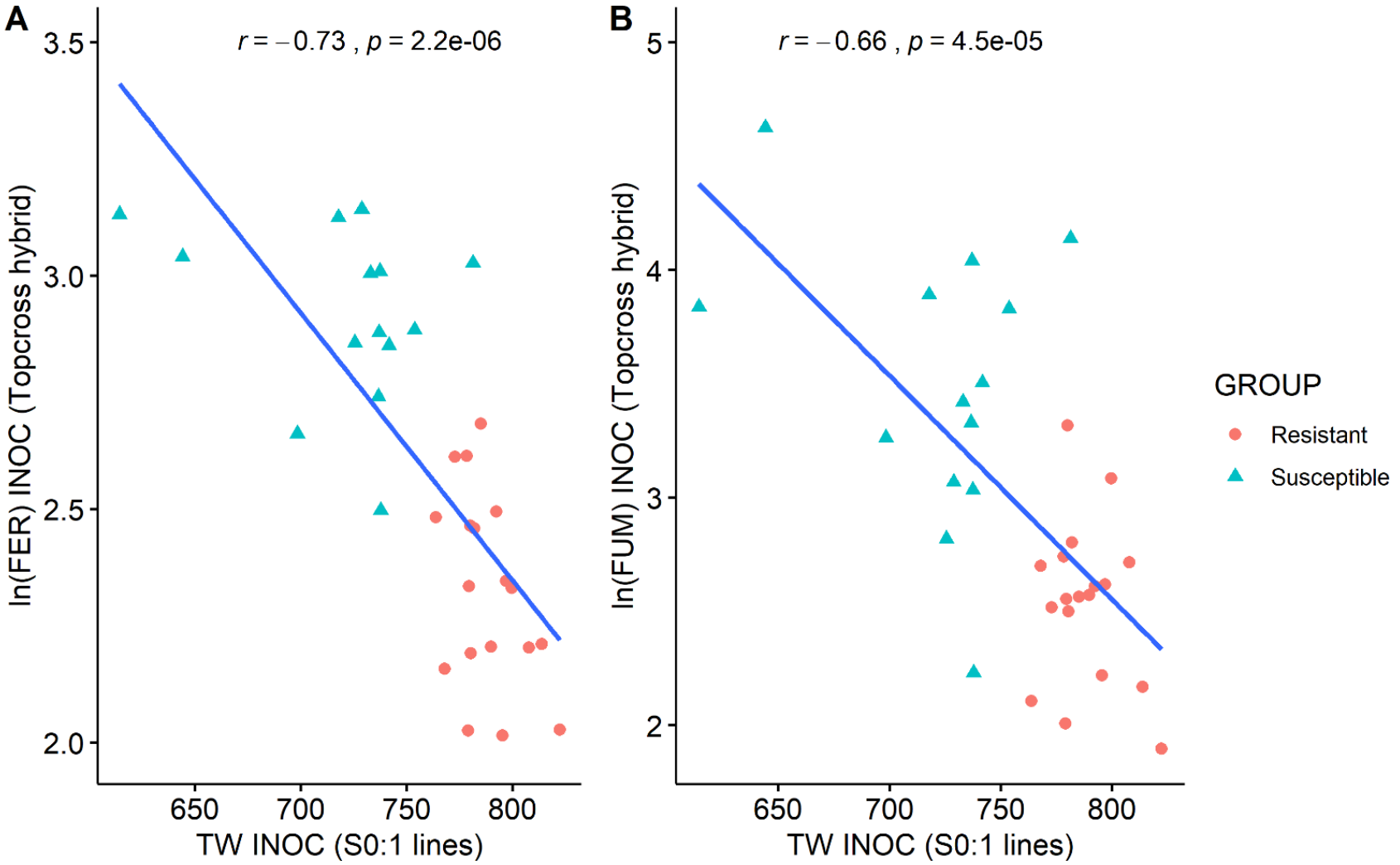


Figure 2.4. The estimated correlation between grain test weight (TW) of resistant and susceptible $S_{0:1}$ line groups and the natural log-transformed Fusarium ear rot [ln(FER)] and fumonisin [ln(FUM)] of their topcross hybrids when artificially inoculated.



Chapter 3: Empirical comparison of genomic and phenotypic selection for resistance to Fusarium ear rot and fumonisin in maize

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ABSTRACT

Fusarium verticillioides is a common maize fungus that causes Fusarium ear rot (FER) and produces fumonisin (FUM), a mycotoxin associated with various maladies in humans and animals that consume contaminated grain. This study aims to empirically compare genomic and phenotypic selection ability to improve FER and FUM resistance and assess GS and PS impact on other agronomic traits. Five rounds of genomic selection (GS) were achieved compared to two rounds of phenotypic selection (PS) in the same time frame and common base population. A subsample of each genomic and phenotypic cycle was evaluated in three North Carolina environments in 2020. From the based population, C3, FER reduced ($p < 0.05$) from 27% to 13.2% and 14% for the final cycle of GS and PS, respectively. In C3, FUM content was nearly 10 μg g^{-1} but reduced to 3.8 and 4.8 for the final cycle of GS and PS, respectively. Head-to-head comparison of GS and PS in one calendar year (i.e., 2017; PS C4 and GS C5), GS reduced FER and FUM more than PS ($p < 0.05$). However, both methods performed similarly in terms of gains per cycle (i.e., from C3 to PS C4 and GS C4). We observed a greater decrease in genetic variation in GS than PS. Balancing rapid genetic gain and loss of genetic variation will be important in recurrent GS. Lastly, selecting for resistance to FER and FUM using GS and PS increased grain test weight but reduced hand-harvest yield.

INTRODUCTION

Fusarium verticillioides Nirenberg (formerly *F. moniliforme* Sheldon, teleomorph *Gibberella moniliformis* Wineland) is one of the most commonly reported maize pathogens worldwide. *F. verticillioides* is the causal agent of Fusarium ear rot (FER). Infected kernels can be asymptomatic or display a range of symptoms from "starbursting"; which can manifest as a pink or white streak on the kernels that mirrors the colonization of the kernel by the fungus, to severe rotting that destroys much of the kernel tissue (Munkvold, 2003a; Duncan and Howard, 2010; Morales et al., 2018). In addition, FER and FUM contamination reduce yield and grain quality (Logrieco et al., 2002).

F. verticillioides produces mycotoxins, including fumonisin (FUM), fusarin C, and fusaric acid (Munkvold, 2003a; Blacutt et al., 2018). Among these mycotoxins, FUM (FB_1, FB_2, and FB_3) has the most cancer-promoting activity (Gelderblom et al., 1988; Bacon et al., 2008). Fumonisin has adverse health effects on humans and animals that consume contaminated maize (Marasas et al., 1988; Gelderblom et al., 1988; Ueno et al., 1997; Haschek et al., 2001; Marasas, 2001b; Gelineau-van Waes et al., 2005; Kimanya et al., 2010; Shirima et al., 2015). Due to the maladies associated with FUM, the International Agency for Research on Cancer classified FUM as possibly carcinogenic to humans (International Agency for Research on Cancer, 2002). As a result, international entities and individual countries maximum or recommended limits of FUM content in maize products for humans and animal consumptions. The United States Food and Drug Administration recommends a limit of no more than 4 $\mu g\ g^{-1}$ of the toxin for various maize grain products (Center for Food Safety and Applied Nutrition, 2001). However, many maize hybrids grown under typical conditions often exceed this recommended amount (Shelby et al., 1994; Abbas et al., 2006), especially in regions conducive to FER.

Resistance to FER and FUM is complex due to the strong environmental influence and polygenic inheritance (Pérez-Brito et al., 2001; Robertson-Hoyt et al., 2006; Ding et al., 2008; Zila et al., 2014). Despite this, the entry-mean heritability of FER and FUM are moderate to high under artificial inoculation with sufficient replication within and across environments in maize various populations (Robertson et al., 2006; Bolduan et al., 2009; Horne et al., 2016).

It is difficult to assess the usage of marker-assisted selection (MAS) in breeding for resistance to FER and FUM content in maize programs. Due to the largely polygenic nature of resistance to FER and FUM, discovered QTL regions are likely too large to successfully implement in MAS (Robertson-Hoyt et al., 2006; Chen et al., 2012). Genomic selection (GS) is likely a better method to take advantage of genetic marker data to improve quantitative traits. First proposed by Meuwissen et al. (2001), GS combines all available markers distributed across the genome and phenotypic data of individuals (training set) to develop a statistical model that predicts genomic estimated breeding values (GEBVs) of new untested individuals with only marker data (selection set) (Lorenz et al., 2011; Heslot et al., 2015). Unlike MAS, GS does not rely on large effect QTL for accurate predictions of the breeding values (Meuwissen et al., 2001; Jannink et al., 2010; Bernardo, 2016). The accuracy of GS depends on the number of markers and training size, the heritability of the trait of interest and statistical model, and the relationship between training and selection sets (Heffner et al., 2009; Jannink et al., 2010; Crossa et al., 2017).

Compared to PS, GS is advantageous because it reduces the breeding cycle as the selection set can be genotyped in greenhouses or off-season nurseries; thus, increasing gains per unit of time (i.e., year). However, in terms of gains per cycle, PS is often equal to or greater than GS depending on the genetic architecture of the trait of interest and levels replication across

multiple environments (Lorenzana and Bernardo, 2009; Heffner et al., 2010). Although there is a significant initial investment in creating a training population, GS can be cheaper than PS over multiple cycles depending on the crop and trait of interest. In the case of FER and FUM, evaluation trials are costly and laborious; maize ears are artificially inoculated, hand-harvested, visually scored for ear rot, and then ground into a fine powder for FUM assay via enzyme-linked immunosorbent assay (ELISA) (Horne et al., 2016). With a GS scheme such as rapid recurrent selection, labor and cost could be significantly less than PS, especially over multiple generations of selection (Jonas and De Koning, 2013; Crossa et al., 2017; Beyene et al., 2019).

In plants, numerous studies have demonstrated the potential utility of GS in terms of predictive accuracy measured with cross-validation in experimental populations (Heslot et al., 2012; Lin et al., 2014; Crossa et al., 2017). However, empirical studies comparing the performance of GS and PS in crops are limited at this time, especially beyond two generations. For instance, in two maize bi-parental populations, Vivek et al. (2017) advanced one cycle of GS and PS; they concluded that GS was super to PS for improving grain yield. In oats, Asoro et al. (2013) advanced two GS, MAS, and PS cycles for β-glucan content and concluded that GS was a superior method for increasing the mean β-glucan over PS. They also found that GS maintained more genetic variance for β-glucan content than PS.

In contrast, Sallam & Smith (2016) reported similar gains from one cycle of GS and PS for resistance to Fusarium head blight and deoxynivalenol contamination in barley. Rutkoski et al. (2015) demonstrated that two cycles of GS and one cycle of PS resulted in similar genetic gains per year when selecting for resistance to stem rust in wheat. Beyene et al. (2019) compared the grain yield of maize hybrids evaluated under well-watered conditions and drought stress;

hybrids were developed from lines selected based on PS and GS. They found that the top 15% of hybrids advanced through GS and PS performed similarly in the two conditions.

Beyond a handful of generations of GS, inbreeding and loss of diversity will occur relatively faster in GS than in PS due to reduced time for breeding cycles and higher selection accuracy of more closely related individuals each generation (Jannink, 2010; Rutkoski et al., 2015). In addition, changes in linkage disequilibrium relationships between QTL and markers will also occur. Optimal contribution selection (OCS) is one method that can be used to address the loss of genetic diversity in GS. OCS works by balancing gains from selection while maintaining genetic variation within selected populations (Meuwissen, 1997). It identifies individuals with high GEBVs while imposing a threshold for an acceptable inbreeding rate based on pedigree or genomic relationship among a selected group of individuals (Meuwissen, 1997; Woolliams et al., 2015).

Phenotypic selection has been the primary method used to reduce ear rot and mycotoxins in maize breeding programs to date; however, genetic gains can be relatively slow due to the cost and time required to obtain accurate evaluations of ear rot and mycotoxin contamination. Genomic selection could be more effective at increasing resistance to ear rot diseases and mycotoxins contamination, but no empirical studies of the relative effectiveness of GS compared to PS for these traits have been reported in maize. Therefore, it is important to compare the genetic gains from multiple cycles of GS compared to PS for resistance to FER and FUM, also measure the indirect effects of these selection procedures on other important traits and genetic variation within populations.

The objectives of this study were to empirically compare GS and PS for (i) resistance to FER and FUM contamination over multiple selection cycles within a common time frame and

starting from a common base population, (ii) the effects of GS and PS on other important agronomic traits, and (iii) their effects on both marker and trait genetic variation within populations.

MATERIALS & METHODS

ReFus population

Briefly, the Resistance to Fusarium ear rot (ReFus) population was developed from 22 inbred founders. Half of the lines were selected as potential donors of resistance alleles, and the other half was selected for overall agronomic performance. After a few rounds of selection and self-fertilization, 494 ReFus Cycle 3 $S_{0:1}$ lines were produced. Details on the creation of the ReFus population can be found in Eller (2009) and Horne et al. (2016).

Phenotypic Selection

ReFus Cycle 3 $S_{0:1}$ lines were phenotyped over three years (2014, 2015, and 2016) in three North Carolina locations (Clayton, Kinston, and Lewiston-Woodville; Holland et al. (2020)). Plots were artificially inoculated twice with a mixture of local isolates of *F. verticillioides*, and up to ten ears per plot were rated for percent of FER (0-100%, increments of 5%). Bulked grain samples from all ears within each plot were assayed for FUM content using ELISA. Details of the phenotyping trials were presented by Holland et al. (2020). A multivariate linear mixed model was fit to the natural log-transformed FER and FUM content data (Isik et al., 2017):

$$Y_{ijklm} = T_i + E(T)_{ij} + R(ET)_{ijk} + B(RET)_{ijkl} + G(T)_{im} + GE(T)_{ijm} + \varepsilon_{ijklm}$$

Where T_i is the fixed effect of a trait (FER or FUM); $E(T)_{ij}$ is the random effect of the environment within trait; $R(ET)_{ijk}$ is the random effect of replication block within environment and trait; $B(RET)_{ijkl}$ is the random effect of the incomplete block within rep, environment, and trait; $G(T)_{im}$ is the random effect of genotype within trait, $GE(T)_{ijm}$ is the random effect of genotype-by-environment interaction within trait, and ε_{ijklm} is the residual effect associated with

a plot within a trait. The random model effects were modeled with unstructured covariances among traits and independent, identical variances within traits. For example, the covariance structure for genotypes within traits was $G(T)_{ij} \sim \begin{bmatrix} \sigma^2_{G1} & \sigma_{G12} \\ \sigma_{G12} & \sigma^2_{G2} \end{bmatrix} \otimes \boldsymbol{I}_G$, where σ^2_{G1} and σ^2_{G2} are the genotypic variances for traits 1 (FER) and 2 (FUM), respectively, σ_{G12} is the genotypic covariance between FER and FUM, and $\boldsymbol{I}_G$ is an identity matrix with dimensions equal to the number of lines. The residual covariance structures were fit as heterogeneous within environments: $\varepsilon_{ijklm} \sim \begin{bmatrix} \sigma^2_{\varepsilon 1j} & \sigma_{\varepsilon 12j} \\ \sigma_{\varepsilon 12j} & \sigma^2_{\varepsilon 2j} \end{bmatrix} \otimes \boldsymbol{I}_p$, where $\sigma^2_{\varepsilon 1j}$ and $\sigma^2_{\varepsilon 2j}$ are the residual variances for FER and FUM, respectively within environment *j*, $\sigma_{\varepsilon 12j}$ is the residual covariance between FER and FUM within environment *j*, and $\boldsymbol{I}_{pj}$ is an identity matrix with dimensions equal to the total number of plots. The model was fit using ASReml Version 4.2 (Gilmour et al., 2015), with residual variances weighted by the sample size of ears rated per plot. Best linear unbiased predictors (BLUPs) for lines for each trait were predicted from this model; we will refer to these as phenotypic BLUPs (P-BLUPs), as they are based purely on phenotypic information and treat the genotype effects within traits as uncorrelated. We standardized the P-BLUPs within each trait and summed the standardized values to obtain a selection index. The 20 $S_{0:1}$ lines with the lowest selection index values (less FER and FUM is desirable) were selected as parents of PS Cycle 4 (Figure 3.1).

A balanced bulk of seeds of the 20 selected lines was planted in two sets with a four-day delay between the sets in Clayton, NC, in 2017. Pollen was bulked from the first set and applied to silks of the available plants in the second set, and vice versa, to reduce assertive mating due to flowering time. The result of these bulk pollinations was the S_0 generation of PS Cycle 4 (Figure 3.1). In the fall of 2017, PS Cycle 4 S_0 lines were self-fertilized without intentional selection in

Homestead, FL, to develop 199 ReFus Cycle 4 $S_{0:1}$ lines (Figure 3.1). In the summer of 2018, PS Cycle 4 $S_{0:1}$ lines were evaluated for FER in field trials with two replications at three locations in North Carolina (Clayton, Kinston, Lewiston-Woodville) with artificial inoculations of *F. verticillioides*, similar to before (Figure 3.1). A univariate model analogous to the multivariate model using to generate P-BLUPs but fit only to FER was used to predict P-BLUPs for FER in this population. Twenty lines with the lowest FER were selected. In the summer of 2019, a balanced bulk of 20 selected lines was planted in Clayton, NC, in two sets with a four-day delay between sets (Figure 3.1). At flowering time, the same pollination scheme was implemented to form the PS Cycle 5 S_0. In the fall of 2019, PS Cycle 5 S_0 lines were planted in Homestead, FL, and self-fertilized to form 246 PS Cycle 5 $S_{0:1}$ (Figure 3.1).

Genomic Selection

A set of 489 ReFus C3 $S_{0:1}$ lines was phenotyped for FER and FUM was also genotyped using a modified genotyping-by-sequencing method (Manching et al., 2017) to create the training set for GS. Details of the genotyping, SNP calling, and SNP quality control can be found in (Holland et al., 2020). An additive realized genomic relationship matrix (**G**) was estimated from 8,846 biallelic markers remaining after removing with >15% missing call rates, > 25 % missing data, and minor allele frequency below 1% using TASSEL software (Bradbury et al., 2007). Genomic best linear unbiased predictors (GBLUPs) for FER and FUM were predicted using a linear mixed model similar to the P-BLUPs model using ASRemLVersion 4.2 (Gilmour et al., 2015), but including the genomic relationship matrix to model the covariances among line effects. For example, the covariance structure for genotypes within traits was $G(T)_{ij} \sim \begin{bmatrix} \sigma_{A1}^2 & \sigma_{A12} \\ \sigma_{A12} & \sigma_{A2}^2 \end{bmatrix} \otimes \boldsymbol{G}$, where σ_{A1}^2 and σ_{A1}^2 are the additive genetic variances for FER and FUM, respectively, σ_{A12} is the additive covariance between FER and FUM, and $\boldsymbol{G}$ is the additive

genomic relationship matrix, an identity matrix with dimensions equal to the number of lines. The GBLUPs for FER and FUM were centered and standardized to unit variance, then 20 lines with the lowest mean standardized predicted FER and FUM were selected to be the parents of the next cycle of GS (GS Cycle 4) (Figure 3.1).

In 2017, remnant S_1 seeds of the selected lines were planted and a sample of 384 plants was also genotyped using the same protocol as the training set, except that whereas the training set was initially aligned to the AGPv2 reference sequence, all subsequent GS cycles sequences reads were aligned to AGv4 coordinates to match with later cycles. The intersection of SNPs passing quality control filters, similar to before, in both the training and the latest sample (5,023 SNPs) was used to create a genomic relationship matrix including both the training set lines and the individual plants of the selection set. GBLUPs predicted from the same model fit to the training set but with the updated genomic relationship matrix. GBLUPs for FER and FUM for the new samples were centered and scaled to unit variance as before and a selection index was computed as the sum of the two trait GBLUPs.

The genotyped plants were intermated in random pairs. For this and subsequent round of GS, a similar quality control filter was used as previously described. Genotypic data were not available until after harvest; therefore, we computed the GBLUPs of each cross as the mean of the two parental plant GBLUPs. Some plants did not pass genotyping quality control, and these plants did not have GBLUP or relationship information available; thus, we estimated GBLUPs of zero and zero relationships to the rest of the genotyped set (Figure 3.1). An optimal set of 10 crosses representing 20 S_1 individuals was selected with having the lowest mean selection index value while maintaining an expected increase in population inbreeding coefficient less than 0.05 using optimal contribution (Wray and Goddard, 1994; Clark et al., 2013) and a genetic algorithm

in R package kofnGA (Wolters, 2015), Bash, ASREML, and R scripts to perform these analyses are available at https://github.com/ncsumaize/ReFUS-genomic-selection.

These selected crosses represented GS Cycle 4 S_0, planted in 2017 in Homestead, FL, and individual plants were sampled for genotyping. First, crosses were made between individual plants; genotyping was performed as before on 329 plants. The same procedure for selecting a set of 10 best crosses with the lowest mean standardized GEBVs across FER and FUM and not exceeding the expected inbreeding coefficient increase of 0.05 was performed (Figure 3.1). The resulting crosses represented GS Cycle 5 S_0.

In the summer of 2018, GS Cycle 5 S_0 was planted in Clayton, NC, and self-fertilized to form GS Cycle 5 $S_{0:1}$. The 356 parental GS Cycle S_0 lines were genotyped, and the same selection procedure was utilized to select an optimal set of 20 S_0 individuals. However, the progeny of the 20 selected individuals, GS Cycle 5 $S_{0:1}$, were planted in Homestead, FL, in the fall of 2018 and crossed in random pairs (Figure 3.1). The genotyping by sequencing protocol largely failed to produce high-quality SNPs in this generation. Therefore, the sample of 338 plants was submitted to a commercial genotyping service (LGC, Berlin, Germany) for targeted genotyping (SeqSNP method) at a selected subset of 2,000 SNPs from the training set. The same procedure was used for selecting a set of 10 best crosses with the lowest mean standardized GEBVs across FER and FUM and not exceeding the expected inbreeding coefficient increase of 0.05. We also selected the ten crosses with the highest mean standardized GEBVs (least resistance) across FER and FUM and not exceeding the expected inbreeding coefficient increase of < 0.05. This resulted in the "Best" and "Worst" groups of GS Cycle 6 S_0 plants. In the fall of 2019, all of GS Cycle 6 S_0 was planted in Homestead, FL, and self-pollinated to create GS Cycle 6 $S_{0:1}$ lines (Figure 3.1).

Genotyping phenotypic and genomic selection cycles

In 2019, the 22 founders were sequenced with the SeqSNP service of LCG Genomics (Berlin, Germany), a targeted genotyping service at a selected subset of 2,000 SNPs from the training set. In 2020, the PS C4 and C5, GS C4 and C6 (only lines to be evaluated in the field experiment) were also sequenced by LGC Genomics (Middleton, WI) using SeqSNP services at 2,500 SNPs selected from the training set. In addition, 22 lines from the training set (originally sequenced by modified GBS) were also sequenced by LGC Genomics. The SNPs were filtered for biallelic markers, > 20 % missing data, and minor allele frequency below 1%. Using the 22 lines of the training set (C3), the two sequencing methods (LGC SeqSNP and modified GBS) could be compared based on common SNPs. Unexpectedly, their SNPs calls were only 80% identical, on average, suggesting that the two sequencing methods are different in their SNP calling. Therefore, SNPs with conflicting calls were removed, resulting in a final set of 1,129 SNPs.

For each round of GS, a subset of SNPs from the training set was selected for genotyping, and the resulting SNPs data for that specific round of GS underwent various filters (for example, removing SNPs with > 20% missing data) before being incorporated into GS. However, this meant that GS cycles vary in shared SNPs. In addition, PS cycles and GS C6 were genotyped at a lower density than others cycles. As a result, 1,129 SNPs were used as a common set for the founders and the rest of GS and PS cycles. This avoided the need to impute large amounts of missing data. Furthermore, GS C5 had more missing SNPs in the common set than the rest of the cycles; as a result, we further reduced this set to a smaller 541 SNPs to have a complete set. The 514 and 1,129 sets were not used for GS but were intended to examine population structure and genetic variation within cycles.

Experimental design

A field study was performed to compare the performance of $S_{0:1}$ from the based cycles (C3) and subsequent GS and PS cycles. A random sample of the base population and each cycle of PS and GS were selected to represent each cycle. Fifty lines were randomly sampled from C3, GS C6 "Best," and PS C5; 25 were sampled from PS C4, GS C4, and GS C5; and 11 were sampled from GS C6 "Worst." In addition, four checks (FR1064, B73, GE440, and GEMS0002) were included as entries in the trial. In total, 240 entries were evaluated. The experimental design was a 15 × 16 α-lattice design. The experiment was planted in Clayton, Kinston, and Lewiston-Woodville, NC, with two complete replications per location in the summer of 2020. Each plot was a single 3.05-m row with a 1.22-m alley between the ranges and the inter-row spacing of 0.97, 0.76, and 0.91 m for CLY, KIN, and LEW, respectively. Each plot was planted with 25 kernels and thinned to a uniform stand of 20 plants, resulting in population densities of ~56, 652, 71,759, and 59, 799 plants ha^{-1} for Clayton, Kinston, and Lewiston-Woodville, respectively.

Artificial inoculation

Inoculum consisted of four North Carolina isolates of *F. verticillioides* (NC-C19, NC-K17, NC-N20, NC-I25) visually selected for rapid growth on agar medium. Approximately seven days after 50% of the plants in a location were silking, 5 ml of a solution containing spores from all isolates at a concentration of 2×10^6 spores mL^{-1} was injected into the silk channel in each plant in a plot. After seven days, the same plants were inoculated with the same volume and concentration through the husk to simulate infection caused by insect damage. Inoculations were performed with a modified Solo (Newport News, VA) backpack sprayer with a syringe to hold and deliver conidial suspensions. One drop of Tween-20 was added to each liter of inoculum suspension to break the surface tension.

Phenotyping

Days to silking (when at least 50% of the plants had visible silk) and days to anthesis (when at least 50% of the plants had anthers) were recorded for each plot at Clayton. At all locations, plant height was measured on three plants in each plot as the distance from the soil to the flag leaf node and ear height was measured as the distance from soil to the primary ear-bearing node on the same plants.

In all locations, husk coverage and tightness were scored on a plot basis (giving one score for a plot). For husk coverage, it was scored on a scale from one to five; one indicated that kernels were visible/exposed on an ear, three suggested that the husk leaves cover the tip of a maize ear, and five indicated that the tip was enclosed by long husk leaves extending beyond the end of the ear. Husk tightness was also scored on a scale from one to five; one indicated that husk leaves were very loose, three indicated moderately tight husk leaves, and five suggested very tight husk leaves (Rector et al., 2002). At Clayton only, husk leaf strength was recorded using a rind penetrometer (N), commonly used to measure stalk strength (Flint-Garcia et al., 2003). Husk leaves from one plant per plot were harvested and folded in half, measuring 5 cm from the base of the husk leaves; a rind penetrometer was used to record the peak force required to penetrate the double layers of husk leaves. At harvest and in all locations, two intact maize ears per plot were harvest, and the number of husk leaves was counted.

Fusarium ear rot was scored as the percentage of kernels exhibiting visible symptoms of infection on a scale from 0 to 100% (in increments of 5%). The ears from each plot were shelled, the grain was bulked, and the total weight was recorded. Hand-harvested yield, measured in g $plant^{-1}$ was calculated by dividing the total weight of the plot by the number of ears measured in the plot. Test weight, expressed in kg m^3, was measured with the Dickey- John GAC 2000 grain

analysis computer (Auburn, IL) adjusted to the predicted value at a constant 15.5% grain moisture.

Grain samples from each plot in all locations were ground to a fine powder using a Romer II Series Mill (Romer Labs, Union, MO). A 20-g sample of the ground powder was used to measure FUM concentration using an ELISA kit from Helica Biosystems Inc (Santa Ana, CA). The kit included the following: two plates (a dilution mixing and antibody-coated 96-well plate), Conjugate solution A and B, phosphate-buffered saline (PBS), and six standards (0, 2.5, 7.5, 20, 50, and 150 ng mL^{-1}).

Briefly, an extracting solvent of 40 ml (90% methanol) was added to each sample. One ml of that was used as the stock solution. Then depending on the range of FUM expected in each sample, it was diluted accordingly. After the samples were diluted, they were subject to the manufacture protocol and measured by a microplate reader with an absorbance filter of 450 nm; a fourth-order polynomial regression model was fitted to the six known standards. This model was utilized to predict FUM in the unknown samples.

Statistical analysis

Using the R package MASS (Ripley et al., 2018), a Box-Cox transformation was used for FER ($\lambda = 0.38$) and FUM ($\lambda = 0.26$) to reduce the relationship between predicted values and residual variance. Using ASReml-R (Butler et al., 2017), the following linear mixed model was used to estimate the best linear unbiased estimators (BLUEs) for cycles population:

$$Y_{ijklm} = \mu + L_i + R(L)_{ij} + B(RL)_{ijk} + C_l + CG_{lm} + CGL_{ilm} + \varepsilon_{ijklm}$$

where Y_{ijklm} is the observed trait value, μ is the overall mean, L_i is the random effect of location *i,* $R(L)_{ij}$ is the random effect of replication *j* in location *i,* $B(RL)_{ijk}$ is the random effect

of incomplete block *k* nested within replication *j* and location *i,* C_l is the fixed effect of cycle *l,* CG_{lm} is the random effect of line *m* within cycle *l,* CGL_{lim} is the random effect of the interaction between the cycle *l* and line *m* and location *i,* and ε_{ijklm} is the error effect. The difference between cycle means was considered significant at $p < 0.05$ if their absolute value was greater than 1.96 times the standard error of the difference.

To evaluate whether the selection methods had any effect on the genotypic variance of FER and FUM. We fitted a model with equal variance and a heterogeneous variance model $CG_{lm} \sim N(0, \sigma^2_{Gn})$ where σ^2_{Gn} is the genotypic variance within cycle *n*. The GS and PS cycles remained the same, but the four checks in this study were combined into one group, "checks," to estimate genetic variance. Then we conducted a likelihood ratio test of the two models for FER and FUM using ASReml-R and determined the genetic variance change based on the *p*-value associated with the chi-square distribution. From the heterogenous variance models, we access the trend of the genetic variance of each cycle due to selection.

The following linear mixed model was used to estimate BLUEs for each line within a cycle:

$$Y_{ijkm} = \mu + L_i + R(L)_{ij} + B(RL)_{ijk} + G_m + GL_{im} + \varepsilon_{ijkm}$$

where all the terms are the same as before, expect G_m is the fixed effect of line *m,* GL_{im} is the random effect of the interaction between the line *m* and location *i,* and ε_{ijkm} is the error effect. The BLUEs of each line were used to estimate the correlation coefficients of traits measured in the field evaluation.

To visualize the population structure of the ReFus population. A realized kinship coefficient matrix was created in TASSEL software version 5.2.54 using the default parameters for only the 22 founders, then subjected to principal component analysis (PCA) and visualized

using the prcomp function in R core (R Core Team, 2019). Next, to assess the overall population structure change due to selection, the SNP data of 22 founders combined with lines from the base population and all the tested GS and PS Cycles were subjected to a PCA in the same manner as before. The expected heterozygosity of the base population and PS and GS cycles was calculated using the adagenet R package software (Jombart, 2008).

To access the predictive ability of our GS models. First, we took the 50 lines of C3 evaluated in the 2020 field experiment and compared their FER and FUM means to that of the GBLUPs used in 2016 to advance C3. The correlation coefficient from that comparison indicated the predictive ability of our initial GS model across years in the same lines. We also computed the correlation between GBLUPs of the 25 GS C5 lines and their observed FER and FUM BLUEs from the 2020 field experiment.

RESULTS & DISCUSSION

Population structure and genetic variation of the ReFus population

The first and second PCs of the SNP data among the 22 founders explained 24.12% and 11.77% of the variance, respectively (Figure 3.2). One distinct sub-group, which consisted of four founders derived from North Carolina State University, was observed. For the SNP data of 22 founders, C3, and lines from all the tested GS and PS cycles, the first and second PC explained 39.25% and 7.81% of the variance of the SNP variance, respectively (Figure 3.3). The 22 founders were clustered within the training set (C3) (Figure 3.3). We observed a substantial overlap of the C3, PS C4 and C5, and GS C4. The later cycles of GS C5 and C6 ("Best" and "Worst") were clustered together with minimal overlap with C3, indicating the effectiveness of GS to drive allele frequencies in the direction of the initially selected individuals from C3 faster than PS (Figure 3.3).

Direct responses to GS and PS

B73 and FR1064 (identified previously as relatively susceptible lines) had higher ($p < 0.05$) mean FER than GE440 and GEMS0002 (more resistant lines) (Table 3.1). The susceptible checks also had higher ($p < 0.05$) FUM than the resistant checks or any GS or PS cycles (Table 3.1). These results indicated that our field-based disease evaluation trials were sufficient to discriminate FER and FUM means of our more resistant and susceptible checks. The base population, C3, had FUM content of nearly 10 μg g^{-1}, surpassing the recommended amount of FUM content in maize products by the FDA (4 μg g^{-1}), it indicated that our field trials revealed a high potential for mycotoxin contamination in the evaluated materials (Table 3.1).

The mean FER for the base population (C3) was 27%. From C3 to PS C4, FER decreased to 19.2% ($p < 0.05$, Table 3.1). In the following selection round from PS C4 to C5, FER decreased again to 14% ($p < 0.05$). From C3 to GS C4, FER reduced to 18.1% ($p < 0.05$; Table 3.1 & Figure 3.4 A). For the next round of GS, FER decreased again from 18.1% to 15.2% ($p > 0.05$). Lastly, from GS C5 to GS C6, FER decreased to 13.2% and 12.2% for GS C6 "Best" and "Worst," respectively ($p > 0.05$, Table 3.1 & Figure 3.4 A). From the base population to the final cycle of GS and PS, the percent changes of FER were -53% and -48%, respectively. However, the final cycles of GS and PS do not differ significantly ($p < 0.05$) for mean FER.

From C3 to PS C4, the FUM content reduced from 9.8 to 6.6 μg g^{-1} ($p < 0.05$) (Table 3.1 & Figure 3.4 B). The reduction in FUM from PS C4 to C5 was 6.6 to 4.8 μg g^{-1} (Table 3.1). From the C3 to GS C4, FUM content decreased to 4.9 μg g^{-1} ($p < 0.05$) (Table 3.1). From GS C4 to C5, there no gains were made; their FUM content was nearly identical. Lastly, from GS C5 to GS C6, FUM content decreased to 3.8 and 4.6 μg g^{-1} for "Best" and "Worst" ($p > 0.05$; Table 3.1). The percent change from C3 to GS C6 "Best" and PS C5 were -61% and -51%, respectively. Although GS final cycle had less FUM content than PS final cycle, both final cycles did not differ ($p < 0.05$) in their FUM content.

There were a few factors that could have impacted the gains made using GS. First, the prediction ability of the GBLUP model for C3 was 33% and 44% for FER and FUM, respectively. These values were similar to those reported by Holland et al. (2020) for FER but lower for FUM for multivariate GBLUP. At GS C5, the prediction ability dropped to 27% and 24% for FER and FUM. The drop in prediction ability is likely due to not updating the training set; our GS models were built from the same training set for each selection round, which impacted our genetic gains (Jannink, 2010; Neyhart et al., 2017). For each round of GS, a subset

of SNPs from the training set were chosen for genotyping a particular cycle. For example, we used a subset of 2,000 SNPs from the training SNPs to genotype GS C5. The SNPs were not consistent from one cycle to another; there was no significant overlap in common SNPs amongst all GS cycles. This could have impacted the genetic gains for GS because each cycle had a potentially different marker-QTL association, especially as the marker density decreased with each GS cycle (Yabe et al., 2013; Neyhart et al., 2017). Lastly, obtaining high-quality marker data has an enormous impact on the effectiveness of GS. We switched genotyping platforms due to the inability to obtain high-quality markers in GS C5. As a result, we lost one round of GS that could have occurred in the summer of 2019 (Figure 3.1).

Changes in marker and trait genetic variance

The base population C3 had an expected heterozygosity value of 0.32 (Figure 3.4 C). A small but consistent decrease in expected heterozygosity was observed over cycles of selection. For instance, the expected heterozygosity in the last cycles of selection, GS C6 (“Best) and PS C5 were 0.26 and 0.29, respectively (Figure 3.4 C). In this study, the decline in genetic variation is small even under GS, likely due to in part to the use of the OCS method utilized in GS to select individuals with best GEBVs while not exceeding the 5% threshold for expected inbreeding coefficient in progenies (Meuwissen, 1997; Goddard, 2009; Clark et al., 2013; Gorjanc et al., 2018).

The likelihood ratio test of each genotypic variance within populations was significant ($p < 0.05$) for FER but not for FUM, indicating that the genetic variance due to selection changed for FER but not for FUM. For FER, the trait genetic variance for the GS and PS cycles decreased with selection, on average (Table 3.2). Concordant with the results of Rutkoski et al. (2015), who reported a reduction in genetic variance due to GS and PS for resistance to stem rust in

wheat. However, the final cycle of PS had less genetic variance than GS, similar to Asoro et al. (2013), who observed lower genetic variance for β-glucan in the final cycle of PS than GS.

Effects of GS and PS on other agronomic traits

GE440 had the lowest hand-harvested yield; this was expected as this line produces small ears. The base population, C3, had a yield of 71.8 g plant^{-1}. With the selection, hand-harvested yield was 69.3 and 67.3 g plant^{-1} for GS C4 and PS C4, respectively. With one more round GS and PS, the hand-harvested yield was 63.3 and 59.5 g plant^{-1} for GS C5 and PS C5 (Table 3.1). At the final cycles of both GS and PS, the hand-harvest yield reduced compared to C3 ($p < 0.05$; Table 3.1). These results are contrary to previous studies, whose results suggested that PS for FER and FUM would not significantly affect yield (Eller et al., 2010; Horne et al., 2016).

Grain TW had been shown to have a moderate to high negative correlation with FER and FUM (Morales et al., 2018, 2019; Butoto et al., 2021). We hypothesized that more resistant lines would have greater grain TW. The base population, C3, had a mean TW of 763.9 kg m^3; as we selected for lower FER and FUM, TW increased with selection on average. At final cycles of GS C6 "Best" and PS C5, TW was 784 and 792 kg m^3, respectively (Table 3.1). Reiterating that TW could be used to indirectly reduce FER and FUM, especially since it is a relatively more straightforward trait to measure, as suggested by Morales et al. (2018) and Butoto et al. (2021).

For husk and ear traits, husk coverage increased with selection, and husk tightness and husk number decreased with selection, on average (Figure 3.5). The husk strength measured by the rind penetrometer remained unchanged in PS but slightly increased with GS (Figure 3.5). Selection for lower FER and FUM reduced DTA for both selection methods. At C3, DTA was 71 but was reduced ($p < 0.05$) to 68 and 69 days by GS C6 and PS C5, respectively (Table 3.1). In the ReFus population, early flowering had a small and significant correlation to less FER and

FUM (Figure 3.6) (Horne et al., 2016; Holland et al., 2020); this likely contributed to the reduction in FER and FUM as the final cycles of PS and GS flowered earlier. We did not observe any clear trend of plant height due to selection for FER and FUM (Table 3.1).

Trait correlations for GS and PS

FER and FUM had a positive and significant correlation ($r = 0.74$, $p < 0.01$). The correlation between FER or FUM and TW was significant and negative ($r = -0.68$ for FER; $r = -0.63$ for FUM, $p < 0.01$) (Figure 3.5). The correlation coefficient between average husk coverage and FER or FUM was small and negative ($r = -0.3$ for FER; $r = -0.29$ for FUM; $p < 0.01$) (Figure 3.5), suggesting that maize ears where kernels are visible tend to have more FER and FUM. Exposed kernels are prone to more fungal colonization and insect damage, contributing to greater FER and FUM (Warfield and Davis, 1996; Rector et al., 2002; Parsons and Munkvold, 2010).

FER or FUM and husk tightness had a small and positive correlation ($r = 0.25$ for FER; $r = 0.2$ FUM; $p < 0.01$; Figure 3.6), suggesting that maize ears with tightly enclosed husk tend to have more FER and FUM. This contradicts Butrón et al. (2006) and Cao et al. (2014), who reported the importance of husk tightness to fumonisin content. However, in this study, the correlation between FER or FUM and husk tightness is confounded by flowering as later cycles such as GS C5 and C6 and PS C5 tended to flower earlier. Thus, their husk leaves tended to be looser at the time of scoring. Husk coverage and husk likely contributed to the resistance to FER and FUM in the ReFus population.

CONCLUSIONS

GS and PS are highly effective methods to reduce FER and FUM in maize. Head-to-head comparison of GS and PS in one calendar year (i.e., 2017; PS C4 and GS C5), GS reduced FER and FUM significantly ($p < 0.05$) more than PS (Table 3.1 & Figure 1). However, both methods performed similarly in terms of gains per cycle (i.e., from C3 to PS C4 and GS C4) (Table 3.1). For GS, we observed most of the gain for FER and FUM in the first two cycles. We observed a significant reduction in FER and FUM content from the base population to the final cycles of GS and PS. Although the final cycle GS had less FER and FUM than PS, that difference was not statistically significant ($p < 0.05$).

The best method to use, GS or PS, will depend on many factors. The implementation of GS in the breeding program will depend on obtaining high-quality genomic data. In one year, we failed to obtain high-quality markers; as a result, we did not conduct GS in the summer of 2019. Further, we had some individuals used in intermatings whose genotypic data did not pass quality control filtering. The inclusion of such individuals reduces the selection intensity and prevents the optimal contribution method from accurately estimating the change in inbreeding coefficient due to selection. Finally, the time required to sample leaf tissue, prepare and sequence libraries, perform bioinformatics analysis to obtain SNP calls, perform quality control of marker data, and fit genomic prediction models was greater than the time from leaf sampling to flowering. This resulted in the use of individuals with failed genotyping in the intermatings, which we avoided in later GS cycles by first self-fertilizing progenies one generation, selecting the best parents of selfed lines, and then intermating the selfed progenies to form a new cycle. This procedure has the same expected genetic gain as selection imposed after flowering and after intermating random individuals. Genotyping methods that can quickly and reliably return SNP calls in the

time frame between tissue sampling and flowering could allow the imposition of selection before flowering, and this single change would result in doubling of the genetic gain from GS.

Balancing rapid genetic gain and loss of genetic variation will be important in implementing GS. Utilizing the OCS or alternative methods such as weighting rare favorable alleles should be considered to help maintain genetic variation over a long-term period (Meuwissen, 1997; Jannink, 2010; Woolliams et al., 2015). Selection using GS and PS reduced hand-harvested yield compared to the base population. To our knowledge, our study is the first to demonstrate the impact of GS and PS over multiple cycles when selecting for FER and FUM. Thus, more empirical studies with proper yield trials are needed to clarify the impact of yield and other agronomic traits when strictly selecting for FER and FUM. In the meantime, yield needs to be monitor when selecting for FER and FUM.

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TABLES

Table 3.1. Means for four checks inbreds, the base population (C3), four GS cycles, and PS two cycles for Fusarium ear rot (FER), fumonisin (FUM), hand-harvested yield (YIELD), grain test weight (TW), plant height (PHT), and days to anthesis (DTA). Within a trait, means not sharing any letter are significantly different at $p < 0.05$.

Material	FER (%)	FUM (μg g^{-1})	YIELD (g $plant^{-1}$)	TW (kg m^{-3})	PHT (cm)	DTA (d)
B73	70.4^{a}	37.9^{a}	40.7^{ab}	575^{a}	178^{abc}	71^{abd}
FR1064	65.3^{a}	63.5^{a}	30.6^{b}	612^{a}	145^{bd}	70^{abd}
GE440	18.3^{bcef}	15.3^{abc}	12.6^{b}	NA	196^{ac}	75^{abd}
GEMS0002	33.3^{bd}	21.5^{ac}	45.0^{b}	722^{bcd}	133^{d}	69^{acd}
C3	27.0^{b}	9.8^{c}	71.8^{c}	764^{c}	182^{a}	71^{bc}
PS C4	19.2^{dc}	6.6^{d}	67.3^{cde}	774^{c}	171^{bc}	70^{ac}
PS C5	14.0^{f}	4.8^{bd}	59.5^{ae}	792^{d}	183^{a}	69^{a}
GS C4	18.1^{ce}	4.9^{bd}	69.3^{cd}	793^{bd}	166^{b}	69^{ad}
GS C5	15.2^{ef}	5.0^{bd}	63.3^{ac}	780^{b}	175^{ab}	69^{a}
GS C6 “Best”	13.2^{f}	3.8^{b}	62.0^{ad}	784^{bd}	167^{b}	68^{d}
GS C6 “Worst”	12.2^{f}	4.6^{bd}	63.7^{ac}	790^{bd}	187^{a}	71^{bc}

* The FER and FUM estimates are reported as back-transformed means.

Table 3.2. Estimated genetic variance and standard error for Fusarium ear rot of the base population (C3), three GS cycles, and two PS cycles. The four checks in this study were combined into one group to estimate genetic variance.

MATERIAL	Variance	Standard error
Checks	6.77	5.87
C3	0.77	0.24
PS C4	0.67	0.31
PS C5	0.54	0.19
GS C4	1.17	0.45
GS C5	0.74	0.32
GS C6 ‘Best’	0.65	0.21

FIGURES

Figure 3.1. ReFus selection scheme for genomic and phenotypic selection. The blue star indicates where PS took place, and the orange star indicates when GS occurred. The selection occurred in North Caroline and Homestead, FL, from 2016 to 2019. All cycles self-fertilized at various points in the selection scheme to create $S_{0:1}$ lines for field evaluation in 2020.

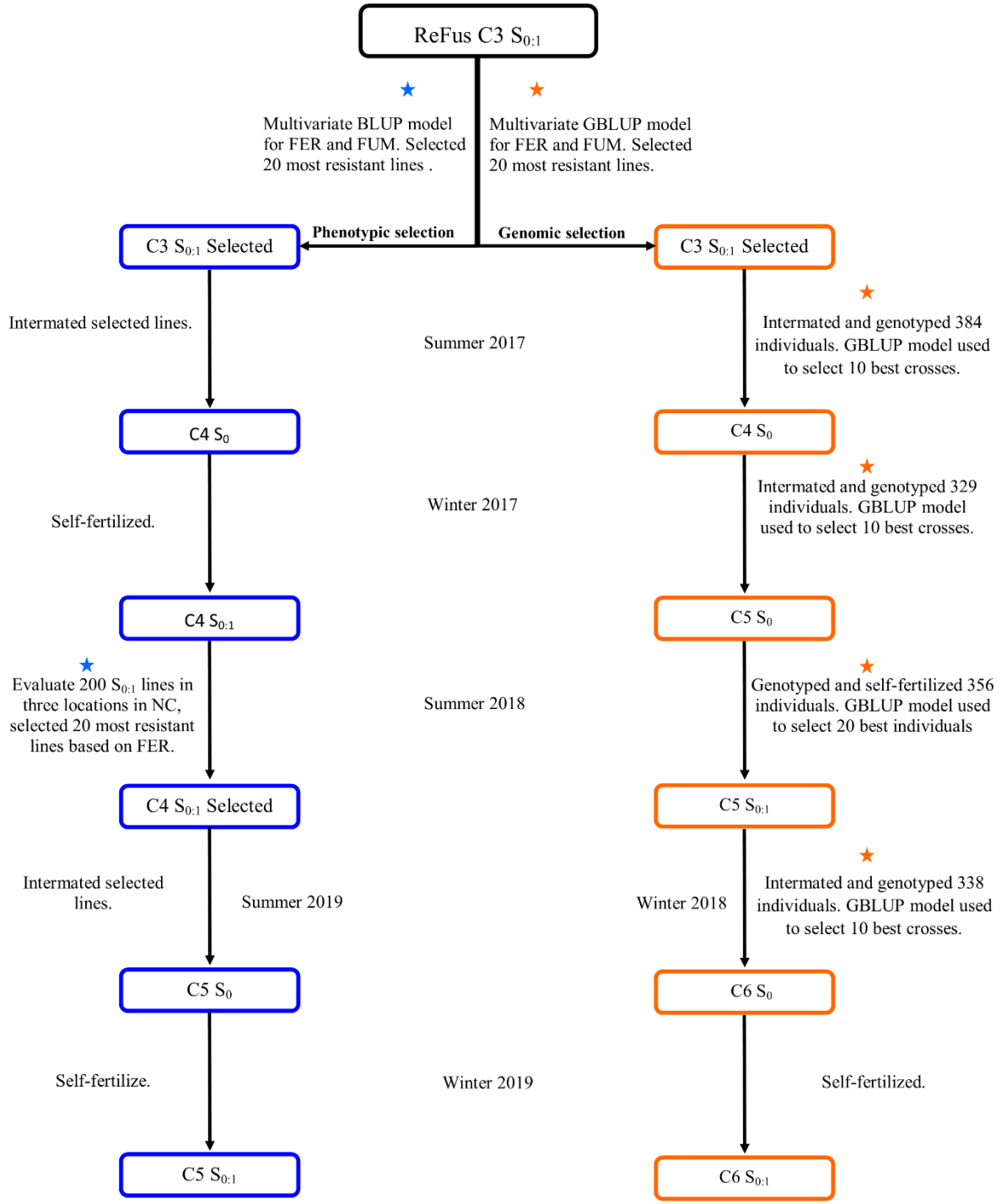


Figure 3. 2. PCA plot of the 22 founders. Half of the founders were selected for potential resistance allele and the other half for agronomic performance.

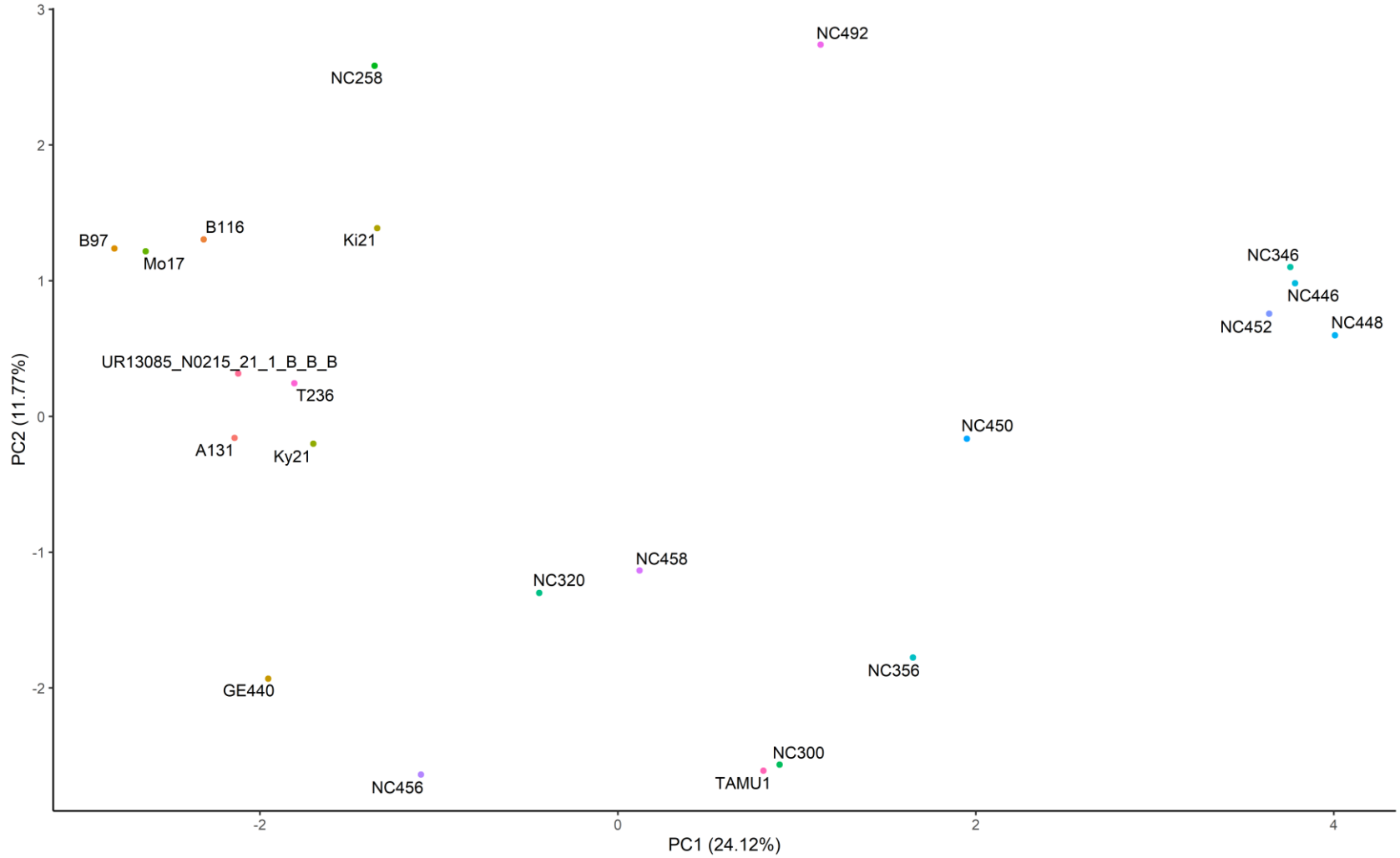


Figure 3.3. PCA Founder, C3 (training set), and GS and PS cycle based on 1,1129 SNPs. The GS C5 and C6 clustered together with little overlap with the training set (C3). The population is pushed in the direction of the selected individuals of C3.

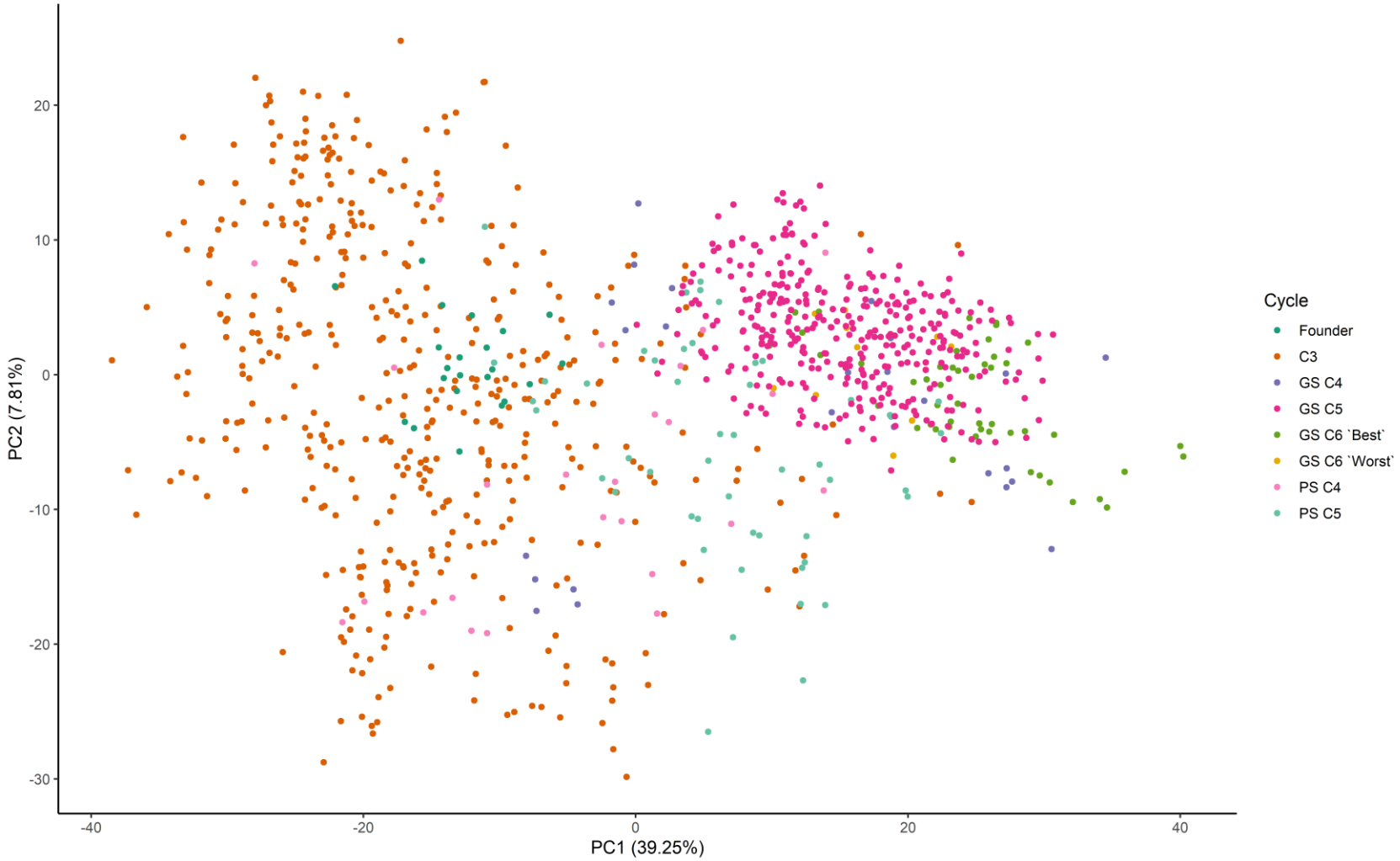
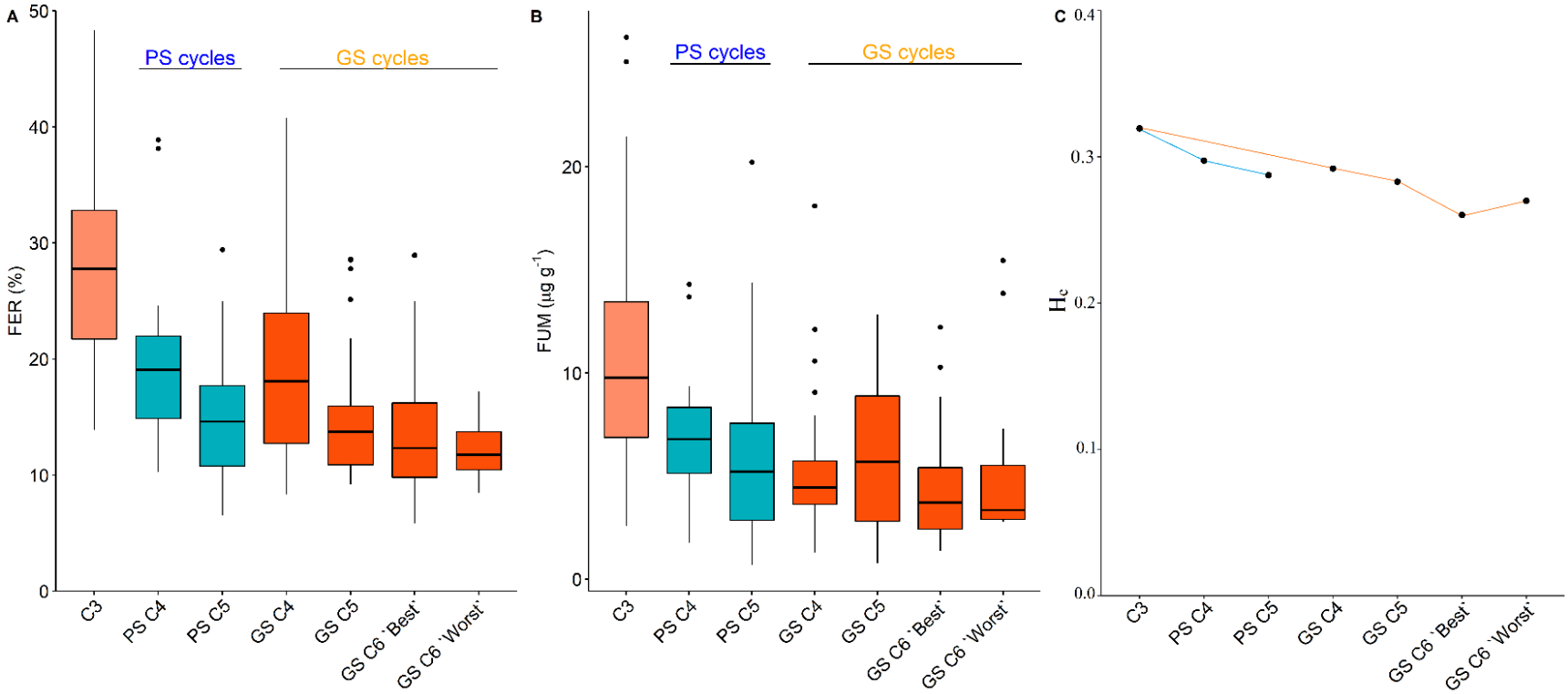


Figure 3.4. (A) Mean change for GS and PS cycles of Fusarium ear rot (FER) and (B) fumonisin due to selection. (C) The expected heterozygosity of the founders, base population (C3), and GS and PS cycles using a smaller subset of 514 SNPs.



*The FER and FUM estimates are reported as back-transformed means.

Figure 3.5. Box plots of husk coverage (HUSK_COVERAGE), husk tightness (HUSK_TIGHTNESS), average husk number (HUSK_NUMBER), and husk strength measured by the rind penetrometer (HUSK_STRNG) for PS and GS cycles. The red diamond represents the mean of the cycle.

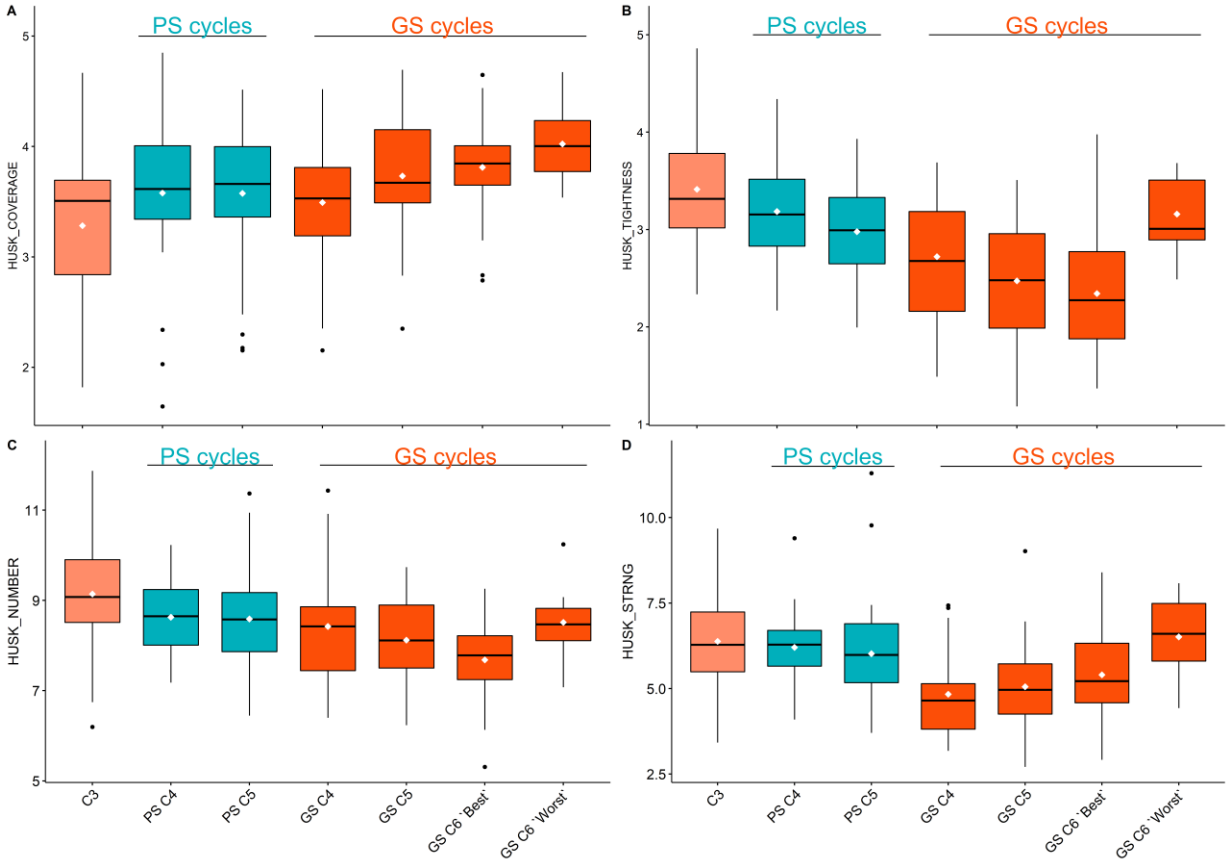
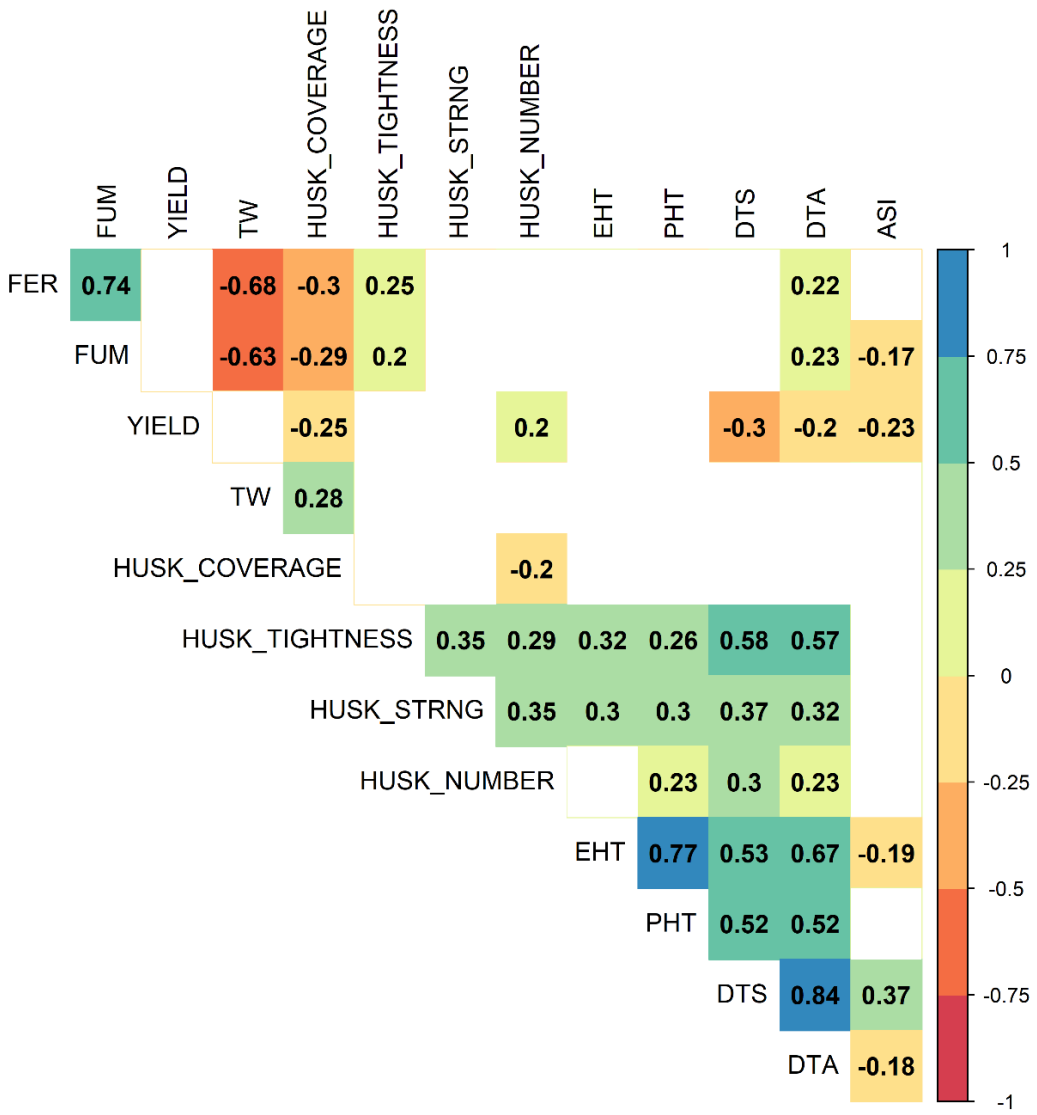


Figure 3.6. The trait correlation matrix for back-transformed Fusarium ear rot (FER) and fumonisin, hand-harvest yield (YIELD), grain test weight (TW), husk coverage (HUSK_COVERAGE), husk tightness (HUSK_TIGHTNESS), husk strength measured by the rind penetrometer (HUSK_STRNG), average husk number (HUSK_NUMBER), plant (PHT) and ear (EHT) height, days to silking (DTS), days to anthesis (DTA), and anthesis-silk interval. Blank coefficients are not significant at $p < 0.01$.



APPENDIX

APPENDIX A: Supplemental Material for Chapter 2

Table A.1. The genetic variance, standard error of the prediction, and residual variance within treatment-location combinations.

Source	ln(FER)	ln(FUM)	Grain yield	Test weight
Genetic Variance				
INOC	0.05	0.25	3.67	489.51
NOT INOC	0	0	14.51	180.00
Standard Error of the prediction (SED)				
INOC	0.20	0.41	2.64	10.99
NOT INOC	0.00	0.00	4.96	6.16
Residual Variance (checks included)				
CLY INOC	2.14	3.89	3436	1849
KIN INOC	0.86	4.21	1892	5782
LEW INOC	2.88	6.05	2389	1386
CLY NOT INOC	3.32	3.39	3350	724
KIN NOT INOC	1.22	2.01	1329	927
LEW NOT INOC	4.06	5.38	2448	809

Figure A.1.The estimated correlation of the resistant and susceptible $S_{0:1}$ line groups for the natural log-transformed Fusarium ear rot [ln(FER)] and fumonisin [ln(FUM)], grain test weight (TW) under artificial inoculation and their topcross hybrids under natural conditions.