

Improving Resistance to Fumonisin Contamination in Maize

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Colonization of the maize ear by *Fusarium verticillioides* (formerly *F. moniliforme*, teleomorph *Gibberella fujikuroi*) can cause Fusarium ear rot. This disease is prevalent in the warm, dry conditions, common in the southern United States and lowland tropics. However, *F. verticillioides* can be found in grain or crop residue of virtually all mature corn fields in the United States, and high levels of resistance are not present in commercial hybrid corn (Munkvold et al., 1997). Fusarium ear rot is of particular concern because *F. verticillioides* and the related fungus, *F. proliferatum*, can produce mycotoxins called fumonisins that contaminate corn grain and are suspected of being carcinogens (Prelusky, 1994; Miller, 1994). Ear rot and fumonisin contamination are distinct aspects of the disease, but, as will be discussed below, they are at least partly related.

Research and breeding efforts aimed at improving resistance to these two aspects of the disease have focused on accurately measuring Fusarium ear rot and fumonisin concentrations, identifying sources of resistance, and characterizing the inheritance of ear rot and fumonisin accumulation. Recently, quantitative trait loci (QTLs) have been identified for both resistance to ear rot, and resistance to accumulation of fumonisin. Techniques to ensure accurate phenotyping of Fusarium ear rot and fumonisin contamination have been developed and validated, and DNA marker technologies have matured and become economically feasible for some DNA marker-assisted selection programs. We consider the application of these two approaches (which are not exclusive) to breeding for reducing susceptibility to Fusarium ear rot and fumonisin contamination.

I. Measuring Fusarium ear rot and fumonisin – field techniques, lab assays for fumonisin

Optimal phenotyping of both ear rot and fumonisin concentration rely on accurate assays in the field and laboratory. Bush et al. (2004) studied the onset of fungal growth and toxin accumulation and determined that maximum infection occurs around 20% kernel moisture, while fumonisin first appears in the dent stage of development at 35 to 40% moisture. In hybrids, the peak for kernel infection is at seven weeks after pollination and the peak for fumonisin concentration is nine weeks after pollination (Bush et al., 2004). This is the optimal time for measurement because fumonisin levels can fluctuate unpredictably if harvest is delayed (Bush et al., 2004).

Artificial inoculation is needed used to ensure that a pathogen is equally distributed among plants throughout the field. For *F. verticillioides*, infection through silks is a more important pathway for kernel infection than through seeds, stalk, or crown; therefore, silk

inoculations are best for evaluating genetic resistance to *Fusarium* ear rot (Munkvold et al., 1997). However, not all ear and silk inoculation techniques are equally effective. A comparison of four techniques found that only inoculum injection through the husk significantly increased rot severity, and fumonisin concentration. This technique also effectively differentiated levels of susceptibility and resistance between lines (Clements et al., 2003). Bush (2001) conducted a similar study to compare the potential of five inoculation techniques for determining resistance or susceptibility of a variety. Inoculation by penetrating husks with pin bars or by injecting inoculum down the silk channel were best able to discriminate different levels of and resistance to fungal infection and fumonisin accumulation. Husk penetration mimics natural inoculation by insect and silk channel infection mimics spores splashed onto husks by rain. Because ears and kernels that are infected are a mixture of tissues that are genotypically 100% maternal (cob, glume, and aleurone), 50% maternal and 50% paternal (embryo), and 67% maternal and 33% paternal (endosperm), we were concerned that the paternal (pollen) genotype could affect disease severity. Previous studies indicated that *Fusarium* ear rot resistance is controlled by the maternal parent (Headrick and Pataky, 1991; Nankam and Pataky, 1996; Scott and King, 1984), but no information on the effect of pollen source on fumonisin content was available. Therefore, we conducted an experiment in one environment to compare *Fusarium* ear rot and fumonisin content of self-fertilized ears and open-pollinated ears. We grew 143 recombinant inbred lines from the cross of B104 and NC300 in two replications in Clayton, NC in 2002 and selfed six plants per row. About two weeks after the population median silk date, we removed tassel bags from selfed plants and injected inoculum down the silk channels. At the same time, we also inoculated six open-pollinated plants in each row and marked the inoculated plants. We observed no significant differences in fumonisin concentration due to pollen source for either fumonisin concentration ($P = 0.44$) or for ear rot ($P = 0.17$), and pollen source by genotype interaction was only significant for ear rot ($P = 0.03$). This interaction could be due to a lack of perfect correlation between genotypic effects or due to differences in genotypic variance of ear rot under the two different methods (Cockerham, 1963). We estimated a significant genotypic correlation of 0.82 between the two methods, and found that the genotype-by-pollination interaction variance that remained after subtracting the component due to differences in genotypic variances was not significant at $P = 0.05$, in agreement with previous studies cited above. These results indicated that pollen source did not have a significant effect on either *Fusarium* ear rot or fumonisin concentration; therefore, we were confident in working with open-pollinated plants for all our other *Fusarium* resistance experiments.

Accurate and cost-efficient assays for fumonisin are required for evaluating large numbers of genotypes for genetics and breeding programs. High-performance liquid chromatography (HPLC) can be used to very accurately estimate fumonisin concentrations (Bush et al., 2004), but this is too expensive to use for large-scale breeding programs (for example, a laboratory at NC State University charges at least \$20 per sample for HPLC analysis of fumonisin). A good alternative to HPLC is an ELISA assay adapted by Chris Maragos of the USDA-ARS Mycotoxin Research Unit in Peoria, IL, from a procedure developed at the USDA-ARS-NCAUR (Peoria, IL) and the Immunological Resource Center at the University of Illinois. Don White at the University of Illinois has used this assay to conduct accurate, high-throughput analysis of fumonisins from grain samples. This ELISA is based on a rabbit polyclonal antibody, and will detect $\approx 1 \mu\text{g/g}$ ground corn, of fumonisins B1, B2, and B3 (structurally different toxins in the fumonisin family of compounds), but does not detect hydrolyzed fumonisins (Clements, 2003). This ELISA detects relative differences in fumonisin concentration between grain

samples, and compares favorably with HPLC (Clements, 2003). We are indebted to Drs. Maragos and White for helping us to analyze our samples with this system (in Dr. White's lab); without their assistance, none of our research on this topic could have been accomplished.

II. Identifying sources of resistance

Resistance to *Fusarium* ear rot is under genetic control (King and Scott, 1981), but no complete resistance has been identified in maize. Shelby et al. (1994) reported significant variation among commercial maize hybrids for fumonisin content, but no hybrid was found to be immune. Furthermore, the hybrid with lowest mean fumonisin content across 11 locations grown without the use of artificial inoculum had 5.78 parts per million (PPM, $\mu\text{g g}^{-1}$), which is above the threshold content level for human food or horse feed provided by the FDA's guideline for industry (Anonymous, 2001). To incorporate acceptable levels of resistance into commercial maize, lines with acceptable resistance levels must first be identified.

To identify sources of resistance, Clements et al. (2004) evaluated *Fusarium* ear rot and fumonisin accumulation in testcrosses of 1,589 public inbred lines to the susceptible tester, FR1064. They identified several inbreds with superior resistance to production or accumulation of fumonisin. Resistance in these lines appears to be dominant, as it is evident both in the inbreds, and in hybrids between 35 selected resistant lines and FR1064 (Clements, 2004).

To supplement the germplasm screens conducted by Clements et al. (2004), we have been conducting screening trials for both *Fusarium* ear rot and fumonisin concentration using material selected by Mike Blanco from the Germplasm Enhancement of Maize (GEM) project and advanced lines from Major Goodman's breeding program at N.C. State University. Each year we screen approximately 50 lines, in two replications, at one location. Inoculations are performed 10 and 17 days post mid-silk, following Robertson et al. (2006). The first inoculation is an injection of 10 ml spore suspension down the silk channel and the second is an injection of 10 ml suspension through the husk. This screen has identified several lines with good levels of resistance to both *Fusarium* ear rot, and fumonisin accumulation (Table 1).

III. Inheritance of *Fusarium* ear rot and fumonisin contamination

Once suitable sources of resistance have been identified, inheritance of resistance should be considered before selecting a breeding strategy. Clements et al. (2003) found moderate, positive correlations between incidence of *Fusarium* ear rot and fumonisin concentration, 0.54 ($P < 0.0001$) in Illinois and 0.60 ($P < 0.0001$) in North Carolina. Because these correlation estimates were only moderately large, they concluded that the two traits should be considered separately in breeding programs, since improvement of ear rot resistance may not result in gains in the resistance to fumonisin content.

We revisited the correlation between *Fusarium* ear rot and fumonisin content, noting that previous estimates of this correlation were phenotypic estimates, and may be confounded with variances and covariances of genotype-by-environment interactions and error effects. Robertson et al. (2006) evaluated two populations, 213 BC₁F_{1,2} lines derived from the backcross of (FR1064 × GE440) to FR1064 (developed by Don White and hereafter referred to as the GEFR population), and the 143 line NC300 × B104 recombinant inbred line population (developed by Terence Molnar and Major Goodman and hereafter referred to as the NCB population). Robertson et al. (2006) evaluated these two populations in three to four environments, using double artificial inoculations, and assaying fumonisin content via ELISA. Multivariate analysis

was used to partition the genotypic variances and covariances from the phenotypic variances and covariances, permitting the estimation of the genotypic correlation between Fusarium ear rot and fumonisin content. The estimated genotypic correlations were surprisingly high, $r_g = 0.96$ and 0.87 , respectively, although the phenotypic correlations were not (Table 1; Robertson et al., 2006). High genotypic correlations suggest that genotypes with greater resistance to Fusarium ear rot also tend to have lower fumonisin contamination. Further, it suggests that the genetic components of resistance are largely the same for the two traits, even though they are not highly phenotypically correlated (Robertson et al., 2006). Although surprising, this does not contradict the observation that fumonisin can sometimes accumulate to high levels in kernels with little ear rot (Munkvold and Desjardins, 1997), because although the genetic controls of resistance seem to be similar, the environmental factors that promote ear rot do not appear to be the same as those that promote fumonisin production.

Given the high genetic correlations between Fusarium ear rot and fumonisin, we asked the question: how effective would indirect selection aimed solely at reducing ear rot be at reducing susceptibility to fumonisin contamination? Response to indirect selection is predicted to be less effective than direct selection against ear rot because fumonisin concentration had a higher heritability than resistance to Fusarium ear rot in both populations (Table 2). This likely occurred because fumonisin assays are more precise than visual scores of percent ear rot. However, ear rot can be scored quickly in the field, whereas assaying fumonisin content requires harvesting the inoculated ears, shelling grain, grinding grain to a precise particle size, weighing samples, performing fumonisin extractions in the laboratory, and finally conducting ELISA assays in triplicate. Even just considering the labor required to conduct all of these additional steps means that indirect selection against ear rot could conceivably be more economically efficient than direct selection against fumonisin contamination. Because ear rot is easier to evaluate, one could increase population sizes and consequently increase the selection coefficient or increase the number of replications to improve the entry mean heritability of the trait.

IV. QTLs for Fusarium ear rot and fumonisin contamination

Phenotypic scoring of Fusarium ear rot and fumonisin concentration present some practical difficulties. The traits must be scored on grain harvest from mature ears, two inoculations with calibrated liquid spore suspensions are needed to obtain consistent Fusarium ear rot ratings (Clements et al., 2004), and evaluation of fumonisin contamination requires an expensive, laborious, toxin assay on precisely ground and weighed samples. In addition, Fusarium ear rot and fumonisin contamination are often strongly affected by environmental factors.

Given the difficulties of phenotypic evaluation of Fusarium ear rot and fumonisin contamination, the reasonably high heritability of both traits, and the availability of PCR-based DNA markers linked to genes with moderate effects on resistance marker assisted selection may be a more efficient selection strategy (Holland, 2004; Robertson et al., 2005). Therefore, Robertson-Hoyt et al. (2006a) mapped QTLs for ear rot resistance and fumonisin contamination in the same two populations used for heritability and genetic correlation estimation. They identified seven QTLs for Fusarium ear rot resistance in the GEFR population, and five Fusarium ear rot resistance QTLs in the NCB population. They also identified nine and six QTLs for resistance to fumonisin accumulation in the GEFR and NCB populations respectively (Robertson-Hoyt et al. (2006a). Despite the very high correlations between Fusarium ear rot and

fumonisin contamination, the QTLs identified were associated with only 65% (GEFR) and 31% (NCB) of the genetic covariance between the traits. The relatively low proportions of genotypic covariance that were associated with QTLs suggest that not all QTLs were identified. It is possible that a large number of genes with small effects that will be hard to detect (i.e., the polygenic background) may explain much of the remaining genetic covariances. Supporting this idea is the result that the combined effect of QTLs was estimated to account for 39 to 99% of genotypic variation (Robertson-Hoyt et al., 2006a), suggesting that, at least for some traits, some true QTLs were not identified. Greater power to detect QTLs and estimate QTL effects could be gained by increasing population size, improving ear rot phenotyping methods, increasing the number of environments for phenotyping, and increasing the number of markers used (Robertson et al., 2005).

V. Application of Inheritance and QTL Studies for Improving Ear Rot and Fumonisin Contamination Resistance

Having reliable estimates of trait heritabilities and genetic correlations and QTL positions and effects allows us to predict the relative value of traditional phenotypic selection and marker-assisted selection. As mentioned previously, the high genotypic correlation between resistance to Fusarium ear rot and fumonisin implies that indirect selection on ear rot could be used to improve resistance to fumonisin contamination in an economically efficient manner. Because at least some of the QTLs for the two traits appear to be different, we suggest that ear rot evaluations for large numbers of early generation breeding materials be followed by combined ear rot and fumonisin content analysis of fewer selected late-generation lines and hybrids.

Marker-assisted selection could offer several advantages over either indirect selection on ear rot or direct selection on fumonisin concentration. Both phenotypic traits require multiple plants per plot and multiple replications and environments to obtain accurate data. In contrast, if QTLs have been accurately mapped, selection on marker loci flanking QTLs could be effective on individual plants. Furthermore, marker-assisted selection could be conducted in greenhouses and off-season nurseries without concern for genotype-by-environment interaction that would likely reduce (or eliminate entirely) the response to phenotypic selection in these environments.

The GEFR population presents a good test case for the effectiveness of backcrossing resistance genes from a line with good resistance but poor agronomic quality (GE440, which has white seed, small ears, and poor stalk lodging resistance) into an elite genetic background that lacks effective resistance (FR1064). We will use this population to test the hypothesis that selection against ear rot in advanced backcross generations will result in improved resistance to both ear rot and fumonisin contamination. To do this, we selected the ten GEFR BC₁F₁-derived families with lowest mean fumonisin content from the data presented by Robertson et al. (2006). We began with a hierarchical family structure of about ten BC₁F_{2:3} plants within each of ten BC₁F₂ families within each of the ten BC₁F₁ families. Each of these plants was crossed to FR1064 to generate about 1000 BC₂F₁s. As of winter, 2005-2006, we have advanced to the BC₄F₁ generation, maintaining balanced family bulks at each generation deriving from about 75% of the original 100 BC₂F₂ families. We will self these lines two generations and evaluate BC₄F_{2:3} lines in replicated trials in the summer of 2007.

The BC₄F_{2:3} lines will be inoculated with isolates of *F. verticillioides* and *F. proliferatum*, and the twenty lines with the best resistance to Fusarium ear rot will be selected. These twenty lines will be topcrossed to the unrelated non-Stiff Stalk tester, FR615 × FR697, to produce hybrids for replicated disease evaluations (ear rot and fumonisin contamination) and

yield trials. The inbreds themselves will also be evaluated for disease resistance. These 20 inbreds will be genotyped using the same markers as Robertson-Hoyt et al. (2006a) to determine which regions from GE440 were maintained as introgressions during the backcrossing and selection process.

If we recover GE440 alleles at all of the QTL regions identified by Robertson-Hoyt et al. (2006a), it can be inferred that both marker assisted selection and phenotypic selection against *Fusarium* ear rot would be equally effective at reducing susceptibility to fumonisin contamination. If we can process DNA markers for nine chromosomal regions on upward of a thousand lines between harvesting winter nursery in spring of 2007 and planting the next winter nursery in fall of 2007, we will also implement a parallel marker-assisted selection program to provide a direct comparison to the phenotypic selection program. In any case, the BC₄-derived lines will provide excellent genetic materials for future fine-mapping experiments to better localize the fumonisin contamination resistance QTLs.

An obvious question that we hope to answer with the backcross-derived introgression lines is: can we improve the disease resistance of FR1064 without losing too much of its agronomic utility? Robertson-Hoyt et al. (2006b) conducted an additional experiment to provide information that can be used to predict the effect on agronomic traits of selection for increased fumonisin contamination resistance. Each of the 213 BC₂F₁-derived GEFR lines that was used for heritability estimation and QTL mapping was also testcrossed to FR615 × FR697 and evaluated for yield potential and agronomic performance in eight North Carolina environments. Robertson-Hoyt et al. (2006b) measured correlations between *Fusarium* ear rot and fumonisin contents in the lines per se and agronomic traits in their testcrosses. Grain yield was not correlated with fumonisin concentration, which indicates that selection for fumonisin contamination resistance should not affect yield potential. Grain yield had a low but significant positive correlation ($r = 0.28$, $P < 0.0001$) with *Fusarium* ear rot resistance, however, and in this case a positive correlation is unfavorable because it indicates that lower ear rot scores (greater resistance) are associated with lower yield. In addition, both *Fusarium* ear rot and fumonisin contents were negatively correlated ($r = -0.22$ to -0.30 , $P < 0.001$) with grain moisture. This suggests that improving resistance to fumonisin contamination and ear rot may result in slower grain dry-down.

To address the longer term goal of incorporating new sources of germplasm with fumonisin contamination resistance into the adapted gene pool that can be easily accessed by breeders in the USA, we have also initiated a recurrent selection program. We are in the process of creating this population by mating 11 lines identified in our screening trials or in the data of Clements et al. (2004) as having good ear rot or fumonisin contamination resistance to 11 lines that have good yield potential (and may or may not have some level of resistance) using a Design II mating scheme. The pedigrees of these lines include tropical and temperate, non-Stiff Stalk germplasm (Table 3). Following the Design II mating, we have conducted one generation of random intermating. Selection for *Fusarium* ear rot resistance will begin after a second generation of random intermating and a generation of selfing. To combine selection for *Fusarium* ear rot and fumonisin resistance with yield potential, we may develop a selection index to combine information on these traits measured in inoculated and replicated evaluations. We hope that this population will serve as a source of lines with higher levels of resistance due to new combinations of resistance genes without sacrificing agronomic quality.

Table 1. Fumonisin concentrations and percent ear rot for screening trial in Clayton, NC. 2003-2005.

fumonisin concentrations (ppm)						
	2005		2004		2003	
Mean		11.0		29.5		6.9
Lowest	<i>GE440*</i>	1.1	NC458	1.7		
	<i>Ames 26531</i>	1.2	<i>GE440*</i>	2.6	<i>02GEM00252</i>	0.1
	Ki21	1.3	<i>02GEM00252</i>	3.9	NC444	0.3
	<i>NC484</i>	1.5	<i>03GEM00200</i>	5.2	NC458	0.6
	NC458	1.5	NC434	5.3	<i>NC454</i>	0.8
	Ames 26513	1.6	NC300	7.6	9366-005/97	0.8
Highest	FR1064+	22.6	<i>NC348</i>	70.0		
	NC460	27.9	B73	76.0	02GEM00115	19.2
	<i>TAMU1</i>	28.9	03GEM00169	78.9	NC432	21.6
	NC468	36.3	FR1064+	87.2	1367-001/97	23.5
	NC464	39.3	<i>3520-blk/03</i>	106.6	<i>C161-7</i>	34.4
	<i>NC478</i>	75.9	<i>NC378</i>	119.2	<i>NC336</i>	35.9
LSD (.05)	25.0		36.7		13.4	
% ear rot						
	2005		2004		2003	
Mean		6.2		16.4		6.599
Lowest	NC464	1.6	<i>GE440*</i>	1.1		
	<i>NC484</i>	1.6	9532-2	1.9	<i>02GEM00252</i>	0.6
	PI632413	1.6	NC444	2.2	<i>NC454</i>	1.1
	<i>Ames 26531</i>	1.7	<i>03GEM00200</i>	2.9	02GEM00270	1.3
	<i>GE440*</i>	1.9	NC358	3.2	NC434	1.4
	Ames 27215	2.0	<i>02GEM00252</i>	3.3	NC456	1.6
Highest	FR1064+	2.2	FR1064+	26.7		
	04GEM00494	14.1	03GEM00163	33.1	00GEM06331	13.2
	<i>NC478</i>	14.5	02GEM00123	41.3	NC436	15.1
	NC486	15.2	<i>NC348</i>	41.5	1937-002/98	15.8
	04GEM00518	26.4	<i>NC378</i>	42.1	<i>C161-7</i>	20.2
	<i>TAMU1</i>	47.4	<i>3520-blk/03</i>	42.5	<i>NC336</i>	21.9
LSD (.05)	8.0		22.2		11.3	
* resistant check			Entries in <i>italics</i> appear in the same set (low or high) for both			
+ susceptible check			traits in the same year.			

Table 2. Estimates of heritability on a line mean basis for Fusarium ear rot and fumonisin content resistance, of the genotypic and phenotypic correlations between ear rot and fumonisin content, and of the predicted ratio of response of fumonisin content to indirect selection on ear rot to response to direct selection on fumonisin content in two maize populations (adapted from Robertson et al., 2006).

Parameter estimate	GEFR population	NCB population
Ear rot line mean h^2	0.47	0.86
Fumonisin line mean h^2	0.75	0.88
Genotypic correlation (r_g)	0.96	0.87
Phenotypic correlation (r_p)	0.40	0.64
Indirect selection response ratio	0.76	0.86

Table 3. Parent lines for Design II mating of Fusarium ear rot and fumonisin contamination resistant lines and agronomically superior lines.

RESISTANT PARENTS		AGRONOMIC PARENTS	
Inbred	Pedigree	Inbred	Pedigree
A131	A12×A39	B116	B97×B99
GE440	Hasting's Prolific	B97	BSCB1(C9)
UR13085:N0215-21-1-B-B-B	UR13085:N02 (GEM program)	NC258	TZ(2)× [(NC248×NC246)×C103]
Ki21	Pacific 9-S8-45 (Thailand)	NC320	SC76-type×B52
Ky21	Boone County White	NC346	PionX105A×H5
Mo17	CI187-2×C103	NC446	Ku2301× NC296 BC
NC300	PionX306B×(PionX105A×H5)	NC448	PionX105A×H5
NC356	TROPHY low moist. rec. selection	NC450	101-12-2-1×NC296
NC458	Ku2301×PM703	NC452	NC296×NC304
T236	T115/(I205×L289)//T115-S11	NC456	PIJ100×X304
TAMU1	NC300/CML288 - B-4-B-B-B-B	NC492	NC258×NC296

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