Responses to Recurrent Index Selection for Reduced Fusarium Ear Rot and Lodging and for Increased Yield in Maize

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ABSTRACT  
Fusarium ear rot, caused by the pathogen Fusarium verticillioides, damages maize (Zea mays L.) grain production and is associated with contamination of grain by fumonisin, a mycotoxin harmful to both humans and animals. 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 S₀:₁ lines for Fusarium ear rot, grain yield, and lodging were implemented in a genetically broad-based population. Direct intrapopulation response to selection in the target traits was measured by comparing lines sampled from Cycle 0 and Cycle 3 populations. In addition, indirect response for fumonisini contamination was also measured along with indirect response for agronomic traits when crossed to commercial inbred tester, FR1064. 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 fumonisini contamination both within the selection population and in topcrosses. Our results are the first demonstration of the indirect effect of selection against Fusarium ear rot on reduction of fumonisini contamination. Heritabilities for target traits did not decrease over generations, indicating potential for further genetic gain from selection in this population.

Maize 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 common occurrence 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 fumonisini contamination of maize (Robertson et al., 2006). Fusarium 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).

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, fumonisini, which poses a threat to both animals and humans. Ingestion of maize kernels contaminated by fumonisini 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 fumonisini in various corn flour and

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Abbreviations: ELISA, enzyme linked immunosorbent assay; PDA, potato dextrose agar; ReFus, Resistance to Fusarium population.
other milled corn products used for human consumption to a concentration between 2 and 4 µg g\(^{-1}\) (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 (Munkvold and Desjardins, 1997; Robertson-Hoyt et al., 2006; Zila et al., 2013, 2014).

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 enzyme linked immunosorbent assay (ELISA). However, direct measurement of fumonisin contamination is both costly and time-consuming compared to indirect selection on ear rot (Robertson-Hoyt et al., 2006), so evaluation and selection based on Fusarium ear rot symptoms 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 (Robertson et al., 2006; Löffler et al., 2010, 2011), selection against Fusarium ear rot alone is predicted to result in 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 (Hallauer and Miranda, 1988). 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 multitrait index can be an 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 primary consideration in 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 also reflect the heritability and genetic correlations among the measured traits (Baker, 1986).

We created a genetically broad-based maize population 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 responses of these traits represent indirect responses to selection.

The objectives of this study were to: (i) 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; (ii) test for indirect improvement in resistance to fumonisin contamination; (iii) test for indirect responses in traits evaluated on topcrosses to an unrelated tester line, and (iv) estimate changes in genetic variances and correlations due to selection.

**MATERIALS AND 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 S1). 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 performed by planting a balanced bulk of seeds from the first intermating on two dates, 8 d 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 × 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 interrow spacing of 0.965 m at Clayton, and 0.914 m at Lewiston-Woodville. Plots were overplanted 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 at Clayton and 68,750 plants per hectare 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-n22) cultured independently on potato dextrose agar (PDA, Fisher Scientific, Pittsburgh, PA). Isolates used for the inoculations were submitted to the Fusarium Research Center collection for identity verification and storage (http://www.fusariummdb.org/intro.php, verified 2 Oct. 2015). Isolates used for inoculations were cultured separately in Petri dishes containing PDA (Fisher Scientific, Pittsburgh, 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. Before inoculation, the concentration of conidia was adjusted to approximately $2 \times 10^6$ mL$^{-1}$ water. Two inoculations were conducted at 7 d apart to minimize escapes and to simulate common modes of infection. A solution of 5 mL of $2 \times 10^6$ conidia per mL was introduced into the silk channel of the primary ear of the first 12 plants in each row about 1 wk after the median silking date of each experiment. A second inoculation of the same volume and spore concentration but injected through the husk directly into the base of the ear was performed on the same ears 7 d 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) and anthesis date (when 50% of the plants within a plot had shed pollen) 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 plot had reached physiological seed maturity, respectively, the first 10 primary ears of each inoculated plot were hand harvested, dried to a constant moisture with forced air, and visually assessed for percentage ear and kernel rot. Percentage ear and kernel rot was scored in increments of 5%, from 0 to 100%, based on the level of disease symptoms present. Grain yield was measured on the same sample of inoculated ears used to measure ear rot.

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 percentage ear rot, grain yield, and percentage lodging. The optimal selection index weights, $b$, were estimated using the equation $b = P_{Ga}^{-1}$ (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 nonnegative 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 $S_0$ seed and intermated twice to form the $S_{0,1}$ 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 reestimated 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 with a standard phenotypic deviation increase in yield or decrease in lodging. The 20 highest ranking lines according to the index were selected for intermingling to form the Cycle 2 population. Only a single generation of intermingling 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
Three distinct experiments were conducted to evaluate selection response in this population. The first experiment evaluated randomly sampled individual S₀₁ lines from each of Cycle 0 and Cycle 3. The second and third experiments involved topcrosses of the lines tested in the second experiment to a common unrelated tester. These topcrosses were evaluated primarily for disease resistance in the second experiment and for agronomic performance in the third experiment.

Experiment 1: Evaluation of S₀₁ Lines from Cycle Zero and Cycle Three
To test the hypothesis that population means, variances, and trait correlations did not change under selection, we evaluated 82 Cycle 0 S₀₁ lines and 85 Cycle 3 S₀₁ lines at three locations in North Carolina (Clayton, Kinston, and Lewiston-Woodville). The 167 experimental entries were arranged in 12 × 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. Planting dimensions, including alley space, were the same as outlined in the selection trial previously, resulting in population densities of 42,458 plants ha⁻¹ at both Clayton and Kinston, and 44,833 plants hectare⁻¹ at Lewiston. Experimental plots were planted with 25 kernels at each of the three locations and thinned to a uniform stand of 20.

Each plot was evaluated for flowering time, days to anthesis, days to silk emergence, Fusarium ear rot, yield, and lodging as described for the selection trials. Additionally, plant height, ear height, and percentage stand were recorded for each experimental plot at all three locations. Plant heights were measured on three random plants per plot as the height from the soil line to the flag leaf node; ear height was measured on the same three random plants per plot as the height from the soil line to the node connected to the primary ear. Percentage erect plants was calculated by subtracting the root lodging and stalk lodging scores from the corresponding stand count and then dividing by stand count.

Inoculation Technique for Evaluation Trials
Inoculation of experimental plots was performed as explained for the selection trials, with the exception that local North Carolina isolates of F. verticillioides (NC-n16, NC-n17, 36D, 40A, 40J, and 40N) were used to inoculate each plot. 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 et al. (2008) from maize fields infected with F. verticillioides. The four remaining isolates used are described by Zila (2014) and were selected based on their increased level of pathogenicity compared to the original isolates used by both Robertson et al. (2006) and Eller et al. (2010). Ear rot, fumonisin content, and grain yield were measured on 10 inoculated ears per plot.

Experiments 2 and 3: Evaluation of Topcrosses of Cycle 0 and Cycle 3 Lines
Each of the 82 Cycle 0 and 85 Cycle 3 S₀₁ 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 four plants from each line, with an average of six plants, was used in topcrosses, and seed from all crosses of an experimental line was bulked for use in topcross evaluation experiments. The 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 S₀₁ lines. A second experiment was conducted to evaluate agronomic performance of the topcrosses. The check hybrids FR1064 × FR615 F₁, FR1064 × NC358 F₁, Pioneer brand hybrids 31G66, 31G98, and Dekalb brand hybrid DK697 were also included in 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 S₀₁ lines per se (Clayton, Kinston, and Lewiston-Woodville).

The testcross disease evaluation plots were inoculated with F. verticillioides and evaluated for the same traits as the S₀₁ lines 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 of the same size and spacing as used for the other experiments. Plots were planted with 50 kernels per plot 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 percentage lodging and mechanically harvested to record total grain yield and moisture. Yield measurements for topcross evaluations were adjusted for the number of dropped ears during harvest and adjusted to a common moisture content of 15.5%.

Fumonisin Assay
After rating Fusarium ear rot in Experiments 1 and 2, all of the ears from a plot 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 20-g sample of powder from each plot was extracted in 90% methanol and assayed for fumonisin concentration using ELISA kits obtained from Helica Biosystems Inc. (Santa Ana, CA). A standard curve was generated using six known calibrators each containing a different amount of fumonisin concentration so that a fourth-order polynomial regression 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 B₁ contained in each plot. Microwells were measured optically using a microplate reader at 450 nm (OD₄₅₀). 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 FB₁, while a high intensity of color indicates low amounts of the toxin FB₁. Fumonisin assays were purchased from Helica Biosystems, Inc.

Statistical Analyses

A variable cycle with three levels was introduced to distinguish entries from C0, C3, and check groups for the analyses of each experiment. Combined analyses across three locations were performed for each of these experiments using a mixed linear model fit using ASReml 3.0 software (Gilmour et al., 2009):

\[
Y_{ijk} = \mu + L_i + C_k + E(C)_{j(k)} + C \times L_{ij} + \text{Rep}(L)_{j(k)} + B(\text{Rep} \times L) + E(\text{C} \times L)_{j(i)} + \epsilon_{ijk},
\]

where cycle (C) was a fixed effect, and entry (E) within C, location (L), C × L interaction, Rep within L, block (B) within Rep × L, and E within C × L 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, cycle was fit as a fixed effect, and complete replications, blocks nested within replication, and entry within cycles as random effects.

Fumonisin and yield per plant analyses for Exp. 1 and 2 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.

Heritability on an entry mean-basis was estimated using the following formula:

\[
H(\text{entry mean – basis}) = \frac{\hat{\sigma}_G^2}{\hat{\sigma}_G^2 + \hat{\sigma}_{G \times L}^2 + \hat{\sigma}_E^2},
\]

where \(\hat{\sigma}_G^2\) is the estimated genetic variance for Cycle \(k\), \(\hat{\sigma}_{G \times L}^2\) is the estimated entry × location variance for Cycle \(k\), \(\hat{\sigma}_E^2\) is the average error variance across three environments, \(I\) is the harmonic mean of the number of locations for each line, and \(r\) is the harmonic mean of the total number of replications across environments in which each line was measured (Holland et al., 2003).

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:

\[
\hat{r}_{gik} = \frac{\hat{\sigma}_{Gik}}{\hat{\sigma}_G \hat{\sigma}_{Gjk}},
\]

where \(\hat{\sigma}_{Gik}\) is the estimated genotypic covariance between traits \(i\) and \(j\) in Cycle \(k\), \(\hat{\sigma}_G\) 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:

\[
\hat{r}_{pik} = \frac{\hat{\sigma}_{Pik}}{\hat{\sigma}_P \hat{\sigma}_{Pjk}},
\]

where \(\hat{\sigma}_{Pik}\) is the estimated phenotypic covariance between traits \(i\) and \(j\) in Cycle \(k\), \(\hat{\sigma}_P\) 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 substantially 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 Fusarium ear rot or lodging resistance. In later cycles, greater weight was placed on selection for reduced 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 estimates in each cycle, but heritability estimates for ear rot and lodging decreased over the three cycles whereas the estimated yield heritability increased from Cycle 0 to 1 and then decreased by half in Cycle 2 (Table 1). The estimated genotypic correlation between ear rot and yield were slightly negative for the first two cycles, but became moderately high and positive in C3 (Table 2). Fusarium ear rot and lodging had a negative correlation estimate in all three cycles (Table 2). Genotypic correlation estimates for grain yield and lodging were small (Table 2).

Experiment 1: ReFus S₀₁ Line Evaluations

The inoculation treatment was successful in clearly separating the Fusarium ear rot resistance levels of the more susceptible check inbreds FR1064 and FR615 (80 to 88% mean ear rot) from the more resistant check inbred NC358 (47% mean ear rot). Mean Fusarium ear rot decreased significantly \((P < 0.05)\) from C0 to C3 by 13.9% (Fig. 1; Table 3). Fumonisin content also decreased significantly \((P < 0.05)\) from C0 to C3 by 13.9% (Fig. 1; Table 3). Neither mean grain yield nor lodging resistance changed significantly between C0 and C3 (Fig. 1; Table 3). Neither mean grain yield nor lodging resistance changed significantly between C0 and C3 (Fig. 1; Table 3).

Heritability estimates on an entry mean–basis for Fusarium ear rot were 0.77 to 0.78 for C0 and C3. Estimates of heritability on an entry mean–basis for fumonisin were 0.74 and 0.67 (Table 4). The estimated genotypic correlation between Fusarium ear rot and fumonisin...
The topcrosses of C0 and C3 did not differ significantly for percentage erect plants. The mean percentage erect plants for C0 and C3 was 96 and 97%, respectively (Table 3); consequently, the heritability estimates for percentage erect plants were mostly low or not significant (Tables 4 and 5).

### Experiment 2: Topcross Disease Evaluation

Mean Fusarium ear rot in topcrosses decreased significantly from 37.1 to 28.9% from C0 to C3 (Fig. 1; Table 3). Fusomisin 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 percentage erect plants.

Heritability on an entry mean-basis for Fusarium ear rot and yield ranged between −0.47 and −0.57 across cycles (Table 5). Very little variation for lodging was observed among the three evaluation environments; mean percentage erect plants for C0 and C3 was 96 and 97%, respectively (Table 3); consequently, the heritability and genotypic correlation estimates involving lodging were mostly low or not significant (Tables 4 and 5).

### Experiment 3: Topcross Agronomic Evaluation

Grain yield, grain moisture content, and percentage erect plants of topcrosses did not change significantly between C0 and C3 (Table 3). Heritability on an entry 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 0 to −0.35 (0.21) for Cycle 3. Like the S₀₁₁ evaluation trial, we observed very little lodging in the topcross trial as well.

### DISCUSSION

Significant favorable changes in mean Fusarium ear rot and fumonisin contamination from C0 to C3 were documented with this experiment. The moderately high genotypic and phenotypic correlations for ear rot and fumonisin (Table 5; Fig. 2) were similar to those reported by Robertson et al. (2006), who originally suggested that selection on ear rot may reduce fumonisin contamination, and by Löffler et al. (2010, 2011). Our results are the first demonstration of the indirect effect of selection against Fusarium ear rot on reduction of fumonisin contamination. Although yield did not change significantly between Cycles 0 and 3, the trend was favorable, 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 S₀₁₁ lines with the inbred tester FR1064 showed significant decreases in both ear rot and fumonisin content from Cycle 0 to 3. This result and the strong relationship between S₀₁₁ and topcross generation values for these traits (Fig. 2) demonstrate that selection among partly inbred generations based on Fusarium ear rot can result in reductions in both ear rot and fumonisin contamination.
Figure 1. Histograms of grain yield, Fusarium ear rot, and fumonisin content from ReFus C0 (dark gray) and C3 (light gray) populations. Population means are displayed as vertical lines (C0 dashed line, C3 dotted line). (A) Mean values for $S_{0:1}$ lines per se. (B) Mean values for topcrosses of $S_{0:1}$ lines to FR1064.
Fusarium ear rot and fumonisin content in hybrid generations. The genotypic and phenotypic correlations between Fusarium ear rot and fumonisin content were similar in inbred and topcross generations, but the heritabilities were slightly reduced in topcross generations, congruent with the results of Hung and Holland (2012). This trend suggests that conducting initial selections for Fusarium ear rot resistance in inbred generations is more economically efficient than having to create topcrosses in early generation materials and evaluate the topcrosses directly. In the case where doubled haploids are produced directly from breeding crosses, however, Löffler et al. (2011) and Martin et al. (2012) suggested that evaluation of topcrosses for ear rot diseases will often be more efficient. The maintenance of relatively high heritability estimates for Fusarium ear rot consistently across three cycle generations suggests that a similar rate of gain is expected if selection is conducted in the latest cycle population.

In contrast to the very favorable responses to selection observed for Fusarium ear rot and fumonisin content, the target agronomic traits of grain yield and lodging resistance were not improved during the selection program. The lack of gain in lodging resistance likely reflects the very low expression of lodging in the evaluation environments. In the absence of higher levels of lodging expression, it remains unclear whether any genotypic improvement for lodging resistance was achieved. The lack of gain in grain yield cannot be attributed to a lack of genotypic variation for the trait in the evaluation environments, however, as substantial phenotypic variation among line means was observed (Fig. 1) and heritability estimates within the evaluation environments were above 80% (Table 4). Furthermore, the estimated heritability for grain yield was relatively high within each selection trial (Table 1).

Given the high estimated heritabilities for grain yield within both selection and evaluation trials and the large cumulative selection differential for grain yield, why was greater response in grain yield not observed in this experiment? Possible reasons for this lower-than-expected response in grain yield include upwardly biased estimates of heritability within both selection and evaluation trials and the large year interaction to phenotypic variation, it remains unclear whether any genotypic improvement for lodging resistance was achieved. The lack of gain in grain yield cannot be attributed to a lack of genotypic variation for the trait in the evaluation environments, however, as substantial phenotypic variation among line means was observed (Fig. 1) and heritability estimates within the evaluation environments were above 80% (Table 4). Furthermore, the estimated heritability for grain yield was relatively high within each selection trial (Table 1).

Given the high estimated heritabilities for grain yield within both selection and evaluation trials and the large cumulative selection differential for grain yield, why was greater response in grain yield not observed in this experiment? Possible reasons for this lower-than-expected response in grain yield include upwardly biased estimates of heritability within both selection and evaluation trials. All of these experiments were conducted in a single year, so the contribution of genotype × year interaction to phenotypic variation could not be estimated, possibly leading to serious upward biases in the heritability estimates. In addition, the numerator of these heritability estimates is the genotypic variance among S₀ lines, which includes contributions

Table 3. ReFus cycle means with inoculation treatment for S₀ 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. Cycle topcross means for grain yield grain moisture, ear and plant height, percentage erect plants, days to anthesis, and days to silking under noninoculated conditions.

<table>
<thead>
<tr>
<th>Cycle</th>
<th>Fusarium ear rot</th>
<th>Fumonisin content</th>
<th>Grain Moisture</th>
<th>Grain yield</th>
<th>Ear height</th>
<th>Plant height</th>
<th>Erect plants</th>
<th>Days to anthesis</th>
<th>Days to silking</th>
</tr>
</thead>
<tbody>
<tr>
<td>S₀₀ lines per se</td>
<td>%</td>
<td>µg g⁻¹</td>
<td>%</td>
<td>g plant⁻¹</td>
<td>cm</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>47.7</td>
<td>29.6</td>
<td>-</td>
<td>84.3</td>
<td>93.0</td>
<td>185.5</td>
<td>96.3</td>
<td>66.4</td>
<td>68.1</td>
</tr>
<tr>
<td>3</td>
<td>33.8</td>
<td>21.4</td>
<td>-</td>
<td>86.5</td>
<td>85.4</td>
<td>179.1</td>
<td>97.4</td>
<td>65.0</td>
<td>67.1</td>
</tr>
<tr>
<td>C3 – C0 difference</td>
<td>-13.9**</td>
<td>-8.2*</td>
<td>-</td>
<td>2.2</td>
<td>-7.6**</td>
<td>-6.4*</td>
<td>1.2</td>
<td>1.4**</td>
<td>-1.0*</td>
</tr>
</tbody>
</table>

** Significant at \( P = 0.01 \).
*** Significant at \( P = 0.001 \).
† Trait not measured.

Table 4. Estimates of heritability (and their SE) for Fusarium ear rot, fumonisin content, grain yield, erect plants, and grain moisture on an entry mean-basis for S₀ lines per se and for their topcrosses for Cycles 0 and 3 of ReFus population. All traits were measured under inoculation treatment except grain yield, erect plants, and grain moisture in topcross hybrids.

<table>
<thead>
<tr>
<th>Cycle</th>
<th>Fusarium ear rot</th>
<th>Fumonisin content</th>
<th>Grain yield</th>
<th>Erect plants</th>
<th>Grain moisture</th>
<th>Erect plants</th>
<th>Grain moisture</th>
<th>Grain moisture</th>
<th>Grain moisture</th>
</tr>
</thead>
<tbody>
<tr>
<td>S₀₀ lines per se</td>
<td>%</td>
<td>µg g⁻¹</td>
<td>%</td>
<td>g plant⁻¹</td>
<td>cm</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0.78 (0.04)</td>
<td>0.74 (0.05)</td>
<td>0.82 (0.04)</td>
<td>0.61 (0.07)</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>0.77 (0.04)</td>
<td>0.67 (0.06)</td>
<td>0.85 (0.03)</td>
<td>0.32 (0.13)</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Topcrosses to inbred FR1064</td>
<td>%</td>
<td>µg g⁻¹</td>
<td>%</td>
<td>g plant⁻¹</td>
<td>cm</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0.21 (0.15)</td>
<td>0.46 (0.10)</td>
<td>0.58 (0.08)</td>
<td>0.16 (0.06)</td>
<td>0.64 (0.06)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>0.53 (0.09)</td>
<td>0.54 (0.09)</td>
<td>0.56 (0.07)</td>
<td>0.08 (0.04)</td>
<td>0.50 (0.08)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

† Trait not measured.
Table 5. Genotypic (above diagonal) and phenotypic (below diagonal) correlation estimates (and their standard errors) for \( S_{0:1} \) lines per se and their hybrid topcrosses from Cycles 0 and 3 of ReFus for ear rot, fumonisin, grain yield, and percentage erect plants. All traits were measured under inoculation treatment except grain yield, erect plants, and grain moisture in topcross hybrids.

<table>
<thead>
<tr>
<th>Cycle</th>
<th>Trait</th>
<th>Ear rot</th>
<th>Fumonisin</th>
<th>Grain yield</th>
<th>Erect plants</th>
</tr>
</thead>
<tbody>
<tr>
<td>ReFus S(_{0:1}) lines</td>
<td>Cycle 0</td>
<td>0.74 (0.08)</td>
<td>-0.47 (0.10)</td>
<td>0.09 (0.16)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ear rot</td>
<td>0.54 (0.04)</td>
<td>-0.33 (0.13)</td>
<td>0.15 (0.16)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Fumonisin</td>
<td>-0.51 (0.05)</td>
<td>-0.31 (0.06)</td>
<td>0.03 (0.16)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Erect plants</td>
<td>0.12 (0.06)</td>
<td>0.14 (0.05)</td>
<td>-0.01 (0.06)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cycle 3</td>
<td>0.87 (0.06)</td>
<td>-0.57 (0.09)</td>
<td>0.39 (0.22)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ear rot</td>
<td>0.56 (0.04)</td>
<td>-0.49 (0.11)</td>
<td>0.31 (0.24)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Fumonisin</td>
<td>-0.53 (0.05)</td>
<td>-0.30 (0.05)</td>
<td>0.04 (0.21)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Erect plants</td>
<td>0.08 (0.05)</td>
<td>0.10 (0.05)</td>
<td>0.04 (0.05)</td>
<td></td>
</tr>
<tr>
<td>Hybrid topcross</td>
<td>Cycle 0</td>
<td>0.74 (0.27)</td>
<td>-0.19 (0.15)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ear rot</td>
<td>0.52 (0.03)</td>
<td>-0.50 (0.30)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Fumonisin</td>
<td>-0.50 (0.03)</td>
<td>-0.34 (0.04)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Erect plants</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cycle 3</td>
<td>0.78 (0.09)</td>
<td>-0.54 (0.19)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ear rot</td>
<td>0.64 (0.03)</td>
<td>-0.60 (0.20)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Fumonisin</td>
<td>-0.54 (0.03)</td>
<td>-0.46 (0.03)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Erect plants</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

† Trait not measured.

Figure 2. Scatterplots of mean values from ReFus C0 (black) and C3 (light gray) populations. (A) Fusarium ear rot vs. fumonisin content mean values for \( S_{0:1} \) lines per se. (B) Fusarium ear rot measured in \( S_{0:1} \) lines per se vs. Fusarium ear rot measured in topcrosses of \( S_{0:1} \) lines to FR1064.
of the variation in genotypic correlation estimates is clearly due to genotype × year interactions. This can be seen by comparing the genotypic correlation estimate in the selection trial of C0 in year 2007 (∼0.16; Table 2) to the reestimate of the same parameter in the evaluation trial of C0 in year 2014 (∼0.53; Table 5). The selection index depends on the estimates of genotypic covariances as well as heritabilities; thus, the variability in these estimates due to limited sampling of years during the selection trial will reduce the gain from selection below predicted values.

Given the possibly unreliable estimates of genotypic correlations involving yield and the low realized heritability for yield, future selection cycles may instead simply select against Fusarium ear rot, leaving yield selection to advanced generations of lines derived from the populations. Further selection on Fusarium ear rot for the indirect improvement of fumonisin content should 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. Increased yield is desirable, but selection for reduced moisture as yield increases may be required to avoid higher incidence of fumonisin from delayed harvest.

Supplemental Information Available
Supplemental information is included with this article.

Table S1. Pedigrees of 22 founder lines used to create ReFus population.

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References