ABSTRACT

HORNE, DAVID WILLIAM. Effects of Three Generations of Recurrent Index Selection for Fusarium Ear Rot Resistance, Grain Yield, and Lodging Resistance in Maize. (Under the direction of Dr. James B. Holland).

Fusarium ear rot, caused by Fusarium *verticillioides* (Sacc.) Nirenberg (Syn. = *F. moniliforme* J. Sheld.), is common wherever maize is grown in areas with hot and humid conditions like that of the Southeastern United States. A genetically broad-based population ('ReFus') was developed and recurrent index selection for resistance to Fusarium ear rot, combined with yield and resistance to plant lodging, was undertaken for three generations in this population. A guiding hypothesis for the selection study was that the positive genetic correlation between Fusarium ear rot and grain contamination by fumonisin, a mycotoxin produced by *F. verticillioides* observed in previous studies would permit efficient indirect improvement of fumonisin contamination resistance via selection on Fusarium ear rot resistance, a trait that is much easier to measure. A series of experiments was conducted to evaluate the direct and indirect responses to selection in this population. Direct responses were measured for Fusarium ear rot, grain yield, and lodging in $S_{0:1}$ generation lines. Indirect responses were also measured for these same traits in topcrosses of the $S_{0:1}$ generation lines to an unrelated tester line. Indirect responses were also measured in both $S_{0:1}$ and topcross generations for grain contamination by fumonisin. Mean Fusarium ear rot in $S_{0:1}$ lines was reduced significantly by 13 percentage points by three cycles of selection. Grain yield and lodging mean values changed in the desired directions, but neither of these changes was significant. Similarly, the topcross generations demonstrated a significant indirect response to selection for reduced mean Fusarium ear rot but no significant changes for grain yield or lodging. Significant indirect responses of reduced fumonisin content in grain were also

observed in both $S_{0:1}$ lines (by 8.2 μg g^{-1}) and their topcrosses (by 10.0 μg g^{-1}). Estimates of genotypic correlations between Fusarium ear rot and fumonisin contamination were significant and strongly positive in both the initial and cycle 3 populations *per se* as well as their topcrosses (range: 0.74 to 0.87). Heritability estimates indicated significant genetic variation for all target traits was maintained in the populations after selection, providing capacity for further genetic gain in this population.

Effects of Three Generations of Recurrent Index Selection for Fusarium Ear Rot Resistance, Grain Yield, and Lodging Resistance in Maize

by
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DEDICATION

To my mom and grandparents, you were both extremely influential in all that I have done through your constant pushing of me to do well in school, work, and life choices. To all my family members, you have all helped mold me into the person I am now due to your love, guidance, and prayers. Each of you has meant so much to the pursuit of my degree. To Brittany, thank you for all your help on those long nights of studying, preparing meals, and for the constant words of encouragement.

BIOGRAPHY

David William Horne was raised in Waxhaw, North Carolina. He was inspired to do great things and pursue his dreams by his mother Michelle E. Horne and his grandparents Bill and Linda Edwards. After graduating high school in 2006, David attended college at North Carolina State University where he started in the Agriculture Institute.

During his time at NC State David was fortunate to work with many great plant breeders who would help to guide him on the path to where he is today. It was during his first job with Dr. Arthur Weissinger that he found his passion for plant breeding. He would go on to work in multiple crops at the University as an undergraduate student while taking classes such as; cotton, peanuts, tobacco, and corn. In 2008, David worked for Dr. Vasu Kuraparthy in cotton genetics. He conducted various field experiments and assisted in lab work focusing on genetics and breeding to enhance cotton productivity. David was fortunate in 2009 to begin work with Dr. Jim Holland in Maize breeding where he was given an individual selection trial to manage, as well as the opportunity of running fumonisin screenings in the lab. During his time with Dr. Holland, David developed a greater interest in applied breeding and decided to experience a more in depth role with it at an industry level. David spent time working under the direction of Dr. Sara Gehlhar at a Monsanto breeding internship in Williamsburg, Iowa during the summer of 2010 where he would focus on corn production through the use of doubled haploids.

In 2013, David began working on his Master's at NC State with Dr. Jim Holland where he analyzed the use of three generations of recurrent index selection for reduced fusarium ear rot, reduced lodging, and increased yield. Upon graduation David will continue his education at Texas A&M University with Dr. Bill Rooney in grain sorghum breeding.

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Introduction

Fusarium verticillioides (Sacc.) Nirenberg (Syn. = *F. moniliforme* J. Sheld.) and *F. proliferatum* (Matsushima) Nirenberg are two important hemibiotrophic species of *Fusarium* that cause Fusarium ear and kernel rot of corn (Clements et al. 2004; Zila et al. 2013). *Fusarium verticillioides* is one of the most prevalent fungi associated with maize; it causes direct damage to corn production through Fusarium ear rot disease (Atanasova-Penichon et al. 2014), and also produces mycotoxins that contaminate grain with detrimental effects on both humans and animals (Nelson, 1992).

F. verticillioides produces two forms of conidia spores, microconidia and macroconidia, that are both capable of infecting maize plants via wind, rain, and insect damage (Gary P. Munkvold and Desjardins 1997). Both conidia types are asexual, and they are distinguished by morphology. Macroconidia are sickle-shaped and thin-walled and produced on macroconidiophores that are both branched and unbranched, and microconidia are produced in long chains within branched and unbranched microconidiophores (Nelson, 1992).

Losses in grain yield and quality due to ear rot are two major economic concerns to producers, processers, and consumers of maize (Afolabi et al. 2007). Infection of maize roots, stalks, and ears by Fusarium species can result in severe reductions in crop yield, estimated at between 10% and 30% under typical infection (Logrieco et al. 2002). Fusarium ear rot most commonly affects maize grown in the Southeastern United States and lowland tropics where conditions tend to be hot and humid, but with stress due to limited water

availability during grain filling (Eller et al. 2010). Reid et al. (1999) found that higher temperatures were optimal for growing conditions of the pathogen *F. verticillioides.* Fusarium species produce phytotoxic compounds that can exhibit broad activity in biological pathways and inhibit metabolic reactions (Abbas et al. 1991). Symptoms associated with Fusarium ear rot typically include white localized or pink mycelia, a "starbursting" pattern on kernels affected, and often complete destruction of kernels when heavily infected (Eller, 2008). The distribution of ear rot symptoms on an infected ear typically includes the tips of ears, but may also include the entire ear where symptoms are severe (Parsons and Munkvold, 2012).

Fungal Infection

Infection of maize by *F. verticillioides* can occur at all stages of plant development and are associated with disease in roots, stalks, and kernels (Munkvold and Desjardins, 1997). Duncan and Howard used a strain of *F. verticillioides* expressing a fluorescent protein to track the pathway of infection in corn ears. They injected conidial suspensions in the silk channel and followed fungal colonization by visualizing fluorescence hyphae. In these studies hyphae were observed to enter through an open stylar canal (2009). Munkvold et al. (1997) analyzed different pathways of infection by *Fusarium moniliforme* (the former scientific name for *F. verticilliodes*) through seeds, stalks, and silks, finding that inoculation of conidia through the silks resulted in the highest incidence of *F. moniliforme* with a mean of 83.7%. However, seeds contaminated with *F. verticillioides* are often symptomless; furthermore, seed-borne *F. verticilloides* can infect seedlings by moving from seed tissue

through the seedling roots (Wilke et al. 2007) . This process occurs across a broad range of temperatures at germination, as reported by Wilke et al. (2007), who found no significant differences in the rate of transmission of F. verticilliodes from seeds to seedlings from 4 degrees Celsius to 30 degrees Celsius at planting. C. W. Bacon and Hinton, (1996) found hyphae growing intercellularly and throughout the plant when symptomless, whereas hyphae grew both intercellularly and intracellularly in plants that exhibited symptoms of infection. Damage due to insects tends to be strongly correlated with ears showing signs of Fusarium ear rot (Miller, 2001). Infection courts created by the European corn borer (*Ostrinia nubilalis*), are often initial sites of infection as noted by Munkvold et al. (1997) in a three year study comparing the use of CryIA(b) genes in maize hybrids to hybrids without CryIA(b) where significant moderate to strong correlations of 0.88, 0.53, and 0.66 between incidence of Fusarium ear rot and incidence of European corn borer were observed.

Fumonisin production

Two of the most frequently observed producers of fumonisins in maize are *F. verticillioides* and *F. proliferatum* (Wilke et al. 2007). Maize ears infected by the pathogen *Fusarium verticillioides* may become rotted and result in kernels contaminated with a mycotoxin known as fumonisin, a possible carcinogen that is harmful to humans and animals (Thiel et al. 1992). Bush et al. (2004) found that the first appearances of fumonisin occurred between four to five weeks and continued to increase to nine weeks after pollination demonstrating that earlier harvest times may limit production of fumonisin. Mycotoxins are produced by fungi and elicit toxic responses when introduced to animals and other higher

vertebrates at low concentrations via natural modes of entry (Bennett 1987). The first animal diseases described as associated with Fusarium species were noticed in 1904 (Munkvold and Desjardins, 1997), but the first report of fumonisins produced by cultured *F. verticillioides* did not occur until 1988 by W.C.A. Gelderbon (Munkvold and Desjardins, 1997). A series of four fumonisins, differing in their molecular structure, have been described and named as A, B, C, and P. Among the different types of fumonisin, the fumonisin B series includes the most biologically toxic fumonisin, FB_1 (Logrieco et al. 2002). Fumonisin B1 can be found in both fungal culture and naturally contaminated grain samples and can cause multiple lesions of the liver and blood clots within the vessels of the heart in rats, porcine pulmonary edema (PPE), and equine leukoencephalomalacia (ELEM) (Nelson, 1992). (Ross et al. 1990) conducted an analysis examining 98 samples of feed associated with ELEM and 83 samples associated with PPE and found that among these samples, 44 and 42 of them had the highest concentration of FB_1 contained in corn screenings alone ranging from less than 1 $\mu g/g^{-1}$ to 126 $\mu g/g^{-1}$ for ELEM and less than 1 $\mu g/g^{-1}$ to 330 $\mu g/g^{-1}$ for PPE in feed samples. High amounts of human esophageal cancer in Transkei, South Africa as well as areas of China where corn is considered a dietary staple have been reported to be correlated with the consumption of moldy corn containing fumonisin with levels of FB_1 ranging from 3.45 to 46.9 $\mu g/g^{-1}$ (Sydenham et al. 1990). Since the discovery of the mycotoxin, the U.S. Food and Drug Administration has released a "Guidance for Industry" protocol for acceptable levels of fumonisin in various corn flour and other milled corn products used for human consumption to a concentration between 2 and 4 $\mu g/g^{-1}$ with European regulations set at $1ug/g^{-1}$ for human

consumption and 0.2 ug/g^{-1} for food feed to infants (Center for Food Safety and Applied Nutrition 2015; Kyprianou, 2007).

Management

Environmental conditions favorable for Fusarium ear rot can cause grain producers to experience higher incidences of mycotoxin production (Miller and Trenholm, 1994). Conidial spores of *Fusarium spp*. overwinter on the soil surface and remain on crop debris, thus reduction of spores on the soil surface may be reduced in subsequent rotations of crops through tillage practices that bury existing inoculum (Munkvold, 2003). Mechanical combine harvesting predisposes kernels to physical damage due to moving parts and increases the potential for mycotoxin contamination, therefore, adjustment of the combine's cylinder speed, seed cleaning, and proper drying and storage practices all may help to limit the majority of kernels with *F. verticillioides* and deter further pathogen growth (Munkvold and Desjardins, 1997). Bush et al. (2004) suggested that harvesting ears early from the field (followed by forced-air drying) when years are optimal for fumonisin could help to lower incidences of the mycotoxin. Proper drying and storage regimes include maintaining dried corn at ten to fifteen degrees Celsius and cooled at temperatures of one to four degrees Celsius for storage (Munkvold, 2003). Insuring that hybrids are grown in areas suitable for their adaptation, drought stress is limited, and insect populations are controlled, may be the most suitable strategies to help reduce fumonisin (Miller, 2001). Currently there are no registered fungicides for the control of ear rot in the United States (UCIMP, 2014).

Other means of control have been suggested, such as treating kernels with a bio-control like antagonistic rhizobacteria (Charles W. Bacon and Williamson 1992). Lew et al. (1991) found that when reducing the appearance of European corn borer (*Ostrinia nubilalis*) in maize, the amount of fumonisin decreased. The use of Bt hybrids for the control of Lepidoptera insects may prove to be another alternative to controlling the increase of fumonisin in corn (Bowers et al. 2014). While there are production and harvest practices that may help reduce the occurrence of ear rot, as mentioned above, the best control strategy is to plant hybrids with better resistance to Fusarium ear rot (Logrieco and Leslie, 2014).

Breeding

The main objective of plant breeding is to genetically improve the performance of cultivars within a species in the most efficient manner possible (Fehr 1987). Sources of resistance to Fusarium ear rot have been identified, but are polygenic in nature and difficult to incorporate into hybrids (Gary P. Munkvold 2003; King and Scott 1981). Under natural conditions (without inoculation), ears infected with the pathogen *F. verticillioides* tend to be randomly distributed throughout the field due to spatial variability in inoculum pressure rather than due to genetic differences, requiring breeders to find a more precise method of evaluation for determining resistance or susceptibility (Miller and Trenholm, 1994). Evaluating large numbers of maize hybrids for resistance or susceptibility to ear rot or fumonisin accumulation can be achieved with the use of artificial inoculations through the husks (Clements et al. 2003). An experiment conducted by (Kleinschmidt et al. 2005) discovered that when using inoculation methods to evaluate hybrids for resistance and

susceptibility a more precise way of measuring accurate levels of fumonisin would be to plant hybrids in locations where environments favored pathogenicity of *Fusarium verticillioides*.

QTL mapping with the use of marker assisted selection to identify markers associated with resistance genes to Fusarium ear rot may help to enhance efforts at breeding for resistance (Ding et al. 2008). However, as discovered by Robertson-Hoyt et al. (2006) when comparing QTL mapped in two different maize populations, QTLs identified only represented 47% and 31% of the phenotypic variation within each population and mapped to mostly distinct genomic regions across populations. They hypothesized that because the QTL identified had such small to moderate additive effects and significant genotype by environment interaction, marker assisted selection might be less effective than traditional breeding methods like recurrent selection (Robertson-Hoyt et al.,2006). More recently, a genome-wide association study (GWAS) in a maize diversity panel of 267 inbreds across two environments was conducted by Zila et al. (2013) to locate SNPs conferring resistance to Fusarium ear rot. They were able to identify three loci associated with improved ear rot resistance but these only explained a small proportion of the phenotypic variance. These three loci might be useful for targeted marker-based selection, but selection on these loci alone is not expected to produce substantial genetic gains, so breeders should use such targeted selection in combination with phenotypic and whole-genome selection schemes (Zila et al. 2013). A subsequent GWAS in a much larger panel of about 1,700 inbred lines from the U.S.D.A. maize inbred collection conducted by Zila et al.(2014) identified seven single nucleotide polymorphisms (SNPs) associated with resistance genes, however, these

SNPs were all distinct from those identified in the initial study, demonstrating the complexity of resistance . The authors suggested that selection for these alleles believed to be conferring disease resistance may be useful when combined with the use of genomic selection to select for improvements in the polygenic background.

An evaluation of 18 inbred lines using a diallel mating design study was conducted to determine if significant combining ability along with resistance to Fusarium ear rot should be assessed prior to development of superior hybrids used for the identification of resistance (Hung and Holland 2012). This study identified a high correlation between resistance measured in inbred lines and their GCA (0.79-.78) for ear rot and fumonisin content, suggesting that selection at the inbred stage of development for resistance may increase variation of inbred lines and reduce the cost of extensive topcross evaluations (Hung and Holland, 2012).

Genotypic correlations between ear rot resistance and fumonisin content were 0.96 and 0.87, indicating that visual selection for ear rot resistance should be an effective method at minimizing susceptibility to fumonisin contamination. Heritability on an entry mean basis was estimated to be 0.47 for ear rot resistance and 0.75 for fumonisin content in a backcross population (GE440xFR1064) x FR1064 (Robertson et al., 2006). Although resistance to fumonisin concentration is more heritable than resistance to Fusarium ear rot, (Robertson et al. 2006) noted that indirect selection against ear rot alone might be more economically efficient for reducing susceptibility to fumonisin contamination because ear rot evaluation is less labor-intensive and time consuming than measuring grain fumonisin concentration, which requires enzyme linked immunosorbent assays (ELISA). Robertson et al. (2006) and

(Eller et al. 2010) demonstrated that backcross breeding can be an effective method at introducing quantitative ear rot resistance into susceptible elite genetic backgrounds. However, backcross breeding limits the potential for long term improvement because lines obtained from backcrossing will not be improved for any character except the one being transferred from the donor parent (Fehr, 1987).

Pedigree-based inbreeding methods are most suitable for many applied field breeding programs, but the limited recombination and rapid approach to homozygosity may limit the long-term genetic gain possible. An alternative strategy is to use recurrent selection (Bernardo, 2002). Populations developed from the use of recurrent selection permit the sampling of many more unique haplotypes because of the use of multiple parental lines and the more frequent recombinations of alleles from intermating that is enforced between each selection generation (Fehr, 1987). The continual incremental increases in favorable allele frequencies due to recurrent selection should allow an increase of favorable genotypes to be selected in order for a breeder to develop populations and lines with trait values of particular interest to their program (Hallauer and Miranda, 1988). Recurrent selection continuously repeats three main phases in the development of cycles: (1) select progeny to be used, (2) evaluate selected progenies in replicated trials, and (3) recombine progenies for continual selection of new cycles (Hallauer and Darrah, 1985).

Drawbacks to the use of recurrent selection include the increased number of years needed to generate each selection cycle, resulting in slower cultivar release; the direct products of recurrent selection are typically early generation lines that require numerous

additional generations of selfing and selection before a new cultivar can be produced; and the long-term gain possible may be limited by the choice of founding material.

Efforts aimed at alleviating both Fusarium ear rot and fumonisin production for the enhancement of superior genotypes exhibiting resistance should be centered on the reduction of both traits (Eller, 2008). A main goal of this research is to evaluate the use of recurrent selection for the improvement of Fusarium ear rot and fumonisin, both highly quantitative traits, using an $S_{0:1}$ recurrent selection scheme. Four populations, including an initial base population and populations representing three cycles of selection for reduced ear rot, reduced fumonisin, reduced lodging, and increased yield were evaluated. A second goal of this project is to determine if decreases in ear rot and fumonisin contamination were achieved after three cycles of selection when the populations were crossed to an unrelated tester.

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CHAPTER 2: Response to Recurrent Index Selection for Yield and Resistances to Fusarium Ear Rot and Lodging

Abstract

Fusarium ear rot caused by the pathogen *Fusarium verticillioides* (Sacc.) Nirenberg (Syn. = *F. moniliforme* J. Sheld.) damages corn production and is associated with contamination by fumonisin, a mycotoxin harmful to both humans and animals. Genetic resistance is the most effective and sustainable management strategy for this disease. Recurrent selection may be an effective way to combine improvements in resistance to Fusarium ear rot with improved grain yield and lodging resistance. To test this hypothesis, three cycles of recurrent index selection based on evaluating $S_{0:1}$ lines for Fusarium ear rot, grain yield, and lodging were implemented in a genetically broad-based population. Direct intra-population response to selection in the target traits was measured by comparing random bulk samples of each selection cycle population and individual lines sampled from Cycle 0 and Cycle 3 populations. In addition, indirect response for fumonisin contamination was also measured along with indirect response for agronomic traits when crossed to an elite inbred tester, FR1064. Estimates of genetic variances and correlations were also examined to determine changes in selection from cycle zero to cycle three. Results indicate significant direct gain from selection for Fusarium ear rot, but no significant gains for yield or lodging resistance. Indirect gains were also observed for Fusarium ear rot resistance in topcrosses to an unrelated tester line and for fumonisin contamination both within the selection population and in topcrosses. Heritabilities for target traits did not decrease over generations, indicating potential for further genetic gain from selection in this population.

Introduction

Maize (*Zea mays* L.) kernels infected with the fungus *Fusarium verticillioides* (Sacc.) Nirenberg (synonym *F. moniliforme* Sheldon) and *F. proliferatum* (Matsushima) Nirenberg are a major concern for areas where maize is grown under hot and humid conditions, including the Southeast United States and lowland tropics (Miller, 2001). The conditions of high humidity, hot weather, and drought at or just before flowering make the coastal plain of North Carolina a favorable area for high levels of ear rot and fumonisin contamination of maize (Robertson et al. 2006). *F. verticillioides* is thought to commonly exist both systemically and asymptomatically in most field corn in roots, stalk tissue, and kernels, and the fungus can be passed from parent to progeny by seedborne infection (Wilke et al. 2007). The pathogen may survive on previous corn residue with two other methods of dispersal being common, airborne or water-splashed conidia deposited on the silks and deposited microconidia onto an infection site caused by feeding insects such as the European corn borer (Munkvold et al. 1997).

Symptoms of Fusarium ear rot include white or light pink mold streaks visible under the pericarp of seeds, known as "starbursting". The fungus also can produce a carcinogenic mycotoxin, fumonisin, which poses a threat to both animals and man. Ingestion of maize kernels contaminated by fumonisin has been associated with equine leukoencephalomalacia in horses and porcine pulmonary edema in swine (Marasas et al. 2004). In humans, the consumption of the mycotoxin is associated with some esophageal cancers and neural tube birth defects (Hendricks, 1999). Since the discovery of the mycotoxin, the U.S. Food and Drug Administration has released a "Guidance for Industry" protocol for acceptable levels of

fumonisin in various corn flour and other milled corn products used for human consumption to a concentration between 2 and 4 µg/g (Center for Food Safety and Applied Nutrition 2001).

Currently there is no completely effective control strategy to prevent high levels of fumonisin accumulation in grain (Robertson et al. 2006). Treatments of grain for human consumption have been researched and have been found to eliminate portions of the total fumonisin contamination (Bush et al. 2004) but have proven to be economically infeasible and are not performed for animal consumption. Clements et al. (2004) reported that there is currently no corn inbred known with immunity to either Fusarium ear rot or fumonisin accumulation in grain. Resistance to Fusarium ear rot and accumulation of its mycotoxin fumonisin are quantitative in nature (Gary P. Munkvold and Desjardins, 1997). Robertson et al. (2006) found that both ear rot and fumonisin contamination are specific facets of the disease; they have a fairly low to moderate phenotypic correlation, but high genetic correlation in both partially and highly inbred lines.

Assessment of ear rot for the improvement of genotypes with lower fumonisin content is predicted to be less effective than directly selecting individuals based on the quantification of fumonisin through techniques such as ELISA (Enzyme Linked Immunosorbent assay). However, direct measurement of fumonisin contamination is both costly and time consuming compared to indirect selection on ear rot (Robertson-Hoyt et al. 2006). With the evaluation of ear rot being less time consuming and costly, as compared to selection based on the quantification of fumonisin, ear rot selections permit the use of increased population sizes for evaluation, increased environments for testing, and increased

replications, all resulting in increased selection intensities and higher entry mean heritabilities (Eller et al. 2010). Since Fusarium ear rot and fumonisin contamination share some genetic basis, as demonstrated by their high genetic correlation, selection against Fusarium ear rot alone may produce a desirable indirect effect of reducing susceptibility to fumonisin contamination of maize grain.

Backcrossing has been used to improve the Fusarium ear rot resistance of an elite commercial maize inbred (Eller et al. 2010), but backcrossing has only limited capacity for genetic improvement. Recurrent selection is an alternative breeding strategy that may enable combining long-term improvements in resistance to Fusarium ear rot and fumonisin contamination with improvements in agronomic performance. Recurrent selection, while it is less useful in the short-term for variety development, provides an ideal long-term mechanism for improving quantitative resistance and quantitatively inherited traits in general. Recurrent selection aims to increase the frequency of favorable alleles for target traits, promote recombination to create new allelic combinations, and maintain genetic variability for continued genetic improvement within the population. Using recurrent selection in maize can help to improve sources of germplasm used to extract inbred lines to develop hybrid cultivars.

When genetic improvement for more than one trait is needed, selection on a multi-trait index can be optimal breeding strategy and can be employed along with recurrent selection (Baker, 1986). In maize, yield is often the trait of most importance and is given the highest weight of selection, however, other agronomic traits such as plant and ear height, lodging, and disease resistance may be evaluated and included in a selection index (Hallauer

and Miranda, 1988). Economic weights for different traits in a selection index should relate to their relative importance to the desired end cultivar (Bernardo, 2002). The actual selection index weights can also reflect the heritability and genetic correlations among the measured traits as well (Baker, 1986).

A genetically broad-based maize population was developed to combine favorable alleles for agronomic performance with those for resistance to Fusarium ear rot. Three generations of recurrent selection were conducted based on evaluations of $S_{0:1}$ lines for Fusarium ear rot, grain yield, and lodging resistance. The goal of this breeding program was to develop new germplasm sources combining alleles for resistance to both Fusarium ear rot and fumonisin contamination in adapted genetic backgrounds with good agronomic performance. The selection units were not evaluated for topcross performance nor for resistance to fumonisin contamination, so the response of the populations for these traits is a measure of indirect response to selection.

The objectives of this study were to: (1) measure direct response to three cycles of $S_{0:1}$ recurrent selection based on an index designed to improve grain yield, resistance to Fusarium ear rot, and resistance to lodging, in a genetically broad-based population; (2) test for indirect improvement in resistance to fumonisin contamination; (3) test for indirect responses in traits evaluated on topcrosses to an unrelated tester line, and (4) estimate changes in genetic variances and correlations due to selection.

Materials & Methods

Population Development

The Resistance to Fusarium (ReFus) population was developed from 22 inbred founders, half of which were chosen as potential sources of Fusarium ear rot resistance alleles, and the other half were selected based on their ability to produce agronomically superior hybrids (Table 2.1). Parental lines were chosen from among non-Stiff Stalk temperate and tropical germplasm groups; the temperate Stiff Stalk heterotic group was not included so that lines from the population would have a good chance to perform well as hybrid parents in combination with unrelated Stiff Stalk lines. Using a Design II factorial mating approach (Comstock and Robinson, 1948), each parent line from the group chosen as putative donors of resistance was mated with each parent from the agronomically superior group.

Two generations of random intermating without selection were conducted; the first intermating was performed by bulking pollen from the entire population, mixing it, and applying it to available silks each day. The second generation of intermating was by planting a balanced bulk of seeds from the first intermating on two dates 8 days apart. Bulk pollen collected from plants from the first planting date was applied to silks of plants in the second planting date and vice versa to minimize assortative mating for flowering time. A minimum of 200 ears was harvested from each intermated generation and balanced bulk seed samples were used to propagate the population to minimize genetic drift. Following the second generation of intermating, plants were self-fertilized without intentional selection to create a

total of 206 random $S_{0:1}$ families that served as the base population (Cycle 0) to which selection would be applied.

Base Population Selection Trials

In 2007 the 206 $S_{0:1}$ lines composing the C0 population were evaluated across two North Carolina environments: the Central Crops Research Station at Clayton, and the Peanut Belt Research Station at Lewiston-Woodville. The 206 C0 $S_{0:1}$ lines plus two repetitions each of the resistant and susceptible inbred standards GE440 and FR1064 were planted in randomized 14 x 15 α-lattice design with two replications at each location.

Experimental units were single row plots with length of 3.05 m, 1.22 m alleys between ranges, and inter-row spacing of 0.965 m at Clayton, and 0.914 m at Lewiston-Woodville. Plots were over-planted with 25 kernels per row and thinned to a more uniform stand of 23 plants per row, generating population densities of 65,100 plants per hectare^{-1} at Clayton and 68,750 plants per hectare^{-1} at Lewiston.

Inoculation Methods for Selection Trials

Each plot was inoculated with six isolates of *F. verticillioides* (NC-i6, NC-i7, NC-i9, NC-n16, NC-n17, and NC-22) cultured independently on PDA (Potato Dextrose Agar, Fisher Scientific Pittsburg, PA). Isolates used for the inoculations were submitted to the Fusarium Research Center collection for identity verification and storage (http://frc.cas.psu.edu/). Isolates used for inoculations were cultured separately in petri dishes containing potato dextrose agar (Fisher Scientific, Pittsburg, PA.) and allowed to grow inside a laminar flow hood at room temperature until the agar surface was covered with a mixture of hyphae and

conidia. Conidia were dislodged in sterile water with the aid of a small brush and the resulting suspension was, strained through cheese cloth to remove excess agar. Prior to inoculation, the concentration of conidia was adjusted to approximately $2x10^6$ mL^{-1} water. Two inoculations were conducted at seven days apart to minimize escapes and to simulate common modes of infection. A solution of 5 mL of $2x10^6$ conidia per mL was introduced into the silk channel of the primary ear of the first twelve plants in each row about one week after the median silking date of each experiment. A second inoculation of the same volume and spore concentration but through the husk directly into the base of the ear was performed on the same ears seven days later. A modified Solo backpack sprayer (Solo, Newport News, VA) was connected to syringes to hold and deliver conidial suspensions. To aid in the reduction of surface tension, one drop of undiluted Tween-20 was added to each liter of inoculum suspension.

Trait Measurements and Selection Index

The number of days from planting to silk emergence (when 50% of the ears in each plot showed silk emergence, DTS) and anthesis date (when 50% of the plants within a plot had shed pollen, DTA) were recorded for each plot at the Clayton, NC location only. At maturity, the number of plants lodged (broken below the ear or leaning more than 30 degrees from vertical) was recorded for each plot.

Once plants within each inbred and hybrid disease trial had reached physiological seed maturity, respectively, the first ten primary ears of each inoculated plot were hand harvested, dried to a constant moisture with forced air and visually accessed for percent ear

and kernel rot. Percent ear and kernel rot was scored in increments of 5% from 0% to 100% based on the level of disease symptoms present. Yield measurements for topcross evaluation were adjusted for the number of dropped ears during harvest and to a common moisture content of 15.5%.

Multitrait Selection Index

To accomplish a long term goal of creating germplasm with resistance to Fusarium ear rot and fumonisin contamination resistance combined with good agronomic performance, selection was based on an optimal selection index including percent ear rot, grain yield, and percent lodging. The optimal selection index weights, ***b***, were estimated using the equation $\mathbf{b}=\mathbf{P}^{-1}\mathbf{Ga}$ (Falconer and Mackay, 1996), where **P** is the estimated phenotypic covariance matrix for the three traits, **G** is the estimated genotypic covariance matrix for the three traits, and **a** is the vector of relative economic weights for the three traits. For the first cycle of selection in which adaptation and vigor varied widely, economic weights were chosen so as to maximize gain for grain yield with the secondary goal of then maximizing gain for Fusarium ear rot resistance while ensuring a non-negative selection differential for lodging resistance.

Cycle 1 and Cycle 2 Selection Trials

Following the multivariate analysis and estimation of index weights for Cycle 0, 20 $S_{0:1}$ lines with highest index values were selected to form a balanced bulk from remnant S_1 seed and intermated twice to form the S_0 generation of the Cycle 1 population. A sample of 200 random S_0 plants was self-pollinated to form $S_{0:1}$ families that were used for the Cycle 1

evaluation study. The Cycle 1 population was evaluated in summer 2009 at the same locations with the same experimental design as the Cycle 0 population. The genotypic and phenotypic covariance matrices were re-estimated using only the Cycle 1 data, and a new selection index was created. For both Cycles 1 and 2, the economic weights in the selection index were derived from the selection trial data to put twice as much weight on one standard phenotypic deviation of decrease in ear rot compared to a standard phenotypic deviation increase in yield or decrease in lodging. The 20 highest ranking lines according to the index were selected for intermating to form the Cycle 2 population. Only a single generation of intermating was used in the formation of Cycles 2 and 3. A sample of 200 $S_{0:1}$ lines from Cycle 2 were evaluated in summer 2011 in the same way to identify the highest ranking 20 lines, which were intermated to form Cycle 3.

Evaluation of Selection Response

Four distinct experiments were conducted to evaluate selection response in this population. The first experiment included entries representing balanced bulk samples of S_1 plants from each cycle of selection from zero to three. The second experiment evaluated randomly sampled individual $S_{0:1}$ lines from each of cycle 0 and cycle 3. The third experiment involved topcrosses of the lines tested in the second experiment to a common unrelated tester and evaluated primarily for disease resistance. The fourth experiment included the same topcross entries as the third experiment, but these were evaluated for agronomic performance in larger plots.

Experiment 1: Evaluation of Bulk ReFus Populations

In 2013, the null hypothesis of no differences among cycle population mean values was tested by comparing bulk entries of each of Cycles 0 – 3 of the ReFus population, along with a highly susceptible check entry, FR1064 and a moderately resistance line, NC358 at two locations (Clayton and Kinston) in North Carolina. The Cycle bulk entries were composed of balanced bulks of 500 random S_1 plants representing each cycle population.

The experimental design was an incomplete block design, in which each incomplete block contained a single plot of each ReFus cycle population bulk plus one plot of one check. A total of ten blocks was evaluated at each site, five including FR1064 and five including NC358. In 2014 the experiment was repeated at three North Carolina locations (Clayton, Kinston, and Lewiston-Woodville). Details of plot sizes and planting densities were the same as for the selection trials.

Each of the ReFus bulk populations was evaluated for flowering time, days to anthesis (DTA) and days to silk (DTS), Fusarium ear rot, yield, and lodging as described for the selection trials, however, no lodging data were recorded for 2013. Additionally, plant height, ear height, and percent stand were recorded for each experimental plot at all three locations. Plant heights were measured as the height (cm) from the soil line to the upper most node of the tassel (flag leaf); ear height was measured as the height (cm) from the soil line to the node connected to the primary ear. Experimental plots for 2013 had 10 random plant and ear height measurements from each plot, while experimental plots in 2014 had only 6 random plant and ear height measurements from each plot. Each row of the experimental plots was planted with 25 kernels and thinned to a uniform stand of 20 plants per row. Planting

dimensions, including alley space, were the same as outlined in the selection trial previously, resulting in population densities of 42,458 plants hectare^{-1} at both Clayton and Kinston and 44,833 plants hectare^{-1} at Lewiston. After plants had reached physiological seed maturity, respectively, in both inbred and hybrid disease trials, the same procedure used to access percent ear rot in the selection trial experiment was used.

Inoculation Technique for Evaluation Trials

Inoculation of all experimental plots grown in 2013 and 2014 were conducted following procedures described by Eller et al. (2008). Local North Carolina isolates of *Fusarium verticillioides* (NC-n16, NC-n17, 36D, 40A, 40J, and 40N) were used to inoculate each plot. Both NC-n16 and NC-n17 isolates were selected based on their pathogenicity and high level of fumonisin production. These isolates were collected and identified by (Eller, 2008) from maize fields infected with *Fusarium verticillioides.* The four remaining isolates used are described by Zila (2014) and were selected based on their increased level of pathogenicity to the original isolates used by both (Robertson et al. 2006) and (Eller et al. 2010). For distant locations, contact was maintained with station managers to asses appropriate inoculation times.

Experiment 2: Evaluation of S0:1 Lines from Cycle Zero and Cycle Three

To test the hypothesis that population means, variances, and trait correlations did not change under selection, I evaluated 82 Cycle 0 $S_{0:1}$ lines and 85 Cycle 3 $S_{0:1}$ lines at three locations in North Carolina (Clayton, Kinston, and Lewiston-Woodville) The 167 experimental entries were arranged in 12 ^{x} 14 α-lattice designs with two replications at each

of the three environments. Three inbred checks, FR1064, FR615, and NC358, were randomly assigned to fill the one unassigned plot within each complete replication. In addition, an extra plot was added in a random position to each incomplete block to augment the blocks with a random check entry. After adding the additional check plots, the total number of experimental plots per complete replication was 182. Each individual inbred line was inoculated to assess disease resistance as described previously and measured for other agronomic traits as noted in the phenotypic evaluation section. Experimental plots were planted with 25 kernels at each of the three locations and thinned to a uniform stand of 20. Plot sizes, plant spacing, and plant densities were the same as the Bulk ReFus experiment.

Experiments 3 and 4: Evaluation of Topcrosses of Cycle 0 and Cycle 3 Lines

Each of the 82 Cycle 0 and 85 Cycle 3 $S_{0:1}$ lines evaluated in the line *per se* experiments was also topcrossed to FR1064 to generate F1 hybrid seed for the topcross evaluations. A minimum of 4 plants from each line, with an average of 6 plants, was used in topcrosses, and seed from all crosses of an experimental line was bulked for use in topcross evaluation experiments. The elite commercial inbred line FR1064 is an improved B73 type with superior agronomic performance but with poor resistance to Fusarium ear rot (Eller et al. 2008).

Two experiments were conducted to evaluate indirect response to selection in topcross generations. A topcross disease evaluation experiment was conducted primarily to evaluate Fusarium ear rot and fumonisin contamination of a random sample of Cycle 0 and Cycle 3 $S_{0:1}$ lines. A second experiment was conducted to evaluate agronomic performance

of the topcrosses. The check hybrids FR1064×FR615 F_1, FR1064×NC358 F_1, Pioneer brand hybrids 31G66, 31G98, and Dekalb brand hybrid DK697 were also included the evaluation experiments. The 167 ReFus topcross entries and a check entry were arranged in 12 × 14 α-lattice designs. The check entry plot in each complete rep was filled by a randomly chosen check hybrid, and an extra check plot containing a randomly chosen check hybrid was also added to each incomplete block to produce an augmented design. The two topcross experiments were evaluated in the same three locations used to evaluate the inbred lines per se (Clayton, Kinston, and Lewiston-Woodville). Hybrid disease testcross plots were inoculated with *Fusarium verticillioides* and evaluated for the same traits as the inbred per se experiment. Each experimental unit of the topcross disease trial was planted with 25 kernels and thinned to a uniform stand of 20 plants per plot.

Experimental units in the topcross agronomic evaluation experiment consisted of two rows 3.66 m in length, with a 1.22 m alley way separating ranges of plots. Inter-row spacing was 0.914 m between plots in Lewiston, NC and 0.9652 in Clayton and Kinston, NC. Plots were planted with 25 kernels per row and thinned to a uniform stand of 40 plants per plot resulting in planting densities of 42,458 plants hectare^{-1} at both Clayton and Kinston and 44,833 plants hectare^{-1} Lewiston. When each plot within the hybrid yield trial had reached physiological seed maturity, they were rated for percent lodging and mechanically harvested to record total grain yield and moisture.

Fumonisin Assay

After rating Fusarium ear rot on all of the ears from each plot in experiments 1, 2, and 3, they were bulked together and shelled. Shelled bulk plots were ground to a fine powder in a Romer II Series Mill (Romer Labs, Union, MO). A twenty gram sample of powder from each plot was extracted in 90% methanol and assayed for fumonisin concentration using an Enzyme-Linked ImmunoSorbent Assay (ELISA) (Helica Biosystems Inc.). A standard curve was generated using six known calibrators each containing a different amount of fumonisin concentration so that an appropriate four parameter logistic model could be used to construct a dose response curve to assess concentrations of fumonisin in each sample. The mixture of sample and methanol was placed into corresponding fumonisin antibody-coated microwells where fumonisin competes with bound antibodies for the quantification of Fumonisin B_1 contained in each plot. Microwells were measured optically using a microplate reader at 450nm (OD_{450}). The intensity of color present in each well is proportional to the amount of fumonisin present in each sample, a low intensity of color signifies large amounts of FB_1, while a high intensity of color indicates low amounts of the toxin FB_1 (Helica Biosystems, Inc). Fumonisin assays were purchased from Helica Biosystems, Inc (Helica, Santa Ana, CA).

Statistical Methods- Bulk ReFus Lines

A combined analysis across five environments of the bulk entry experiment was performed using a mixed linear model;

$$Y_{ijkl} = \mu + Location_i + Entry_j + (Location\ x\ Entry)_{ij} + Rep(Loc)_{K(i)} + \varepsilon_{ijkl} ,$$

where entry was considered to be a fixed effect, and location, location by entry, and replication nested within location were considered to be random effects. Analyses of ear rot, fumonisin, and yield were weighted by the number of plants measured in each plot. Ear height and plant height were weighted by the number of ears and plants with which measurements were recorded. Fumonisin violated the assumption of equal variance for residuals; therefore this trait was transformed using a natural logarithmic transformation for analysis. Percent erect plants (lodging), was analyzed in the same manner for both 2013 and 2014 with the exception that a weighted analysis was not performed. Flowering data for Clayton, 2013 and 2014 was analyzed using a mixed linear model with entry as a fixed effect, and year and year by entry interaction were considered to be random. Least square means for entry and their average standard errors of pairwise comparison were estimated from this model. The CORR procedure in SAS was used to obtain an estimate of Pearson correlation coefficients from the plot values for each trait. The check entries NC358 and FR1064 were excluded from the combined analysis so that cycle differences could be tested directly with an F-test.

Statistical Methods- Inbred, Hybrid Disease, and Hybrid Yield Trial

ASReml 3.0 software (Gilmour et al., 2009) was used to analyze data collected from the inbred, hybrid, and hybrid yield trial. Percent erect plants was calculated by subtracting the root lodging and stalk lodging scores from the corresponding stand count and then dividing by stand count. For Experiments 2, 3, and 4, a variable ‘cycle’ with three levels was introduced to distinguished entries from C0, C3, and check groups. Combined analyses

across three locations were performed for each of these experiments using a mixed linear model;

$$Y_{ijklm} = \mu + Location_i + Cycle_k + Entry(Cycle)_{j(k)} + (Cycle\ x\ Location)_{ki} + Rep(Location)_{l(i)} + Block(Rep\ x\ Location)_{m(li)} + Entry(Cycle\ x\ Location)_{j(ki)} + \varepsilon_{ijklm},$$

where cycle was a fixed effect, and entry within cycle, location, cycle by location interaction, and entry within cycle by location interaction were treated as random effects. The model allowed each cycle population to have a unique line within cycle variance, and for each location to have unique error variances. Flowering data were available from only one environment and therefore, they were analyzed fitting cycle as a fixed effect and block nested within replication and entry within cycles as random effects.

For experiments 2 and 3, ear rot, fumonisin, and yield were weighted by the number of ears contained within each plot for the analysis. Both ear rot and fumonisin violated the assumption of equal residual variance and were therefore fitted using a natural logarithmic transformation.

Fusarium Ear rot, fumonisin content, yield, and percent erect plants were calculated both on a plot-basis and entry mean basis (Holland et al. 2002). Heritability on a plot basis for cycle *k* was estimated using the following formula:

$$\hat{H}(per-plot\ basis) = \frac{\hat{\sigma}^2_{Gk}}{\hat{\sigma}^2_{Gk} + \hat{\sigma}^2_{GLk} + \overline{\hat{\sigma}}^2_{\varepsilon}},$$

where $\hat{\sigma}^2_{Gk}$ is the estimated genetic variance for cycle *k*, $\hat{\sigma}^2_{GLk}$ is the estimated entry × location variance for cycle *k*, and $\overline{\hat{\sigma}}^2_{\varepsilon}$ is the average error variance across three environments. Heritability on an entry mean basis was estimated using the following formula:

$$\hat{H}(entry\ mean\ basis) = \frac{\hat{\sigma}^2_{Gk}}{\hat{\sigma}^2_{Gk}+\frac{\hat{\sigma}^2_{GLk}}{l}+\frac{\bar{\hat{\sigma}}^2_{\varepsilon}}{lr}},$$

where l is the number of locations for each experiment, and r is the number of replications per location.

Genotypic and phenotypic correlations were also estimated for each pair of traits measured at all three environments using a multivariate mixed model in ASReml 3.0, following the concepts outlined in (Holland 2006). The same model structure was used as for univariate analysis, but the variances and covariances for each pair of traits were decomposed into variance and covariance components for each of the model terms. The genotypic correlation between traits *i* and *j* for cycle *k* was estimated as:

$$\hat{r}_{gijk} = \frac{\hat{\sigma}_{Gijk}}{\hat{\sigma}_{Gik}\hat{\sigma}_{Gjk}},$$

where $\hat{\sigma}_{Gijk}$ is the estimated genotypic covariance between traits *i* and *j* in cycle *k*, $\hat{\sigma}_{Gik}$ is the estimated genotypic standard deviation for trait *i* in cycle *k*, and $\hat{\sigma}_{Gjk}$ is the estimated genotypic standard deviation for trait *j* in cycle *k* (Holland, 2006).

The phenotypic correlation between traits *i* and *j* was estimated as:

$$\hat{r}_{pijk} = \frac{\hat{\sigma}_{Pijk}}{\hat{\sigma}_{Pik}\hat{\sigma}_{Pjk}},$$

where $\hat{\sigma}_{Pijk}$ is the estimated phenotypic covariance between traits *i* and *j* in cycle *k*, $\hat{\sigma}_{Pik}$ is the estimated phenotypic standard deviation for trait *i* in cycle *k*, and $\hat{\sigma}_{Pjk}$ is the estimated phenotypic standard deviation for trait j in cycle *k* (Holland, 2006).

Results

C0, C1, C2 Selection Trials

Selection differentials (the difference between the mean value of the selected lines and the overall population mean) varied among cycles. The first cycle of selection was aimed at improving adaptation and yield primarily, and the selection differential was greater for yield than for ear rot or lodging resistance. In later cycles, greater weight was placed on ear rot. Over all three cycles, the cumulative selection differentials were -18.0% for ear rot (i.e., selection for lower ear rot), +35.9 g plant^{-1} for grain yield (higher yield), and -12.4% for lodging (i.e., selection for reduced lodging). All traits had moderate heritability in each cycle, but heritability estimates for ear rot and lodging decreased over the three cycles while yield increased from cycle zero to one and then decreased by half in cycle two (Table 2.2). Genotypic correlations for ear rot and yield in both cycles zero and one were slightly negative while cycle two was moderately high (Table 2.3). Fusarium ear rot and lodging had a negative correlation in all three cycles (Table 2.3). Genotypic correlations for yield and lodging, while small, increased from cycle zero to cycle two (Table 2.3).

Experiment 1: Bulk ReFus Lines

Significant ($P<0.01$) differences among cycles were observed for Fusarium ear rot, ear height, and plant height across the five locations in 2013 and 2014. Fusarium ear rot decreased with each cycle of selection, however, fumonisin content only decreased from cycle zero to cycle one and then increased in cycles two and three (Table 2.4). Fusarium ear rot was significantly different between cycles three and zero (11.2%), but fumonisin content

did not differ significantly (Table 2.4). The mean yield increased from cycle zero to cycle one, but then decreased in cycles two and three, whereas percent erect plants decreased from cycle zero to cycle one, but then increased from cycle two to cycle three (Table 2.4). Both yield and percent erect plants showed no significant differences from cycle zero to cycle three (Table 2.4). A small positive (0.24) significant correlation ($P<0.001$) was observed for Fusarium ear rot and Fumonisin content. A negative correlation (-0.44) for yield and Fusarium ear rot was significant ($P<0.0001$). Yield and Percent Erect Plants showed a significant correlation ($P<0.01$), yet negative (-0.18) correlation. Fusarium ear rot and Percent Erect Plants did not have a significant correlation (Table 2.5). Fusarium ear rot, fumonisin content, plant height, ear height, days to anthesis, days to silk and percent erect plants all showed negative selection differentials across cycles. Yield showed a slight positive selection differential (Table 2.4).

Experiment 2: ReFus S0:1 line Evaluations

Mean Fusarium ear rot decreased significantly ($P < 0.05$) from C0 to C3 by 13.9% (Table 2.6). Fumonisin content also decreased significantly ($P<0.05$) by 8.2 g ug^{-1} from C0 to C3. The mean yield for C3 was 2.16 g per plant greater than for C0, but this difference was not significant.

Heritability estimates on a line mean-basis for Fusarium ear rot were 0.78-0.77 for C0 and C3. Cycle zero and cycle three estimates of heritability on a line mean basis for fumonisin were 0.74 and 0.67 (Table 2.7). The estimated genotypic correlation between Fusarium ear rot and fumonisin content was 0.74 (0.08) for cycle zero and 0.87 (0.06) for

cycle three. Phenotypic correlations for Fusarium ear rot and Fumonisin content were 0.54 (0.04) for cycle zero and 0.56 (0.04) for cycle three (Table 2.8). Heritability estimates on a line mean basis for Percent Erect Plants was 0.61 (0.07) for cycle zero and 0.32 (0.13) for cycle three. Line mean basis heritability for yield was 0.82 (0.04) for cycle zero and 0.87 (0.05) for cycle three. Genotypic and phenotypic correlations between Fusarium ear rot and yield ranged between -0.47 and -0.57 across cycles (Table 2.8).

Experiment 3: Hybrid Disease Evaluation

Fusarium ear rot in topcrosses decreased significantly from 37.1 to 28.9% (back-transformed) from C0 to C3. Fumonisin content in topcrosses also decreased significantly from 42.4 to 32.5 g ug^{-1}. The topcrosses of C0 and C3 did not differ significantly for percent erect plants.

Heritability on a line mean-basis for Fusarium ear rot in topcrosses increased from 0.21 (0.15) to 0.53 (0.09) between cycles 0 and 3 (Table 2.9). Genotypic correlations between Fusarium ear rot and Fumonisin content in topcrosses ranged from 0.74 to 0.78 across cycles, whereas the phenotypic correlations ranged from 0.52 to 0.64 (Table 2.10).

Experiment 4: Topcross Evaluation

Grain yield, grain moisture content, and percent erect plants of topcrosses did not change significantly between cycles 0 and 3 (Table 2.7). Heritability on a family mean basis for yield was consistent between cycles, ranging from 0.58 to 0.56. The genotypic correlation between grain yield and grain moisture content changed significantly between cycles, from 0.29 (0.18) for cycle zero to -0.35 (0.21) for cycle three.

Discussion

Results obtained from the comparison of bulk population evaluations of ReFus C0 through C3 populations varied among traits, with the null hypothesis of no differences among cycle means rejected only for Fusarium ear rot, plant height, and ear height. Although ear rot decreased from cycle zero to three by 11 %, fumonisin content only decreased for the first two cycles of recurrent selection, and exhibited an increase in Cycle 3, although not significant. Both yield and lodging data across the cycles repeated the same trend as fumonisin content in cycles zero and one, but did not follow positive trends from cycle two to three.

There are a number of possible explanations for the variability in responses to selection. First, the selection differentials varied across cycles and traits (Table 2.1), because the selection index weights varied due to changing economic weights and due to changing estimates of the components of the genotypic and phenotypic variance-covariance matrices. Second, the selection experiments were necessarily conducted in different years and genotype-by-year interaction would result in fluctuating responses. Third, random sampling in both the selection, intermating, and evaluation process contribute to variability among cycle responses. Finally, both cycles zero and one were intermated twice increasing the amount of recombination between alleles present in the selected germplasm, while cycles two and three only underwent one generation of intermating. Bernardo, (2002) expressed the importance of genetic variation in recurrent selection through repeated recombination, so as to avoid limiting oneself to random fluctuations in allele frequencies. The negative correlations observed between ear rot and yield or yield and lodging, suggest that continued

selection against ear rot and lodging may contribute indirectly to improve yield gains across cycles.

The evaluation of $S_{0:1}$ lines *per se* from Cycles 0 and 3 (Experiment 2) is likely a more precise comparison of the cumulative effects of selection response than is the evaluation of bulk populations (Experiment 1). Each cycle mean value in the bulk population evaluation was estimated based on 50 total plots. By comparison, each cycle mean value in the $S_{0:1}$ lines per se evaluation was based on 480 total plots. Significant favorable changes in mean Fusarium ear rot and fumonisin contamination from cycle zero to three were documented with this experiment. High heritability estimates for cycle zero and three for ear rot and fumonisin provide some evidence that increased replications or increased environments used for testing may continue to reduce genotype by environment interactions for even higher estimates of heritability. The moderately high genotypic and phenotypic correlations for ear rot and fumonisin were much like those reported by Robertson et al. (2004), who originally suggested that selection on ear rot may reduce fumonisin contamination. Our results are the first demonstration of the indirect effect of selection against Fusarium ear rot on reduction in fumonisin contamination. Although yield did not change significantly between cycles zero and three, the trend was in the right direction, and the heritabilities for both Fusarium ear rot and grain yield remained consistent across cycles, suggesting that future cycles of selection may contribute to continued genetic gains in these traits.

Indirect responses to topcross evaluations of the $S_{0:1}$ lines with the inbred tester FR1064 showed significant decreases in both ear rot and fumonisin content from cycle zero

to three. Selection of inbred lines based on ear rot alone shows that reductions in fumonisin and rot can be achieved in hybrid performance. Both genotypic and phenotypic correlations for the two traits remained constant when topcrossed to FR1064 but showed slight decreases in heritability. The difference in heritability estimates between cycle zero and three may suggest that even greater indirect responses to fumonisin may be achieved when future selections against ear rot are made. Therefore, selection imposed on inbreds for Fusarium ear rot may allow breeders to reduce fumonisin content without having to expand resources to generate expensive yield trials. More generations of intermating followed by later stages of inbred testing should increase the amount of additive variance associated with inbred lines resulting in greater genetic gains for indirect response to fumonisin in hybrids.

Agronomic traits such as moisture and yield, however, did not show any significant change across cycles when used as hybrids. Although selection pressure was applied to reduce lodging, very little lodging was observed in the evaluation trials. The genotypic variance for lodging was zero in the topcross evaluation, for example. It is likely that the evaluation environments did not promote lodging sufficiently to permit a good estimation of differences that may exist among genotypes and cycles. Genotypic correlations between grain yield and grain moisture changed from positive to negative between cycles zero and three, which is favorable for selecting for increased yield without increasing grain moisture.

Further selection on Fusarium ear rot for the indirect improvement of fumonisin content should therefore not adversely affect two key agronomic traits such as yield and moisture, but more cycles may be needed to accurately assess any changes that may occur. Increase in yield is desirable, but stronger correlations for reduced moisture as yield increases

would be preferred in order to avoid higher incidence of fumonisin from delayed harvest. Therefore, higher selection intensity for yield and moisture may be warranted to properly distinguish which inbred lines produce higher or lower yielding hybrids with varying levels of moisture. Lodging resistance should also be selected at a higher intensity by either more mechanical means or by planting at locations where environments are conducive for lodging.

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Table 2.1 Pedigrees of 22 founder lines used to create ReFus population.

Inbred	Pedigree
Set I	
B97	BSCB1(C9)
B116	(B97 x B99)-047-1-1-1-1-1-1
NC258	TZ(2) x [(NC248 x 246) x C103]
NC320	SC76-type (non-SS) B52
NC346	Pioneer X105A x H5
NC446	KU2301 x NC296 BC3
NC448	Pioneer X105A x H5
NC450	101-12-2-1 x NC296
NC452	NC296 x NC304
NC456	Pioneer IJ100 x PioneerX304C
NC492	NC258 x NC296
Set II	
A131	A12 x A39
GE440	Hasting's Prolific
Ki21	Pacific 9-S8-45
Ky21	Boone County White
Mo17	CI187-2 x C103
NC300	Pioneer X306B x (Pioneer X105A x H5)
NC300/CML288-B-4-B-B-B-B	NC300 x CML288
NC356	TROPHY low moist. rec. selection
NC458	KU2301 x PM703
T236	T115x(I205 x L289)/xT115-S11
UR13085:N0215-21-1-B-B-B	UR13085 x N02

Table 2.2 Summary of C0, C1, and C2 selection trials. Each trial was evaluated in two complete replications at two locations.

Parameter estimate	Ear rot (%)	Yield (g plant^{-1})	Lodging (%)
	Cycle 0 evaluation (2007)		
h^2	0.74	0.58	0.64
Population mean	12.7	78.8	17
Sel. diff.	-2.1	21.4	0.00
Population SD	5.9	13.0	13.2
N	206		
	Cycle 1 evaluation (2009)		
h^2	0.65	0.61	0.57
Population mean	16.1	109.7	11.5
Sel. diff.	-9.0	10.0	-6.1
Population SD	8.0	14.6	10.1
N	200		
	Cycle 2 evaluation (2011)		
h^2	0.52	0.31	0.54
Population mean	12.2	74.1	24.9
Sel. diff.	-6.9	4.5	-6.3
Population SD	5.7	12.6	12.6
N	200		
Cumulative selection differential	-18.0	35.9	-12.4

Table 2.3 Genotypic variance covariance and correlation estimates from selection trials. Diagonal elements are variance components estimates, off-diagonal elements in lower triangle are genotypic covariance estimates, off-diagonal elements in upper triangle are genotypic correlation estimates.

	Ear rot	Yield	Lodging
		Cycle 0 evaluation (2007)	
Ear rot	25.5	-0.16	-0.30
Yield	-8.3	96.8	-0.08
Lodging	-16.9	-8.4	118.2
		Cycle 1 evaluation (2009)	
Ear rot	40. 8	-0.06	-0.09
Yield	-3.9	124.6	0.05
Lodging	-4.1	4.0	57.3
		Cycle 2 evaluation (2011)	
Ear rot	15.7	0.47	-0.06
Yield	17.0	84.6	0.18
Lodging	2.3	8.9	101.4

Table 2.4 ReFus bulk $S_{0:1}$ means with their standard errors by cycle, along with pair-wise differences from cycle 0 to cycle 3and regression of means on cycle values.

Cycle	Fusarium ear rot (%)	Fumonisin content (µg g^{-1})	Yield (g) per plant	Ear height (cm)	Plant height (cm)	Erect plants (%)	Days to anthesis (d)	Days to silking (d)
0	47.1 (3.1)	44.1 (9.8)	73.4 (4.4)	88.3 (4.8)	178.3 (8.4)	97.0 (1.5)	71.3 (3.1)	73.9 (3.0)
1	40.1 (3.3)	31.9 (9.8)	80.6 (4.4)	86.8 (4.8)	174.9 (8.4)	94.9 (1.5)	70.4 (3.1)	72.9 (3.0)
2	39.1 (3.3)	34.1 (9.8)	79.6 (4.4)	80.9 (4.8)	169.6 (8.4)	95.9 (1.5)	69.7 (3.1)	71.9 (3.0)
3	35.9 (3.3)	41.1 (9.8)	76.7 (4.4)	81.6 (4.8)	171.6 (8.4)	96.1 (1.5)	69.4 (3.1)	72.1 (3.0)
C3 – C0 difference	-11.2* (2.6)	+3.0 (6.1)	+3.3 (3.0)	-6.7* (1.6)	-6.7* (6.7)	-0.9 (1.4)	-1.9* (0.5)	-1.8* (0.5)
$\hat{\beta}$	-3.5*	-0.1	0.9	-2.6	-2.4	-0.2	-0.6*	-0.6
R^2	0.95	0.42	0.13	0.82	0.71	0.07	0.95	0.82

* Significant at P<0.05

Table 2.5 Pearson's correlation coefficients between plot value means for disease and agronomic traits of ReFus bulk populations across five locations in two years.

	Fusarium ear rot (%)	Fumonisin content (μg g^{-1})	Yield (g) per plant	Ear height (cm)	Plant height (cm)	Erect plants (%)	Days to anthesis (d)	Days to silking (d)
Fusarium ear rot (%)	1							
Fumonisin content (μg g^{-1})	0.24***	1						
Yield (g) per plant	-0.44****	NS	1					
Ear height (cm)	NS	NS	0.23**	1				
Plant height (cm)	NS	-0.14*	0.23**	0.84****	1			
Erect plants (%)	NS	NS	-0.18*	NS	NS	1		
Days to anthesis (d)	-0.42****	-0.37***	NS	NS	0.35***	NS	1	
Days to silking (d)	-0.32**	-0.30**	-0.27*	-0.24*	0.28**	NS	0.96****	1

NS is not significant at P= 0.05
*Significant at P =0.05
**Significant at P =0.01
***Significant at P = 0.001
****Significant at P = 0.0001

Table 2.6 ReFus cycle means for $S_{0:1}$ lines per se and their topcrosses to inbred FR1064 for Fusarium ear rot, fumonisin, yield, ear and plant height, erect plants, days to anthesis, and days to silking.

Experiment	Cycle	Fusarium ear rot (%)	Fumonisin content (µg g^{-1})	Yield (g) per plant	Ear height (cm)	Plant height (cm)	Erect Plants (%)	Days to anthesis (d)	Days to silking (d)
ReFus $S_{0:1}$ Lines									
2	0	47.7	29.6	84.3	93.0	185.5	96.3	66.4	68.1
2	3	33.8	21.4	86.5	85.4	179.1	97.4	65.0	67.1
C3 – C0 difference		-13.9**	-8.2*	2.2	-7.6**	-6.4*	1.2	1.4**	-1.0*
Topcrosses to inbred FR1064									
3	0	37.1	42.4	-	94.3	198.6	-	73.1	74.0
3	3	28.9	32.5	-	90.0	195.5	-	72.8	74.0
C3 – C0 difference		-8.2***	-9.9**	-	-4.2**	-3.1*	-	-0.3	0

*Significant at P =0.05
**Significant at P =0.01
***Significant at P = 0.001
****Significant at P = 0.0001

Table 2.7 Estimates of heritability on a line mean-basis for $S_{0:1}$ lines *per se* and for their topcrosses for cycles 0 and 3 of ReFus population.

Experiment	Cycle	Ear rot	Fumonisin	Yield	% Erect Plants	Moisture
2	0	0.78 (0.04)	0.74 (0.05)	0.82 (0.04)	0.61 (0.07)	-
2	3	0.77 (0.04)	0.67 (0.06)	0.85 (0.03)	0.32 (0.13)	-
3	0	0.21 (0.15)	0.46 (0.10)	-	-	-
3	3	0.53 (0.09)	0.54 (0.09)	-	-	-
4	0	-	-	0.58 (0.08)	0.16 (0.06)	0.64 (0.06)
4	3	-	-	0.56 (0.07)	0.08 (0.04)	0.50 (0.08)

Table 2.8 Genotypic (above diagonal) and phenotypic (below diagonal) correlation estimates (and their standard errors) for $S_{0:1}$ lines *per se* from cycles 0 and 3 of ReFus for ear rot, fumonisin, yield and % erect plants.

	Ear rot	Fumonisin	Yield	% Erect Plants
Cycle 0				
Ear rot		0.74 (0.08)	-0.47 (0.10)	0.09 (0.16)
Fumonisin	0.54 (0.04)		-0.33 (0.13)	0.15 (0.16)
Yield	-0.51(0.05)	-0.31 (0.06)		0.03 (0.16)
% Erect Plants	0.12 (0.06)	0.14 (0.05)	-0.01 (0.06)	
Cycle 3				
Ear rot		0.87 (0.06)	-0.57 (0.09)	0.39 (0.22)
Fumonisin	0.56 (0.04)		-0.49 (0.11)	0.31 (0.24)
Yield	-0.53 (0.05)	-0.30 (0.05)		0.04 (0.21)
% Erect Plants	0.08 (0.05)	0.10 (0.05)	0.04 (0.05)	

Table 2.9 Cycle topcross means for grain yield (Mg ha-1), grain moisture (%), ear and plant height (cm), % erect plants, days to anthesis (d), and days to silking (d).

Experiment	Cycle	Grain yield (Mg ha^{-1})	Grain moisture (%)	Ear height (cm)	Plant height (cm)	Erect plants (%)	Days to anthesis (d)	Days to silking (d)
			Hybrid Topcross					
4	0	6.2	18.2	94.1	199.7	96.5	72.5	73.4
4	3	6.3	18.6	90.2	196.2	96.9	72.2	73.3
C3 – C0 difference		0.1	0.4	-3.9	-3.5	0.4	-0.3	-0.1

*Significant at *P* =0.05
**Significant at *P* =0.01
***Significant at *P* = 0.001
****Significant at *P* = 0.0001

Table 2.10 Hybrid disease genotypic and phenotypic correlations with their standard errors by cycle for ear rot, fumonisin, yield.

	Ear rot	Fumonisin	Yield
Cycle 0			
Ear rot		0.74 (0.27)	-0.19 (0.15)
Fumonisin	0.52 (0.03)		-0.50 (0.30)
Yield	-0.50 (0.03)	-0.34 (0.04)	
Cycle 3			
Ear rot		0.78 (0.09)	-0.54 (0.19)
Fumonisin	0.64 (0.03)		-0.60 (0.20)
Yield	-0.54 (0.03)	-0.46 (0.03)	

APPENDICES

Table 2.11 Estimates of heritability on $S_{0:1}$ line *per* se means for cycle zero and cycle three of agronomic traits and their genotypic and phenotypic correlations between ear height and plant height, and days to anthesis with days to silking.

	Cycle 0			Cycle 3		
	Heritability	Genotypic correlation	Phenotypic correlation	Heritability	Genotypic correlation	Phenotypic correlation
Ear Height	0.87			0.88		
		0.73	0.71		0.83	0.77
Plant Height	0.83			0.91		
Days to anthesis	0.91			0.84		
		0.90	0.86		0.91	0.85
Days to silking	0.92			0.84		

Table 2.12 Estimates of heritability for cycle zero and cycle three on hybrid disease and topcross with their corresponding genotypic and phenotypic correlations among ear height and plant height and days to anthesis and days to silking.

Hybrid Disease		Heritability	Genotypic	Phenotypic
Cycle 0	Ear height	0.82	0.81	0.69
	Plant Height	0.81		
	Days to Anthesis	0.59	0.91	0.86
	Days to Silking	0.46		
Cycle 3	Ear height	0.79	0.72	0.61
	Plant Height	0.81		
	Days to Anthesis	0.34	0.99	0.88
	Days to Silking	0.46		
Topcross				
Cycle 0	Ear height	0.86	0.82	0.74
	Plant Height	0.80		
	Days to Anthesis	0.74	0.93	0.80
	Days to Silking	0.68		
Cycle 3	Ear height	0.81	0.82	0.73
	Plant Height	0.86		
	Days to Anthesis	0.63	0.95	0.79
	Days to Silking	0.66		