

## ABSTRACT

BELCHER, ARABY RUTH. The Physiology and Host Genetics of Quantitative Resistance in Maize to the Fungal Pathogen *Cochliobolus heterostrophus*. (Under the direction of Dr. Peter J. Balint-Kurti.)

Quantitative disease resistance, despite widespread use, remains poorly understood. A previous project in the NCSU Maize Disease Resistance Genetics lab has generated 253 near-isogenic lines (NILs) in the background of the maize inbred line B73. B73 has excellent agronomic quality but is highly susceptible to a number of diseases. The NILs are genetically differentiated by combinations of 1-5 of 12 total introgressed regions from the multiple disease-resistant parent NC250P. These 12 NC250P introgressions were selected for study as, following an initial B73 x NC250P cross, they had been retained by a program of recurrent backcrossing to B73 and selection for resistance to the fungus *Cochliobolus heterostrophus*, causal agent of southern corn leaf blight (SLB). Prior research also evaluated the effect of each NC250P introgression in conferring resistance or susceptibility against SLB. Introgressions having an effect can be designated as disease quantitative trait loci, or “dQTLs”.

Presented here is a 2-phase study with the ultimate aim of characterizing the physiological basis for the effect on disease severity of these NC250P-derived SLB dQTLs. The first phase attempts to determine more precisely how infection is altered by the two largest-effect introgressions, termed dQTL 3.04 and dQTL 6.01 (or 3B and 6A). To do so, growth chamber juvenile plant trials were used to compare the interactions between *C. heterostrophus* and 6 select lines - B73, the major-gene resistant line B73rhml (also a B73-background NIL), and four NILs with varying combinations of dQTLs 3.04 and 6.01 - by quantifying spore germination and penetration efficiency, hyphal growth, and host expression of the pathogenesis related genes *PR1* and *PR5*. The second phase investigates dQTL disease specificity by field testing 236 NILs for adult plant resistance to 5 fungal maize pathogens.

Based on the results of the first phase, host genotype was not a significant factor for germination or penetration efficiency before 48 hours post-inoculation (hpi) ( $P \geq 0.27$ ) nor for hyphal growth at 24 hpi. dQTL 6.01 significantly reduced fungal growth at 48 hpi ( $P \leq 0.05$ ). *PR1* and *PR5* were significantly upregulated at 15 and 24 hpi in all lines ( $P \leq 0.01$ ), but were not affected by any dQTLs. Moreover, in a genetic complementation test using mapping population data, it was determined that dQTL 6.01 and *rhm1* represent the same resistance gene.

In the second phase of experiments, a total of 237 NILs were rated for resistance to SLB, northern leaf blight (causal agent *Exserohilum turcicum*), gray leaf spot (causal agent *Cercospora zea-maydis*), aflatoxin production by *Aspergillus flavus*, and fusarium ear rot and fumonisin toxin production by *Fusarium verticillioides*. Correlations for resistance to any given disease pair were low across the NIL population ( $-0.247 \leq r \leq 0.535$ ). However, eight of the twelve NC250P introgressions represented in the NILs were shown to have significant multiple disease resistance effects ( $P \leq 0.058$  for all significant effects). A dQTL in bin 3.04 had a significant effect on resistance for 4 diseases; and the dQTLs in bins 2.06, 5.07-5.09, and 6.01 had significant effects on 3 diseases. The dQTLs in bins 3.00-3.01, 9.01, 9.02-9.03, and 10.02 had significant effects on resistance for 2 diseases.

The combined results of these experiments, by adding to the current knowledge of the timing and specificity of quantitative disease resistance effects within the infection court, can be used to both 1) help plant breeders better deploy quantitative resistance genes through an increased understanding of their effects and 2) provide quantitative resistance and multiple disease resistance gene candidates for future fine-mapping and cloning efforts.

The Physiology and Host Genetics of Quantitative Resistance in Maize to the Fungal  
Pathogen *Cochliobolus heterostrophus*

by  
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Native to the city of Milwaukee, Wisconsin, Araby Belcher first became interested in the agricultural sciences as an undergraduate in Genetics at the University of Wisconsin – Madison. After taking a number of courses in plant pathology, she took a special interest in the genetics of host-parasite interactions. After graduation, she interned as a plant breeding research assistant to become more familiar with the applied aspects of plant genetics. Then, in 2007 Araby applied and was accepted to the Plant Pathology graduate program at North Carolina State University, under the guidance of Dr. Peter Balint-Kurti in the maize disease resistance genetics lab. Following her intended focus of fungal pathogens of cereal crops, she completed a master's thesis at NCSU with the objective of characterizing the resistance phenotypes of quantitative disease resistance in five fungal pathogens of maize. While quantitative disease resistance is ubiquitously employed by plant breeders, the means by which the genes alter the relationship between host and pathogen is still poorly understood. Working closely with Dr. James Holland's lab in Crop Science at NCSU, she was also taught a good deal about statistical methods and about breeding maize for traits outside of disease resistance. For those learning opportunities and the remainder of her professional knowledge obtained at NCSU, Araby will always be grateful.

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## 1. Chapter I – Introduction and Review of the Literature

### 1. 1. Introduction

Southern corn leaf blight (SLB), caused by the necrotrophic fungus *Cochliobolus heterostrophus*, has great value in mycology and agronomy as a model system. Mycologists have used *C. heterostrophus* to discover essential mechanisms for mating, signal transduction and toxin metabolism (11, 18, 25, 46, 88, 89, 90). For agronomists, host resistance to *C. heterostrophus* is a model for quantitative disease resistance (QDR), the most common class of resistance against pathogens of maize and necrotrophic pathogens of all crops. From 1970-1971, SLB caused what was arguably the most damaging plant disease epidemic in modern U.S. agricultural history (35). The extreme losses in that epidemic were due to widespread use of a single highly susceptible maize genotype, Texas cytoplasmic male-sterile (cmsT) corn. A mitochondrial protein, URF13, specific to cmsT cytoplasm conferred acute susceptibility to SLB race T, the race responsible for the 1970-1971 epidemic. Race T is only weakly aggressive on maize genotypes without URF13; almost all SLB symptoms commonly seen in the field are the result of the more prevalent and aggressive race O. Moreover, the germplasm currently used commercially in the United States is resistant to all known races of *C. heterostrophus* (100). Since 1972, significant losses to SLB have been seen mainly in regions of Asia and Africa where ideal climate and cultural conditions exist for disease development (16). Currently, in the United States, SLB is less important as an economic threat, but does still have value as a model QDR system (13, 16).

Quantitative resistance – also referred to as polygenic, horizontal, race-nonspecific, partial, field, or rate-reducing resistance – is any resistance that confers a less than complete reduction in disease symptoms (77). It can be due to a single large-effect gene or the cumulative influence of a large number of small-effect genes (94). This is in contrast to the

other major form of resistance used in breeding, qualitative resistance. Qualitative resistance is defined as a complete or near-complete reduction in disease symptoms conferred by a single host gene. While qualitative resistance is often used synonymously with gene-for-gene resistance (see Section 2), gene-for-gene resistance is merely a subclass of qualitative resistance, albeit the most prevalent one.

As stated, QDR is the primary form of resistance against diseases of corn and necrotrophic pathogens of all crops. Despite the widespread use of QDR, the genes and mechanisms involved are poorly understood. In contrast, many qualitative resistance genes have now been sequenced and characterized, and many of the cellular mechanisms involved in the qualitative resistance response to infection have been reported in detail (7, 40, 53). It is suspected that QDR shares some mechanistic elements of qualitative resistance, but little evidence for or against these theories has surfaced (52, 66, 85, 101).

The use of QDR in breeding would be improved by an understanding of the cellular processes which underlie QDR. As part this research, we explore the possibility of QDR as an active defense, meaning that the molecules executing the resistance are not sufficiently produced until a pathogen is detected. Passive defenses are factors present at an effective level regardless of pathogen attack, including constitutively produced antimicrobial compounds and aspects of plant physiology that affect infection, such as cell wall thickness or plant height (4, 24). While both qualitative resistance and systemic acquired resistance (SAR, another extensively researched form of resistance) are near exclusively active, both active and passive defenses are suspected to be involved in QDR (51).

The research reported here examines the specificity, timing, and physiology of *C. heterostrophus* QDR genes, and it does so in two sets of experiments (see Section 5). The first set of experiments has two objectives. The first objective is to determine basic histological differences during *C. heterostrophus* infection among a group of four near-

isogenic lines (NILs) with varying combinations of QDR genes. The second objective is to explore the possible involvement of an active defense response during *C. heterostrophus* infection of a group of six NILs, using pathogenesis related (PR) gene expression as marker for active defense. All four NILs from the first group are represented in the second, and each NIL contains zero, one, or two of the QDR loci dQTL 3.04, dQTL 6.01, and *rhm*. The QDR loci dQTL 3.04 and dQTL 6.01 were genetically mapped and evaluated for their effect on SLB through previous work in our lab (see Section 6); to the best of our knowledge, *rhm* is currently the only fine-mapped SLB resistance gene found in the literature that has been characterized in its effects beyond various measures of lesion length and number (23, 80, 81). The second set of experiments uses 237 NILs with varying levels of quantitative resistance against SLB to investigate the phenomenon of multiple disease resistance (MDR). MDR is the pleiotropic effect of a resistance gene on multiple pathogens. Field resistance to five fungal pathogens of maize, including SLB, was assessed. Combined, the results from both sets of experiments should significantly add to the current knowledge of the biology underlying QDR loci.

## 1.2. Quantitative Disease Resistance

Approximately 20% of the world's harvest is lost to plant diseases each year. \$400-600 million of those losses are prevented the subsequent year by deployment of resistant germplasm (29). Breeders rely upon two primary classes of resistance: qualitative, or "gene-for-gene", resistance and quantitative disease resistance (QDR). Qualitative resistance in plants, undoubtedly the better understood of the two, is defined as complete prevention of the pathogen from establishing infection conferred by a single dominant resistance ("R") gene. It is also race-specific, effective only against pathogen genotypes carrying the dominant avirulence (Avr) gene complementary to a host R gene. During the qualitative resistance

response, the pathogen-produced Avr gene product is directly or indirectly recognized by the R gene product. Avr-R recognition initiates signaling pathways for a suite of highly conserved cellular processes designed to limit pathogen spread. These include cell-wall reinforcement, antimicrobial compound production and localized host cell death (7, 33, 45).

### *1.2.1. Properties associated with QDR*

In contrast to qualitative resistance, QDR is defined as a partial inhibition of pathogen success via the combined action of one or more genes (2). Historically, it has been regarded as race-nonspecific, synonymous with the “horizontal resistance” coined by Vanderplank (91) although recent evidence contradicts this assumption (3, 15, 58, 71). The standard dogma in plant pathology holds that QDR is durable while qualitative resistance is quickly overcome by pathogen populations (1, 94). This is based partly on breeder and pathologist experience and partly on pathogen population genetics. Because qualitative resistance is more inhibitive to pathogens than quantitative resistance, it theoretically puts a greater selective pressure on pathogen populations to evolve immunity (79). There are examples of both highly durable qualitative resistance genes and very short-lived quantitative resistance, but durability is higher on average for QDR (22, 47).

Unlike qualitative resistance, QDR is often influenced by the environment. Because of its variability, QDR can be difficult to detect and select for in breeding or mapping programs. For example, while the average disease levels of a line carrying a given small-effect QDR gene will be lower than the lines without that gene, a randomly selected plot of that resistant line in a field could have more disease than the adjacent plot of a less resistant line. So, while qualitative resistance genes can be selected visually on a single-plot basis, selection for specific QDR loci is often far more difficult. To identify (map) a particular QDR locus, the lines must be rated over a number of replications, locations and years in the field; and resistance must be associated with marker alleles using complex statistical

methods. Depending on the marker density, QDR gene strength, and population size and linkage disequilibrium (the degree to which marker chromosomal linkage is broken up by genetic recombination and random mating), QDR locus mapping resolution can range from a few thousand basepairs to half the length of a chromosome. This makes identifying the genes underlying a single QDR locus and testing for individual locus effects very difficult. It is not surprising then that very little work has been done in testing the physiological effects of discrete loci. (2, 4, 51, 78, 79)

### 1.2.2. Mechanisms of QDR

There are no general mechanisms associated with QDR (7). Infection in lines with combinations of QDR loci has been shown to differ from susceptible lines in terms of the time required for lesion development, amount of spores produced, lesion size, lesion number and hyphal growth (38, 39, 79). Antimicrobial compounds have been associated with resistant lines in scattered cases, but to our knowledge there is no evidence that inhibitory compounds play a significant role in QDR (34, 52). In the case of southern leaf blight, resistance has been correlated with higher callose deposition and anthocyanin accumulation (14, 36, 69, 73). However, as with antimicrobial compounds, neither callose nor anthocyanin has been demonstrated to be the major contributor to thwarting infection in those host genotypes (69).

To the best of my knowledge, only five QDR genes have been cloned. The rice gene *pi21* confers resistance to rice blast, which is caused by the fungus *Magnaporthe grisea*. *pi21* is not homologous with any known genes (31). The *OsGLP* (*Oryza sativa* germin-like protein) 12-gene cluster on rice chromosome 8 confers resistance to *M. grisea* and the rice sheath blight pathogen *Rhizoctonia solani* (57). Germin-like proteins have been designated as the PR-15 class of pathogenesis-response genes (PR genes; see Section 5), and have been previously shown to contribute to resistance against plant pathogens, likely through the

generation of H<sub>2</sub>O<sub>2</sub> (92, 21). A second multiple disease quantitative resistance gene, the wheat gene *Lr34*, produces a protein similar in structure to the multi-drug resistance subfamily of ATP-binding cassette transporters. The protein is produced in susceptible plants; resistant plants contain an allele with three polymorphisms conferring resistance to the leaf rust fungus *Puccinia triticina*, the stripe rust pathogen *P. striiformis*, and the powdery mildew fungus *Blumeria graminis* (44). The wheat stripe rust resistance gene *Yr36* (*WKS1*) encodes a predicted kinase domain followed by a predicted steroidogenic acute regulatory protein-related lipid transfer (START) domain, suggesting that *WKS1* may be involved in lipid trafficking (30). Lastly, the maize gene *Rcg1* was cloned from a QDR locus for anthracnose stalk rot and anthracnose leaf blight, caused by *Colletotrichum graminicola*. *Rcg1* is predicted to be a NB-LRR protein, one of the five major classes of R gene products (28). Proteins with NB (nucleotide-binding) and LRR (leucine-rich repeat) domains are thought to be directly or indirectly involved in pathogen protein recognition within host cells, based on the association of LRR domains with protein-protein interactions (60).

### 1.2.3. Links to QDR and other forms of resistance

The discovery that the product of QDR gene *Rcg1* is predicted to be R gene-like in structure is only one of numerous pieces of evidence connecting QDR to qualitative resistance. Some speculate that QDR may not actually be a distinct class of resistance, but simply the muted phenotypic expression of less effective qualitative resistance genes (102). In further support of this hypothesis are QDR gene-like R genes. In normal qualitative resistance systems, there is complete resistance when a pathogen carrying a dominant functional copy of an Avr gene infects a plant with a dominant functional copy of the corresponding R gene. However, many R genes confer partial, or quantitative, resistance when the plant is infected by a pathogen with the recessive avr allele (50). Similarly, when infected with pathogens carrying the dominant Avr allele, many mutant and natural R gene variants also confer partial resistance (20, 72). Moreover, R genes have long been known to

occur in gene clusters; and R genes, R gene analogs (RGAs) and QDR loci have been shown to co-localize with each other in a significantly non-random fashion in the rice and maize genomes (43, 102, 103).

General consensus holds that QDR and qualitative resistance differ in race specificity: that R genes are only effective against certain pathogen genotypes, but QDR loci are believed to confer resistance against most or all isolates in a pathogen species (77). Recently, Marcel et al. (58) found significant pathogen isolate by host line interaction effects for resistance to leaf rust on barley. Calenge et al. (15) found isolate-specific QTL for apple scab resistance, and Avila et al. (3) found isolate- and organ-specific QTL for ascochyta blight resistance in faba bean. Kolmer and Leonard (42) selected for increased aggressiveness on the resistant maize cultivar Jarvis with *C. heterostrophus* and found that, while aggressiveness had significantly increased against all subsequent lines tested, the greatest increase in aggressiveness was against Jarvis.

### **1.3. Southern Leaf Blight**

#### *1.3.1. Disease cycle and histology*

*C. heterostrophus* is a necrotrophic ascomycete that invades the chlorenchyma of corn and sorghum, producing necrotic lesions on leaves (39). There are different names for the sexual and asexual stages of the fungus. *Cochliobolus heterostrophus* technically refers to the sexual stage, which produces pseudothecia as an ascocarp and is rarely seen in nature. However, *C. heterostrophus* is commonly used in the literature as a sexual stage-nonspecific name for the fungus. In earlier literature, it is also referred to as *Helminthosporium maydis*. *Bipolaris maydis* is the asexual stage, which spreads by windblown and splash-dispersed conidia during its polycyclic disease development in the field (39, 99). *C. heterostrophus* is

most aggressive in warmer areas, but can be moderately successful in temperate regions. Its optimal temperature range is 25-32C. Humidity is required for initial infection; and long periods of dry, sunny weather negatively affect disease development. In glasshouse studies, light intensity was shown to significantly affect disease severity (67, 68, 98, 99).

The mechanism by which race O kills plant cells is still unclear, but the physiology of its life cycle is known in detail. The season's initial inoculum consists of fungal spores and mycelium released from cornfield debris. Fungal spores adhere nonspecifically to maize leaves, then germinate and form appressoria that directly penetrate the epidermal tissue. Fungal hyphae immediately progress to and proliferate in the chlorenchyma, growing parallel with the vasculature but not penetrating it. Advancing hyphae kill the surrounding host tissue in a consistent pattern. During plant cell death, chloroplast breakdown precedes cell wall collapse. The fungus continues to feed on these necrotic tissues until it sporulates. Under optimal conditions in the field, this process requires around 60-72 hours. (12, 39, 49, 99, 100, 105)

### *1.3.2. Regulation of infection*

The signaling pathways controlling fungal pathogenesis have not been completely determined, but some genetic components have been identified. A heterotrimeric G protein G-alpha subunit gene mutant *C. heterostrophus* strain was unable to form appressoria and initiate infection at the same efficiency as the wildtype strain, but became less different from the wildtype as the leaves senesced (25). A strain with a mutation in the mitogen-activated protein kinase homolog *CHK1* was markedly less aggressive, although that mutant was also unsuccessful outside the host (48). One study reported that genomes of plant-pathogenic ascomycetes had a far greater number of two-component signal-transduction genes than those of non-pathogenic ascomycetes, which suggests these signaling molecule families play an important part in infection (18). Mutation at a locus affecting extracellular matrix

production, *Ecm1*, inhibited full wild-type-length lesion formation but had no detectable affect on germination, penetration, and growth on media (107). Finally, Pascholati and Nicholson (74) found *C. heterostrophus* infection suppressed resistance to *Cochliobolus carbonum* in maize. This could suggest that signaling between the fungus and the plant, not just inside the fungus, is important to infection. While these results create a rough map of the molecular processes behind *C. heterostrophus* infection, there is still no comprehensive model available for the cellular mechanisms that determine fungal pathogenesis.

The host mechanisms involved in resistance to *C. heterostrophus* are also unclear. Seedlings are generally less resistant than adult plants, but yield loss from race O is primarily due to infection during the grain-filling period (99). Actively growing plants are overall highly resistant, but become more susceptible just before anthesis. Most of the resistance in use comes from temperate lines, but other sources of resistance are available (74, 37). For example, several of the SLB resistance loci being investigated in this study (see Section 6) are from a Nigerian composite line.

Of all known SLB resistance loci, the *rhm1* gene (also a Nigerian introgression) has the most extensive phenotypic data available. Its effects are recessively inherited, more prominent in juvenile plants, and include a small decrease in lesion length combined with a highly significant decrease in fungal sporulation (32). Simmons et al. (80) compared the RNA abundance of 8,000-13,000 defense-related genes in *rhm* mutants and wildtype plants at 24 hours after inoculation, but found no consistent differences in RNA levels between mutant and wildtype among replications. However, the study did identify hundreds of genes differing in RNA abundance between inoculated and uninoculated plants. (79, 32, 36, 80)

Published SLB resistance QTL confer small effect decreases in disease levels in the field, and resistant lines have shown effects on hyphal branching, sporulation, and production of resistance-associated secondary plant metabolites (39, 80). While these results might

suggest mechanisms, none of this work establishes any definite mechanism of resistance for any discrete QDR gene or QTL for SLB.

#### **1.4. Multiple Disease Resistance**

While most resistance research examines the effects of alleles on a single pathogen, plants in the field are constantly exposed a broad array of microbes. This requires breeder integration of resistance genes for all diseases potentially affecting that crop. In some cases, this may require the systematic combination of specific resistance genes from different sources, termed resistance gene pyramiding. Resistance gene pyramiding is time and labor intensive. First, for a single resistance gene, the breeder must make crosses between the line carrying the resistant allele, which often has poor qualities outside of resistance, and a line with acceptable agronomic traits, referred to as an elite line. The progeny of this initial cross that inherit the resistance are backcrossed to the elite line, and the progeny of this first backcross that inherit both resistance and good general traits are selected for further backcrossing. With increasing numbers of diseases to breed resistance for, the number of resistance alleles to be incorporated and the number of resistant lines to incorporate them from quickly escalates, rendering backcross resistance breeding to a very formidable process. Furthermore, some studies suggest that each resistance gene added to a line may come with a decrease in fitness (63, 84), further complicating the breeder's task.

A potential means to improve breeding efficiency is to use genes or loci conferring multiple disease resistance (MDR). Scattered examples exist of MDR, although most are indirect or not applicable to many breeding programs. The indirect examples include correlations between resistance ratings for multiple diseases across the lines of large populations (62) and co-localization of resistance genes and QTL for different diseases (102, 103, 106). The correlation studies published are indirect because linkage disequilibrium

limits them to MDR effects across large portions of the genome, versus showing pleiotropic effects for individual genes. The co-localization studies may show MDR lying within much smaller genome fragments, but involve loci either not mapped in the same genetic background or not tested by the same researchers. An unpublished analysis has found correlations for MDR in a maize mapping population with very low linkage disequilibrium - so low that mapping resolution with this population is estimated at the intra-gene level (Wisser, personal communication). If the correlations are retained in the final analysis, then these results do indicate pleiotropic MDR genes. As in the co-localization studies, the putative MDR genes would need to be verified by testing for MDR near-isogenic lines (NILs) differing only by the suspected MDR genes (2).

The studies that do use NILs and show single-allele MDR in the same experiment almost all involve resistance conferred by artificially induced mutations (9, 19, 26, 70). While these mutant studies have indeed identified true pleiotropic MDR genes, there are three major obstacles to their usefulness to breeders. First, many of these mutants also have pleiotropic growth defects. Second, along with growth defects, many of these mutations conferring resistance to one group of pathogens also confer significant susceptibility to additional diseases (63, 70). This is in part due to the roles many of these genes normally play in general resistance mediated via either the salicylic acid or the jasmonic acid signaling pathways. These two pathways have been shown in numerous plant-pathogen systems to be effective against different groups of diseases and to be mutually antagonistic (82). Third, artificially induced mutations do not further our ability to identify and exploit naturally occurring MDR genes. The use of natural MDR genes should more efficiently avoid the issue of pleiotropic growth defects by only deploying genes selected from well-adapted lines.

To our knowledge, there are only two reported examples of naturally occurring MDR genes. The qualitative resistance gene *Mi-1* confers resistance to both aphids and nematodes in tomato (97), and the semi-quantitative (very large effect) resistance gene *Lr34/Yr18*

confers resistance to leaf rust, stripe rust, powdery mildew and various other diseases of wheat (38). It is unlikely that these two genes represent the large number of accessible MDR genes that are suggested by the correlation and co-localization studies. More examples must be found and analyzed before MDR can evolve from a curiosity to a widely-available practical option for breeding programs.

## **1.5. PR Genes and Active Defense**

### *1.5.1. Overview of active defense*

Qualitative disease resistance generally involves an active defense response – the components that directly inhibit pathogen growth are induced or activated after R gene-mediated recognition of the pathogen. It is unknown whether most quantitative disease resistance uses active or passive defenses. Both have been speculated, and certainly each could be widespread. Passive defenses would include any constitutively present facet of host physiology that affects disease. Canopy density, stomata height, phytoanticipin production, and carbohydrate make-up of the cuticle are all plant physiological properties shown to have a quantitative effect on disease progress (59, 61, 87).

The active defenses induced during qualitative resistance, such as chitinase production, cell wall lignification, phytoalexin production and papillae formation also have quantitative effects on pathogens (54, 56, 93, 64). Hence, some conjecture that the genes for these active defenses may underlie quantitative resistance loci (52). However, quantitative effects are not necessarily equivalent to quantitative resistance. “Resistance” is relative. It indicates genetic variation: plant varieties with lower mean disease than alternate varieties. Because the majority of the genes known to be induced during qualitative resistance responses are highly conserved within species, it would seem unlikely that they represent the

genetic variation controlling quantitative resistance. Moreover, null mutations in or overexpression of a number of these genes have failed to produce any significant change in resistance (92).

On the other hand, some experiments have demonstrated a relationship between active defense genes and quantitative resistance. Members of one defense response gene class, the *PR* genes (see following section), have been significantly associated with quantitative resistance effects against a variety of plant diseases, including rice blast (52), tan spot of wheat (27), and potato late blight (85). The cloned QDR gene *OsGLP* is actually a *PR* gene cluster providing resistance to rice blast and rice sheath blight (57). Arabidopsis genotypes varying in content of camalexin, a phytoalexin, demonstrated a corresponding negative relationship with lesion size for the pathogen *Botrytis cinerea* (41). It is important to note then that quantitative resistance may not only be determined by the presence or absence of a given active defense product, but also by the specific degree or speed of induction.

#### 1.5.2. Overview of *PR* genes

A classic marker for active defense is the upregulation of pathogenesis related (*PR*) genes. *PR* genes encode a highly conserved group of proteins that are consistently upregulated after infection during qualitative resistance and systemic acquired resistance (SAR). *PR* genes were first discovered by screens in tobacco for genes upregulated during the qualitative resistance response to *Tobacco mosaic virus*. Since those initial experiments, 17 families of *PR* proteins have been classified. It should be noted that a reference to a gene in the literature in the form “*PR*-[*X*]” (“[*X*]” being a number from 1-17 representing one of the 17 *PR* gene families) is actually referencing a *PR* gene family or a member of that family, not a particular gene. For example, in the investigation of *PR-1* and *PR-5* expression by Morris et al (66), the *PR-1* transcript quantified was mentioned as being a different allele of a *PR-1* maize gene found in another study. Moreover, there is no universal set of criteria used

to classify *PR* gene families. Rather, after the families were originally created to divide known *PR* genes into structural and functional groups, additional members were added to these families based on protein structure, nucleotide sequence homology, serological activity and/or biological function, purely at the discretion of the discoverers. (10, 52, 65, 92)

Most *PR* genes have been found to have some antimicrobial activity. For example, the *PR-3*, *PR-4*, *PR-8* and *PR-11* families are chitinase genes, and the products of *PR-9* family members have peroxidase activity (92, 95). While this suggests they make some contribution to defense, to the best of my knowledge it has never been shown that the product of a single *PR* gene is sufficient to confer resistance levels comparable to what are classically considered “resistance genes”. Furthermore, as stated, *PR* genes are defined by their upregulation during resistance, not by any role they may play. The products of the two *PR* genes used as active defense markers in this research, *PR-1* and *PR-5*, have no known consistent biological function. Benhamou et al. (6) suggested that *PR-1* may contribute to reinforcement of host cell walls, based on cell wall localization studies. *PR-5* proteins are annotated as thaumatin-like, based on their resemblance to the sweet-tasting protein thaumatin from the shrub *Thaumatococcus danielli*, although no *PR-5* proteins are known to have sweet-tasting properties (75, 76). There is currently no definitive function recognized for this family. Various *PR-5* proteins have been shown to have beta-1,3-glucanase activity, chitinase activity, and the ability to alter fungal membrane permeability (95). Regardless of their inconsistent roles in the mechanics of resistance, *PR* genes remain dependable markers of active defense.

### *1.5.3. Role of PR genes in different responses to disease*

Although *PR* genes are often associated with qualitative resistance and SAR, there is also evidence linking them to QDR. Liu et al. (52), using portions of *PR* gene sequences as molecular markers in a QTL mapping study, found *PR* genes to significantly associate with

variation for QDR against rice blast. In a similar study with *Colletotrichum lindemuthianum* in bean, Geffroy et al. (34) associated *PR* genes with resistance and even found isolate-specific QTL for *PR* gene induction. Vleeshouwers et al. (96) positively correlated *PR-1*, *PR-2*, and *PR-5* transcription with quantitative resistance to *Phytophthora infestans* in potato. More directly relevant to the thesis work presented here, Lo et al. (55) found *PR-10* to be upregulated in sorghum after *C. heterostrophus* inoculation, and Morris et al. (66) found *PR-1* and *PR-5* to be upregulated after *C. heterostrophus* inoculation in maize and upregulated earlier and to a greater degree in the resistant than the susceptible lines used.

Combined, these studies are strong evidence that QDR can involve the same active defenses used with qualitative resistance and SAR. However, a key line of evidence is still lacking. These studies demonstrate correlation between QDR and active defense, but give no evidence for causation. It must be shown that, in lines differing only by a single QDR locus, only the resistant lines displays active defense.

## **1.6. Relevant Background Information on the Project Design**

This thesis builds both upon the breeding and genotyping work completed during the doctoral dissertation research of John Zwonitzer at NCSU (108), and of supplementary experiments that validate the methods used in both thesis projects.

### *1.6.1. NIL breeding, genotyping, QTL mapping, and estimation of introgression effects on SLB resistance by John Zwonitzer*

The 253 SLB-resistant NILs used in this research were developed as part of a doctoral dissertation including the genetic analysis of SLB resistance in maize (108). The SLB resistance investigated was that of two inbred sister lines, NC292 and NC330, which had been produced by a prior NCSU SLB resistance selection program (see Fig. 1 for breeding

scheme diagram). The progeny of the original cross between their two parents, NC250P (high SLB resistance, low agronomic quality) and B73 (high SLB susceptibility, high agronomic quality), were selected for SLB resistance and agronomic quality and then backcrossed to B73. After two subsequent cycles of selection and backcrossing, a number of the progeny were selfed, and one of the most resistant and agronomically superior resulting BC<sub>3</sub>F<sub>2</sub> lines was inbred to create NC292. A member of the same BC<sub>3</sub>F<sub>1</sub> family from which NC292 was selfed was again backcrossed to B73, and the progeny of that backcross selfed. One of the resulting BC<sub>4</sub>F<sub>2</sub> lines was inbred to create NC330, a line with similar resistance to but better agronomic qualities than NC292.

A genome-wide SSR marker analysis identified twelve small NC250P-derived chromosomal regions (introgressions) that differentiated B73 from NC292 and NC330, with eight of the twelve shared between NC292 and NC330 (Fig. 2). NC250P does not include B73 in its pedigree, although it does include a related line, B37. The B37 genome is estimated to be 17% identical by descent to that of B73 based on average RFLP similarity (8). Collectively these NC250P introgressions are likely responsible for the phenotypic differences between B73 and NC292 and NC330, particularly disease resistance (Fig. 4). Markers mapping to these regions were then used to select progeny from B73 x (NC292 x B73) and B73 x (NC330 x B73) backcrosses that contained combinations of one to five of the twelve introgressions (see Fig. 3 for breeding scheme diagram). These lines were then selfed and again genotyped with SSR markers, forming 252 NILs with a primarily B73 genetic background. The NILs were tested for SLB adult field resistance in Clayton, NC and Tifton, GA in the summer of 2007 and in Homestead, FL in the winter of 2007/08. These ratings were used to estimate an adult field resistance effect for each introgression (Fig. 5). Of the twelve, only introgressions 3B, 6A and 9B were tested as having a significant effect on adult field resistance, although potential smaller effects were observed from other introgressions. A 45-line subset of the NILs was evaluated in the growth chamber for

juvenile plant resistance to SLB, and a juvenile resistance effect estimate was calculated for each introgression (Fig. 5).

Zwonitzer et al (109) mapped SLB resistance QTL in two populations very closely related to the NILs. The mapping populations were  $F_{2:3}$  lines derived from B73*rhm1* x NC250A and NC250A x B73 crosses. B73*rhm1* is a B73 NIL carrying the recessive SLB resistance gene *rhm1* (83). NC250A is a very closely related (87.5% identical by descent) sister line of NC250P. Of the nine SLB resistance QTL mapped between the two populations, four co-localized with NC250P introgressions in the NILs (introgressions 2B, 3B, and 6A) and three mapped very close to NC250P introgressions (introgressions 5A and 10A, and between introgressions 2B and 2C; Fig. 2). It is also of note that a resistance QTL from the NC250A x B73 population, the 6A NC250P introgression, and the *rhm* gene all co-localize. Despite this QTL in the NC250A x B73 population being the largest-effect and most statistically significant QTL detected in that population, no QTL was detected at that position in the B73*rhm1* x NC250A population (109). These results would be expected if 6A and *rhm* represent the same gene, a hypothesis further supported by the similar effects 6A has to *rhm*: both are mostly recessive, significantly effective with both juvenile and adult plants, and originate in the same highly resistant Nigerian composite line.

### 1.6.2. Research objectives

The basic objective of the following research was to better characterize the resistance in the NIL collection. This broad objective was narrowed with two discrete sets of experiments. The first set of experiments compares the histology of *C. heterostrophus* infection in maize lines expressing different levels of QDR. More specifically, pathogenesis on four NILs (B73, B73*rhm*, NC292, and NC330) was rated as spore germination efficiency, leaf penetration efficiency, and lateral fungus growth through the leaf. Furthermore, the relative strength of a general active defense response was compared between six NILs (the

four used for the spore germination/penetration efficiency and fungal growth experiments, plus the B73 x (NC292 x B73)-derived NILs JZ191 and JZ196) by quantifying transcript levels of maize pathogenesis-related genes PR-1 and PR-5 pre- and post-inoculation with either *C. heterostrophus* or the sterile inoculum solution. The second set of experiments assesses 237 of the 253 SLB-resistant NILs for resistance to five different pathogen species. The 234 NILs were evaluated across 4 field locations in the summer of 2008 for resistance to southern leaf blight, northern leaf blight, gray leaf spot, aflatoxin production by *Aspergillus flavus* and fumonisin production and ear rot by *Fusarium verticillioides*.

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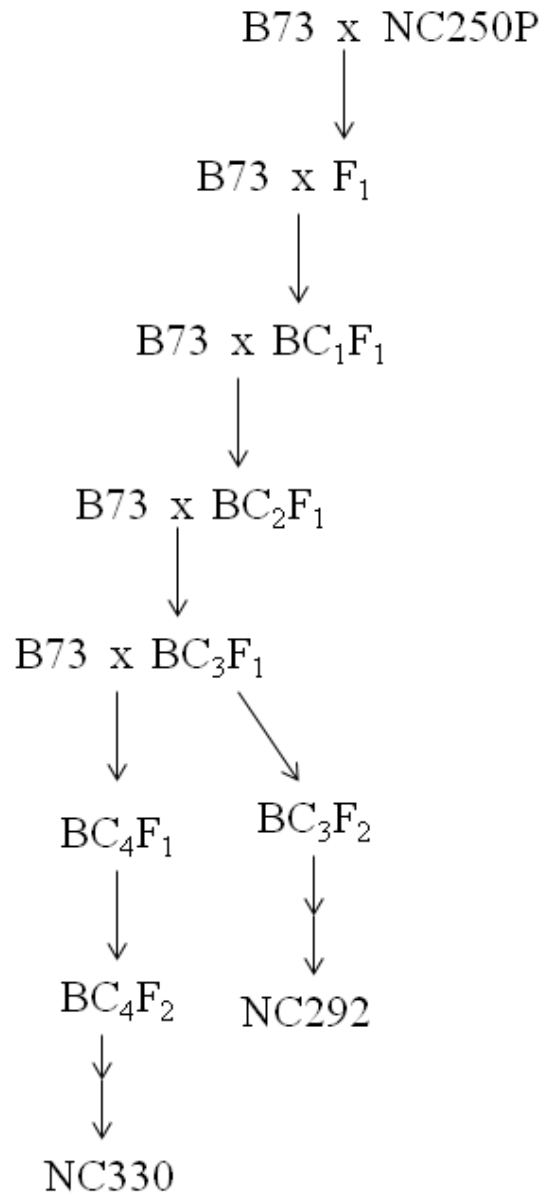
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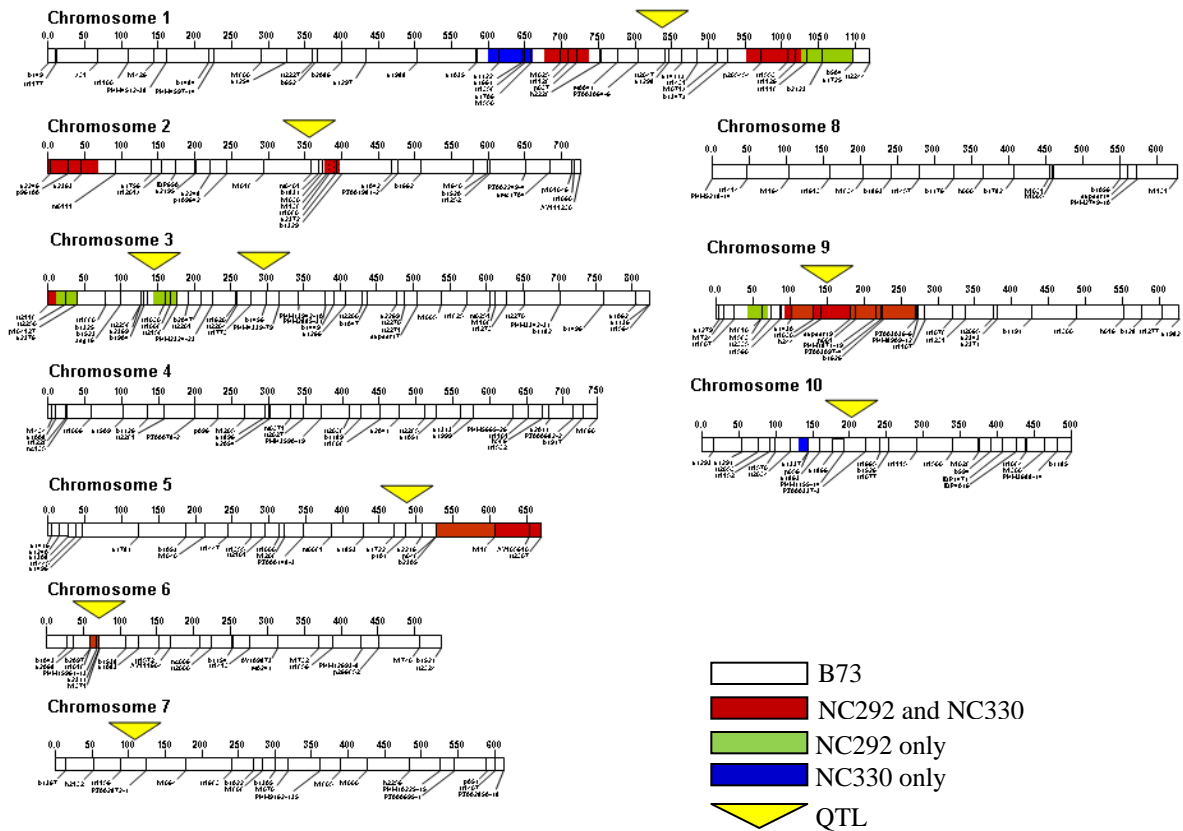
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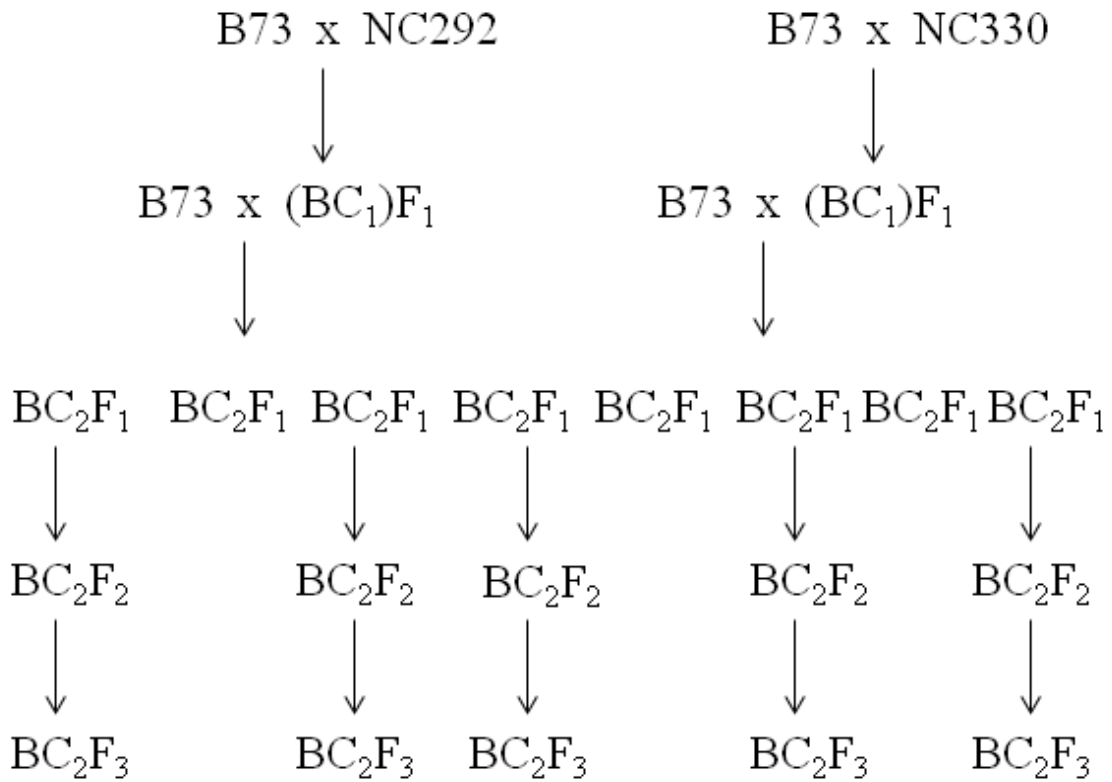


**Figure 1.1. Breeding scheme for inbred lines NC292 and NC330.**



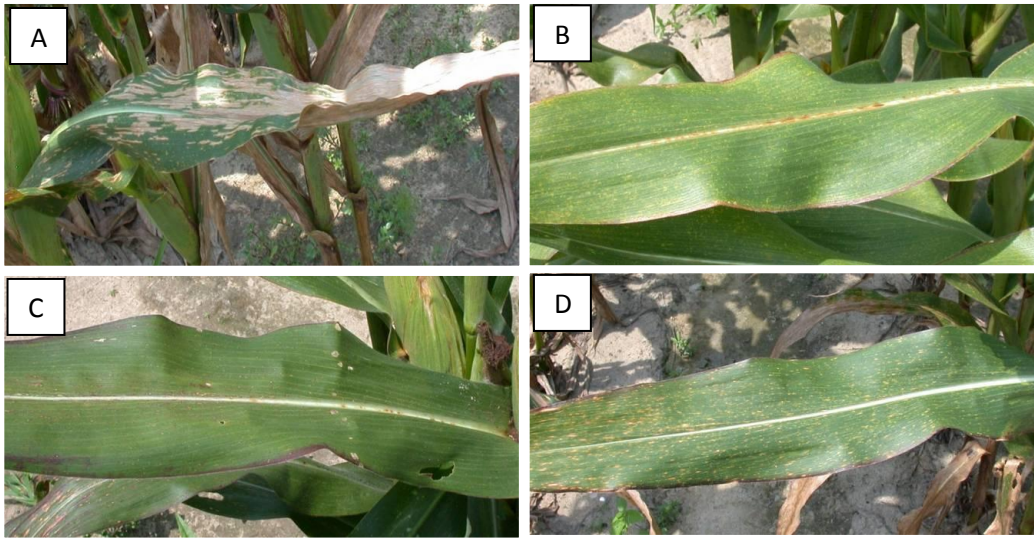
**Figure 1.2. Chromosome map showing NC250P introgressions and SLB resistance QTL estimated with *B73rhml* x NC250A and NC250A x *B73* mapping populations.**

Labels above chromosomes are bins. Labels below chromosomes are SSR markers. Taken from Zwonitzer (108).



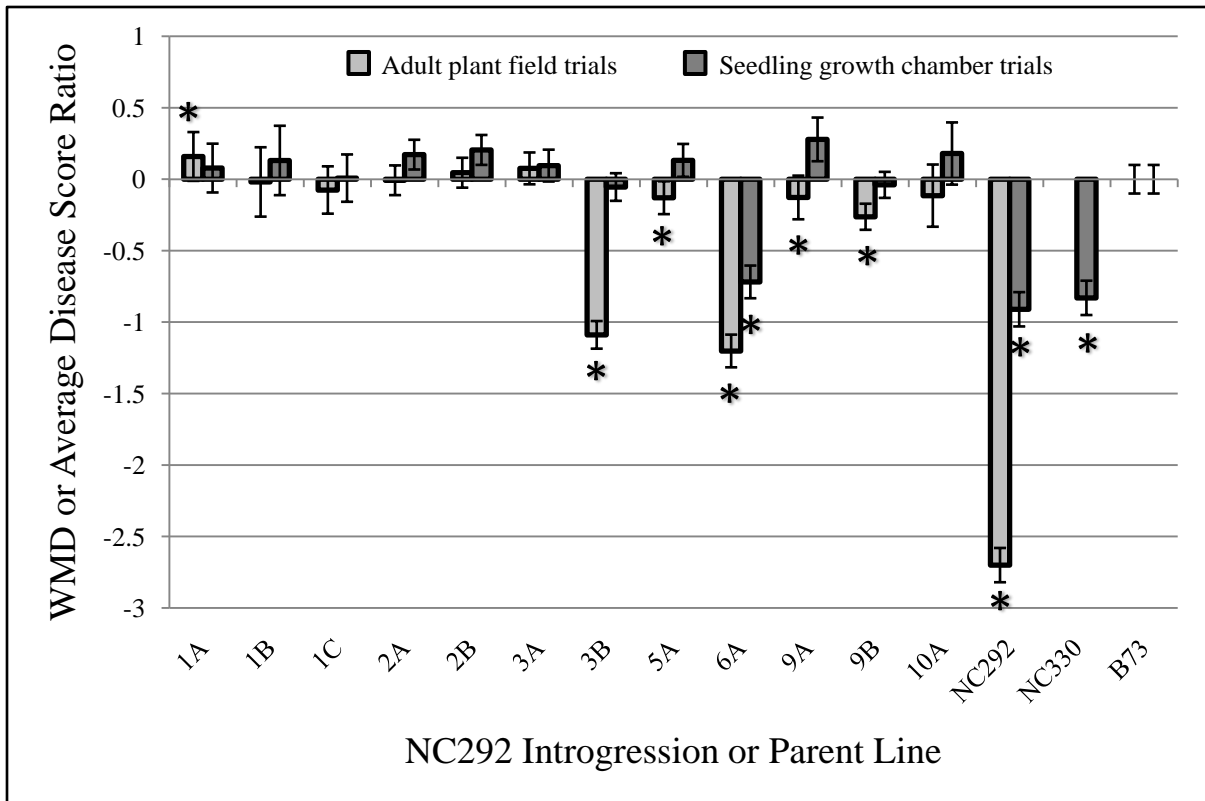
**Figure 1.3. Breeding scheme for the 253 NILs created as part of John Zwonitzer’s doctoral thesis.**

Plants were genotyped with SSR markers at the  $BC_2F_1$  and  $BC_2F_2$  generations. Genotype data were used for marker-assisted selection of those generations.



**Figure 1.4 SLB-infected ear leaves from the NIL parent lines B73, NC292, and NC330, along with an ear leaf from the NIL NC250.**

NC250 (B) is derived from and almost completely allelic to NC250P, the line from which all of the introgressions investigated in the NILs were derived. (see Figures 1.1 and 1.2). Because NC292 (C), NC330 (D), and the NILs are only genetically differentiated from B73 (A) by the NC250P introgressions that they contain, it is assumed that all of the phenotypic differences between the NILs and B73 are conferred by NC250P alleles. Taken from Zwonitzer (108).



**Figure 1.5. Estimates of effects in field and growth chamber studies for the 12 NC250P introgressions present in NC330, NC292, and the NILs.**

Introgression effects are expressed as the estimated mean difference from B73 of a single-introgression line homozygous for NC250P alleles within the genetic marker boundaries of a given introgression. Adult field trial scores are expressed as weighted mean disease (WMD) normalized to the scores for the parent lines NC292 and B73. Seedling growth chamber trial plants were scored as average percent diseased leaf area over the course of the trial normalized to the scores for the parent lines NC292 and B73 in each trial (the “ratio” of Average Disease Ratio is the ratio of entry average diseased leaf area to NC292 and B73 diseased leaf area). Error bars are for standard deviations estimated from least-squares means generated by PROC MIXED in SAS. Taken from Zwonitzer (108).

**-CHAPTER II-**

**Maize Resistance QTLs Associated with Differences in Histology but not *PR* Gene  
Expression During *C. heterostrophus* Infection**

**by**

Araby R. Belcher, John C. Zwonitzer, Rahul Dhawan, Peter J. Balint-Kurti

PLANNED SUBMISSION TO PHYSIOLOGICAL AND MOLECULAR PLANT  
PATHOLOGY

Araby R. Belcher, John C. Zwonitzer, Rahul Dhawan, Peter J. Balint-Kurti

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## 2.1. Abstract

Quantitative disease resistance, despite widespread use, remains poorly understood. We previously introgressed disease resistance quantitative trait loci (dQTL) for resistance to the fungal maize pathogen *C. heterostrophus* (causal agent of southern corn leaf blight) into near-isogenic lines (NILs) in the background of the historically important maize inbred line B73. The objective of this research was to determine how and when two large-effect dQTLs (located in bins 3.04 and 6.01) as well as the previously identified gene *rhm* influence host and pathogen biology during infection of juvenile plants. To achieve that objective, we evaluated the quantitative differences in infection between six B73-background NILs – B73, B73rhm1, and four NILs with varying combinations of dQTLs 3.04 and 6.01 – by quantifying spore germination and penetration efficiency, hyphal growth, and host active defense response (measured as host expression of the pathogenesis related genes *PR1* and *PR5*). Moreover, to verify whether *rhm1* and dQTL 6.01 represent different genes, we performed a genetic complementation test using data from a prior *C. heterostrophus* dQTL mapping project that included a B73rhm1 x NC250A (carrier of dQTL 6.01) population. Host genotype was not a significant factor for germination or penetration efficiency ( $P \geq 0.27$ ). None of the resistance loci had an effect on hyphal growth at 24 hours post-

inoculation (hpi), but dQTL 6.01 NILs did have significantly less fungal growth at 48 hpi ( $\alpha=0.05$ ). *PR1* and *PR5* were significantly upregulated at 15 and 24 hpi in all lines ( $P\leq 0.01$ ), although relative *PR* gene expression between lines did not uniformly correspond with the presence of any resistance locus or with the relative resistance between NILs. Within the dQTL mapping population, chromosome bin 6.01 regions carrying either *rhm1* or dQTL 6.01 failed to genetically complement each other in  $F_{2:3}$  lines segregating for bin 6.01 markers ( $P\geq 0.37$ ). Based on these results, we conclude a) *rhm1* is almost certain to be the gene underlying the resistance in dQTL 6.01, b) dQTL 3.04 does not affect fungal success in juvenile plants, c) *rhm1*/dQTL 6.01 does not reduce fungal success until after 18 hours post-penetration, and d) *PR* gene-associated active defenses have no apparent relationship with the quantitative resistance conferred in juvenile plants by *rhm1*/dQTL 6.01 or dQTL 3.04.

## 2.2. Introduction

Southern corn leaf blight (SLB) is a foliar disease particular to maize caused by the necrotrophic ascomycete *Cochliobolus heterostrophus* (Drechs.) (anamorph *Bipolaris maydis* [Nisikado] Shoemaker; syn. *Helminthosporium maydis* Nisikado). It is most damaging in the hot and humid tropical and sub-tropical regions of southern Asia, Africa, Latin America, southern Europe, and the southeastern US (29, 38). SLB gained prominence from 1969 to 1971 when Texas cytoplasmic male-sterile (cmsT) corn was found to be acutely susceptible to SLB Race T, one of three known races. At the time cmsT hybrids represented around 85% of US acreage (26); losses were estimated at near one billion dollars before cmsT germplasm was phased out (36). Genotype-specific susceptibility to Race T is now known to require both the cmsT-associated URF13 mitochondrial protein in the host and a host-selective toxin (HST) produced by Race T (6). A host-selective toxin is generally defined as a pathogen secondary metabolite that confers dominant qualitative susceptibility against plant cultivars

carrying the corresponding dominant host susceptibility allele (39). Chinese cytoplasmic male sterile (*cmsC*) lines are similarly vulnerable to a HST of SLB Race C (37).

SLB Race O, the predominant and most aggressive race, is not known to produce HSTs. Host resistance to Race O is usually based on the combined influence of multiple race non-specific partial-effect quantitative resistance genes (13). Because these genes often have subtle phenotypes with variable expression in the field, few have been cloned and characterized to date (10, 11, 12, 18, 21; see Chapter 1 – Section 2). Instead, chromosome regions containing quantitative resistance genes (disease quantitative trait loci, or dQTL) are identified by the statistical association of the phenotype with nearby genetic markers (25).

A number of studies have reported the mapping of dQTL for SLB resistance. In a previous study, we identified two large effect SLB resistance dQTL derived from the maize line NC250P located in maize chromosome bins 3.04 and 6.01 (46). One large-effect recessive locus, *rhm1*, has been fine-mapped to bin 6.00 (41). The *rhm1* locus shares a number of properties with the NC250-derived dQTL at 6.01, suggesting both may represent the same gene. For example, they are both largely recessive and both *rhm1* and dQTL 6.01 markedly reduce SLB symptoms in juvenile and adult plants (34, 45). Many cereal QDR genes are only effective in adults (4, 17, 42). Outside of these basic genetic features detected by dQTL mapping, however, little is known about the biological factors that determine resistance and susceptibility to Race O.

The functional basis of plant quantitative disease resistance in general is poorly understood (16, 25). Proposed mechanisms of pathogen inhibition include both passive defenses such as modifications in host developmental and architecture along with active defenses such as smaller-effect R genes, pathogen-induced antimicrobial compounds, PAMP-triggered immunity, and HSTs, where resistance would be conferred by the absence of a toxin's host target (8, 9, 20, 22, 25, 44). All of these mechanisms have been linked to

dQTL in other plant-pathogen systems, but no mode of action has been determined for any SLB dQTL.

To better understand the physiological and molecular processes by which genes confer quantitative SLB resistance, we used juvenile plants from near-isogenic lines (NILs) to characterize the effects of *rhm1* and of the SLB dQTL previously mapped to bins 6.01 and 3.04 for four resistance phenotypes. Spore germination efficiency, penetration efficiency, hyphal growth, and induction of an active defense response (as implied by transcript abundance of the pathogenesis-related genes *PR1* and *PR5*) were quantified and compared over time for the susceptible line B73; the B73-background near-isogenic line (NIL) B73rhm1, containing the *rhm1* resistance locus; and two or four B73-background NILs containing dQTL 6.01 (NC330), dQTL 3.04 (JZ191), or both (NC292 and JZ196). Furthermore, to determine whether the recessive loci *rhm1* and dQTL 6.01 do in fact represent the same gene, we reanalyzed the data from a previously published SLB dQTL mapping study (data courtesy of John Zwonitzer; (46) that utilized a B73rhm1 x NC250A (carrier of dQTL 6.01) population. If they are different genes, then genetic complementation of the two loci would be marked by a significantly higher average disease rating for *rhm1*/dQTL 6.01 heterozygotes than for lines homozygous for either parent allele.

## **2.3. Materials and Methods**

### *2.3.1. Plant materials and growth conditions*

B73 is an inbred line derived by recurrent selection of an Iowa Stiff Stalk Synthetic line (28). While B73 has good agronomic qualities, it is susceptible to a number of common maize diseases, including SLB (3). NC292 and NC330 are inbred lines developed through a North Carolina State University breeding program. The program used an initial B73 x NC250P (SLB resistant inbred) cross followed by recurrent backcrosses to B73 and selection

for SLB resistance at each generation. NC292 and NC330 shared the same BC<sub>3</sub>F<sub>1</sub> family, and were selfed from BC<sub>3</sub>F<sub>2</sub> and BC<sub>4</sub>F<sub>2</sub> plants, respectively (see Chapter 1 – Figures for breeding diagram).

A genome-wide SSR molecular marker analysis of NC292, NC330 and B73 by Zwonitzer (45) identified 12 NC250 introgressions that differentiated NC292 and NC330 from B73: 1a, 1b, 1c, 2a, 2b, 3a, 3b (dQTL 3.04), 5a, 6a (dQTL 6.01), 9a, 9b, and 10a (Fig. 1). JZ191 and JZ196 are both NILs developed from marker-assisted selection of B73 x (NC292 x B73) backcross BC<sub>2</sub>F<sub>2</sub> progeny. JZ196 is homozygous for NC250P introgressions 2b, dQTL 3.04, and dQTL 6.01, while JZ191 is homozygous for dQTL 3.04 alone. Introgression 2b was found to have a significant but small susceptibility effect in adults (see Chapter 3), but no significant effect on juvenile plants (45).

B73rhm1 seed (gift of Jerald Pataky) was obtained from inbred lines originally created as part of a project to backcross *rhm1* into elite germplasm adapted for Illinois by Art Hooker at the University of Illinois at Urbana (31).

The B73rhm1 x NC250A SLB dQTL mapping population data were collected in the field on 193 adult F<sub>2:3</sub> families as described by Zwonitzer et al. (46). NC250A is a closely related (87.5% identical by descent) sister line of NC250P (24), and is allelic to NC250P for dQTLs 3.04 and 6.01 (within the resolution afforded by our SSR marker and disease effect evaluation results; data not shown).

Plants were planted, grown, and infected in 600ml Styrofoam cups in a 1:2 peat-lite and gravel mixture. Plants were watered overhead twice daily with a nutrient solution (Phytotron Procedure Manual 1991). The chamber was kept at a constant temperature of 22°C and under a 14 hour fluorescent and incandescent light/10 hour darkness cycle.

### 2.3.2. Fungal materials and growth conditions

All fungal cultures were of *C. heterostrophus* Race O and were grown on PDA plates either as initial platings of stocks frozen in glycerol or as primary transfers from those plates. Cultures were kept at a constant temperature of 22°C with a 12hr darkness/12hr fluorescent daylight cycle. The germination and penetration efficiency experiments used a GFP-transgenic isolate of *C. heterostrophus* (Fig. 3G, Fig. 4; gift of Charlotte Bronson). The hyphal growth and *PR* gene expression experiments used *C. heterostrophus* isolate 2-16Bm (Fig. 3A-F; 4). Cultures were used for inoculation at 10-13 days after plating.

### 2.3.3. Inoculation

For each inoculation, the adaxial side of the second leaf of each plant was sprayed with *C. heterostrophus* conidia in water with the addition of 0.05% agar, 0.05% Tween 20. The agar and Tween solution alone was used for mock-inoculations in the gene expression experiment (Fig. 3H). To minimize germination of spores prior to leaf contact, plants were inoculated within one hour after spore isolation. Furthermore, the mixture was kept on ice until inoculation to delay germination of the spores. The inoculum mixture was prepared by pouring chilled agar/Tween 20 solution onto plate cultures, dislodging spores with a paintbrush, and then filtering the resulting mixture with two layers of cheesecloth. The concentration of this initial mixture was estimated with a haemocytometer, and the mixture was then diluted to a final concentration of  $1 \times 10^3$  spores/ml. Each leaf was sprayed until run-off with a Paasche H airbrush and a Paasche D200R air compressor set at 23psa.

Immediately after plants had dried from inoculation, they were enclosed in clear autoclave bags and were incubated overnight, approximately 14 hours (Fig. 2). Inoculation generally took place 2-5 hours before the end of the “daylight” period in the growth chamber. In the gene expression experiments, fungus-inoculated and mock-inoculated plants were inoculated and maintained in the same growth chamber, but with approximately 2.5’ of space

between the two groups. All mock-inoculated plants were inspected for disease symptoms from 0-72 hpi (lesions usually appear between 24-48 hpi), and no symptoms were observed (Fig. 3H).

The bags represented the whole plot units of a split-plot design. Each bag/whole plot contained one plant of each genotype, randomly arranged within the bag. All experiments used plants inoculated at the 3-6 leaf stage (10-13 days after planting). All leaf samples were cut from the middle 3.5cm of the second leaf of each plant, always from the same side of the midrib (Fig. 3).

#### 2.3.4. Determination of spore germination/penetration efficiency and rate

Germination efficiency was determined as the proportion of spores with visible germ tubes out of the total number of visible spores on the adaxial side of each 3.5cm leaf segment (Fig. 4A, B). Penetration efficiency was measured as the proportion of spores with at least one germ tube that had successfully penetrated the surface of the leaf divided by the total number of germinated spores on the adaxial side of a leaf segment (Fig. 3C-E). Because spores were visualized by fluorescent microscopy with a GFP-transformed *C. heterostrophus* isolate, only living spores were included in this assay (as dead spores did not fluoresce). A Zeiss Axioskop 2 FS plus was used for all germination and penetration efficiency experiments (Carl Zeiss MicroImaging, Inc., Thornwood, NY, USA). Leaf segment width was also measured to allow analysis of inoculum density (spores/cm<sup>2</sup> leaf) effects.

Germination rate was assessed in three experiments (referred to as Trial 1, Trial 2, and Trial 3) at timepoints ranging from 4-33 hpi: 6-13 hpi, 18-26 hpi, and 28-33 hpi in Trial 1; 4-11 hpi in Trial 2, and 5-9 hpi in Trial 3. For each experiment, all plants in a whole-plot unit were removed from the growth chamber at the same time. Spores were counted immediately after each sample was removed from the plant.

Penetration efficiency was assessed as described above at approximately 24 and 48 hpi during the same three experiments as germination efficiency. During Trial 1, penetration and germination counts were taken from the same leaf segments, although the penetration data used in the analysis were only collected between 18 and 33 hpi (penetration efficiency was at or near 0% from 6-13 hpi). Data were collected for penetration efficiency only between 48 and 55 hpi during Trial 2 and between 24 and 28 hpi and then 47 and 51 hpi during Trial 3.

#### *2.3.5. Analysis of hyphal growth within leaves*

One leaf sample was collected from each of six plants per genotype (B73, B73rhml, NC292, and NC330) at 24 and at 48 hpi. The 3.5cm leaf segment taken from each plant was cleared in KOH and stained with aniline blue according to the methods described by Hood and Shew (14). The segments were examined at 10X magnification with a Zeiss Axiophot fluorescent microscope (Carl Zeiss MicroImaging, Inc., Thornwood, NY, USA). Images of ten infection sites per segment were collected with a Q Capture camera and software (QImaging, Surrey, BC, Canada) and measured using the open source software GNU Image Manipulation Program ([www.gimp.org](http://www.gimp.org)).

For each infection site, the length of hyphal growth along the axes parallel and perpendicular to the leaf vasculature was measured on the images collected (Fig. 5). For infection sites with multiple conidia, the full parallel and perpendicular lengths of an infection site were not used. Instead, the length along each axis was shortened to (summed for the two ends of each length) the distance between the endpoint of the infection site and the conidium nearest to that endpoint.

#### *2.3.6. Quantification of PR gene expression*

Samples were collected at 0, 15, and 24 hpi. Biological replications were collected from three inoculated and three mock-inoculated plants of each genotype (B73, B73rhml,

NC292, NC330, JZ191, and JZ196; Fig. 3) at 15 and 24 hpi, and from three inoculated plants per genotype at 0 hpi. From each sampled plant, the middle third of the second leaf of each plant was removed, inserted into a paper envelope, placed immediately in liquid nitrogen, and then stored at -80°C until RNA extraction.

RNA was extracted using QIAGEN RNeasy Plant Mini-Kits with on-column RNase-free DNase digestion, using the protocols included with the kits (QIAGEN, Hilden, Germany). Extracted RNA was eluted in RNase-free water and stored at -20°C until cDNA synthesis. Prior to freezing, the concentration of each sample was estimated via a NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, DE, USA).

The cDNA was synthesized with 1µg RNA from each sample, using Bioline Tetro cDNA Synthesis Kits (Bioline, London, UK). Oligo dT's (included in kit) were used as cDNA synthesis primers. A reaction time of 60 minutes and temperature of 41°C were selected for the synthesis step in the Bioline Tetro protocol.

Three qPCR analytical replications from each of the three biological replications sampled for each Timepoint\*Treatment (mock- or fungus-inoculated)\*Genotype combination were used in the experiment. Analytical replications were run on separate qPCR plates. The qPCR primers used were as follows: forward- 5'-TCA GCA AAC AAC AAA CAA TGG-3' and reverse-5'-GTA GTC CTG CGG CGA GTT CT-3' for *PR1*, forward-5'-CGA CAT GAA GAC CCA TGC CTG T-3' and reverse-5'-CCT GCA AAA TCC AAA TCA CTA GCC CA-3' for *PR5*, and forward-5'-GCT CAG GCA AAA AGG GGT ATC CT-3' and reverse-5'-CTC ATT GTA GAA GGT GTG GTG CCA T-3' for maize actin (based on transcript sequences from GenBank accessions U82200, U82201, and J01238, respectively).

### 2.3.7. Statistical analyses

The B73*rhm1* x NC250A SLB dQTL mapping population data were analyzed using PROC MIXED in SAS (v.9.1; SAS Institute, Cary, NC, USA). In the initial full model, the average disease rating for each line within each of three rating years was used as the dependent variable, year of rating was included as a random effect class variable, and a fixed

effect class variable was included for each marker within the significant dQTLs detected in the *B73rhm1* x NC250A by Zwonitzer et al (Table 1; 46). Each marker variable had three levels: homozygous for the *B73rhm1* allele, homozygous for the NC250A allele, or heterozygous. “Redundant markers” were dropped from the final model. A marker for a given dQTL was considered redundant if a second marker from that same dQTL contributed similar information (i.e., approximately equal genotypic class effect estimates) for the dQTL but, compared to the other marker(s) for that dQTL, a) had a more significant Type III F-test result, b) led to a greater reduction in experimental error, or c) was associated with less missing marker data.

Fisher’s protected LSD was used to investigate differences between genotypic groups within markers. T-tests were obtained for differences of least-squares means between levels of the two dQTL 6.01 markers, the representative dQTL 3.04 marker, and a representative marker of dQTL 9.02, the dQTL which displayed the largest effect on resistance in the original mapping results (46).

Germination and penetration efficiency were each analyzed with PROC GLIMMIX as a binomially distributed dependent variables with a generalized linear models using logit links. In the penetration efficiency analysis, data from the 24 hpi and 48 hpi time periods were collected from separate combinations of experimental repetitions (Trials 1 and 3 and Trials 2 and 3, respectively), and therefore each period was analyzed separately. Time, density (spores/(mm)<sup>2</sup> sample leaf area), and host genotype were included as fixed main effect factors in the full model for both germination and penetration efficiency, with within-trial error correlations modeled by the SUBJECT= option within a RANDOM statement. The mycelium development experiment was analyzed as a randomized split-plot design with subsampling using PROC MIXED in SAS. Time post-inoculation (24 or 48 hpi) was the whole-plot factor, and incubation bags were the whole-plot units. Host line was the subplot factor, and individual plants were the subplot units. Infection site was the within-plant

subsampling error and was represented in the model as a repeated measure by use of the SUBJECT= option in a RANDOM statement. The raw data were log-transformed to better meet the assumptions of normality and independent distribution of error. Statistically significant differences between groups were determined using Tukey's HSD for multiple comparisons.

An optimal threshold for  $C_t$  values was calculated for each qPCR run using the maximum-curvature approach. The average of these optimized thresholds was used as a common threshold to obtain normalized  $C_t$  values for use in the final analysis. Gene expression data was analyzed using the  $\Delta\Delta C_t$  method as described by the ABI Prism user manual (2).  $C_t$  values for individual *PR* genes relative to actin  $C_t$  values in the same replications were represented by the  $\Delta C_t$  values ( $\Delta C_t = -(C_{t(PR)} - C_{t(actin)})$ ), while the  $\Delta\Delta C_t$  values represent *PR*:actin  $\Delta C_t$  ratios in infected plants normalized to the  $PR_{mean}$ :actin $_{mean}$   $\Delta C_t$  ratio of one of two control groups: a) infected plants at 0 hpi or b) mock-inoculated plants from the same plant genotype and timepoint ( $\Delta\Delta C_t = (\Delta C_{t(Infected)} - \Delta C_{t(Control)})$ ).  $\Delta\Delta C_t$  values were analyzed with the SAS GLM procedure, using a separate model for each *PR* gene-control group combination. For each *PR* gene-control group combination, contrasts were used to investigate differences in *PR* gene expression between the following NIL classes: 1) with versus without dQTL 3.04; 2) with versus without dQTL 6.01; 3) B73rhm1 versus B73; 4) under the assumption that *rhm1* and dQTL 6.01 are the same, with versus without *rhm1*/dQTL 6.01; and 5) B73rhm1 versus the dQTL 6.01 NILs. Figure 8E lists the dQTL carried in each of the six lines included in the *PR* gene expression assays. As the full degree of genetic identity between B73rhm1 and the other five NILs is unknown, it seemed advisable to not include B73rhm1 in contrasts of the first two classes. This reasoning, weighed with assumed sufficiency of the two or more non-B73rhm1 NILs for each comparison group within those contrast classes, led to a final decision to exclude coefficients for B73rhm1 in contrast and estimate computations for classes 1 and 2.

## 2.4. Results

### 2.4.1. Test for genetic complementation of *rhm1* and dQTL 6.01

The SSR markers included in the final model are indicated in Table 1. All dQTL found significant by Zwonitzer et al (46) were found significant in the results of our model ( $P < 0.05$ ). The *rhm1* and dQTL 6.01 resistance loci failed to genetically complement each other, as evidenced by the lack of significant effect for both of the bin 6.01 markers ( $P \geq 0.50$ ). Fisher's protected LSD was used with regard to comparing genotypic classes within markers (Table 2). Because the omnibus F-tests for the 6.01 markers were not significant, the unadjusted p-values given for differences between genotypic classes within the 6.01 markers are not valid statistics per se but merely included for qualitative comparison (see Discussion for further explanation). The least-squares mean for the heterozygote class within each 6.01 marker was intermediate to the respectively homozygote classes of each marker (Fig. 6, bin 3.03 and 9.02 least-squares means were included for comparison).

The two bin 5.05-5.06 markers (Table 1) were originally considered redundant, based on non-significant Type III F-tests for effect ( $P = 0.1867$  for SNPM18 and  $P = 0.1423$  for *umc51a* once included in the final model). However, inclusion of only either SNPM18 or *umc51a* resulted in different F-test and least-squares means results for the 6.01 markers, despite producing few differences for the same respective results between SNPM18 and *umc51a* (data not shown). Because of their influence on the bin 6.01 results and because a dropping either SNPM18 or *umc51a* from the final model resulted in a substantial increase in the Akaike Information Criterion (1), both markers were included in the final analysis.

#### 2.4.2. Spore germination/penetration efficiency and rate

Estimated final germination efficiencies ranged from 97% to 99% (represented by 14 hpi estimates, Table 3). Host genotype was not a significant effect in the germination efficiency model ( $P=0.18$  for the Type III fixed effects F-test).

Penetration efficiency ranged from 50% to 100% at 24 hpi and from 67% to 100% at 48 hpi. The results of the full model for penetration efficiency did not show a significant effect for spore density, time, or host genotype at either the 24 or 48 hpi time periods ( $P \geq 0.22$  for all fixed effect F-tests). A reduced model with only host genotype as a fixed effect was fitted for each time period, to obtain genotype efficiency least-squares means and standard errors with LSMEANS statements in PROC GLIMMIX (Type III F-tests for a genotype fixed effect yielded results of  $p=0.32$  at 24 hpi and  $p=0.40$  at 48 hpi). Calculated efficiencies least-squares means for the four plant genotypes ranged from 81% to 90% at 24 hpi and from 93% to 98% at 48 hpi (Fig. 7; Table 3).

#### 2.4.3. Hyphal growth

Throughout the microscopy work, no obvious qualitative differences in infection were observed between any of the lines. For the three quantitative measurement classes of hyphal growth (the length parallel and perpendicular to leaf veins and the estimated elliptical area), there was no significant host genotype effect at 24 hpi ( $P \geq 0.5190$  for all hyphal growth measurement classes; Fig. 5 and Table 4). A significant host genotype effect was present at 48 hpi ( $P \leq 0.0013$ ), and NC292 and NC330 had significantly less fungal growth for all three measurement classes than susceptible parent B73 (Table 4;  $P \leq 0.05$ ). B73rhm1 leaf samples displayed levels of growth that were intermediate to those of B73, NC292, or NC330 but not significantly different from any other line ( $P \leq 0.05$ ).

#### 2.4.4. *PR* gene expression

*PR1* and *PR5* cDNA  $\Delta\Delta C_t$ 's were evaluated as described above for each control group (Fig. 8). For all comparisons, *PR1* and *PR5* were significantly up-regulated in infected plants at 15 and 24 hpi compared to either control group: inoculated plants at 0 hpi or mock-inoculated plants at the same timepoint ( $P < 0.01$  for all  $t$  tests). Based on the germination and penetration efficiency data, 15 hpi is only a few hours after the fungus has started to penetrate and around 10 hours after it has started to germinate.

Contrasts were used to evaluate each of the five contrast classes for effects of the three resistance loci: dQTL 3.04, *rhm1*, and dQTL 6.01, under both the assumptions that *rhm1* and dQTL 6.01 are representing the same resistance gene and that they are representing different genes (Table 5). Briefly, although 25 of the 40 total contrasts were significant ( $P \leq 0.037$ ; Table 5), the contrast groups in the significant contrasts did not correspond with any apparent patterns in the  $\Delta\Delta C_t$  means (Fig. 8).

## 2.5. Discussion

The primary objective of this study was to uncover the mechanisms by which individual quantitative disease resistance loci disrupt infection. More specifically, the experiments were designed to 1) determine whether *rhm1* and dQTL 6.01 represent the same resistance/susceptibility gene, 2) identify the stage or stages at which infection was inhibited in resistant plants, and 3) determine whether an active defense response was associated with resistance loci. Juvenile plants were used to measure all traits.

Although the end goal of this research is to determine the physiology of adult field resistance in the NIL collection, the use of juvenile plants is justified by the results of a trial conducted by Zwonitzer (45) which rated juvenile growth chamber resistance in 45 NILs

containing various combinations of 12 total putative dQTLs (including dQTLs 3.04 and 6.01). The juvenile ratings were then correlated with adult field resistance ratings. Adult and juvenile resistance was correlated across all lines ( $r=0.63$  between growth chamber and summer 2007 scores). However, only dQTL 6.01 was estimated to have a significant effect on juvenile resistance, and the estimated effect of dQTL 6.01 was not significantly different from the estimated resistance of the resistant lines from which all of the putative dQTLs were derived (Fig. 9, dQTLs 3.04 and 6.01 are referred to as introgressions 6A and 3B, respectively). dQTL 3.04 was also estimated to contribute to resistance, but not significantly. However, Balint-Kurti and Carson (4) did map a SLB dQTL to this region in their juvenile plant study. Based on these previous results, only the effects of dQTLs 3.04 and 6.01 were investigated in these experiments.

The failure of *rhm1* and dQTL 6.01 to genetically complement in our analysis of the B73*rhm1* x NC250A SLB dQTL mapping population data is near-conclusive evidence that these loci do represent the same recessive resistance gene, albeit perhaps separate alleles. The Type III F-tests for genotypic class effects for each of the two bin 6.01 markers were non-significant ( $P \geq 0.50$ ). The least-squares mean for the heterozygous genotypic class was not higher but intermediate in disease severity relative to the parental homozygous classes for each 6.01 marker. The ranking of the parental classes was reversed between the first and second 6.01 markers (Table 2 and Fig. 6), further suggesting that the genotypic classes do not differ in resistance genes.

The standard error for each marker genotypic class least-squares mean (Fig. 6) seems potentially large enough to prohibit this analysis from statistically detecting true differences between 6.01 marker genotypic classes, if the differences were small. However, this type of false negative in our results seems unlikely given a) the intermediate ranking of the heterozygous class for each 6.01 marker, b) the above-mentioned shifts in genotypic class rank between 6.01 markers (although this could also be accounted for by high experimental

error), and c) the highly significant p-values for the bin 3.03 and bin 9.02 *t* tests compared to the decidedly non-significant p-values for the bin 6.01 *t* tests (Table 2), despite equal experimental error.

It is important to note here that - given the use of Fisher's protected LSD - because the omnibus F-tests for the 6.01 markers were not significant, the unadjusted p-values presented for differences between genotypic classes within the 6.01 markers (Table 2) are statistically inappropriate. However, as Fisher's protected LSD is the least conservative common multiple comparison test, any genuine p-values for the 6.01 markers obtained by application of a different test would be even larger than those presented. Therefore, we felt that our unadjusted bin 6.01 *t* tests were legitimate to include for comparison with the bin 3.03 and 9.02 *t* tests; they function well as semi-quantitative evidence against the possibility of experimental error masking a true increase in disease severity for *rhm1*/dQTL 6.01 heterozygotes relative to lines homozygous for either parental allele.

Although this combined evidence strongly suggests a shared *rhm1*/dQTL 6.01 gene, we are awaiting a final conclusion until parallel results are found with *rhm1*/dQTL 6.01 heterozygotes that do not carry additional NC250P introgressions. A field evaluation of progeny from crosses between B73*rhm1* and B73 NILs carrying only the NC250P 6.01 locus is currently underway. The high likelihood that *rhm1* and dQTL 6.01 are the same gene is important in interpreting the remaining results of this project. Mainly, it calls the question of whether any differences in histology or host defense gene expression observed between B73*rhm1* and the dQTL 6.01 NILs (JZ196, NC292, and NC330) are due to different resistance genes, genetic background effects of genes not directly related to the resistance in these lines, or experimental error. Any significant differences reported between *rhm1* and dQTL 6.01 due to either of the two latter effects would therefore be false positives if the same resistance gene underlies both loci.

None of the resistance loci tested showed a significant effect on germination or penetration efficiency (Fig. 7, Table 3). Based on these results, we tentatively conclude that dQTL 3.04, dQTL 6.01, and *rhm1* only influence disease after *C. heterostrophus* enters the leaf.

Fungal growth, as measured by the diameter of stained infection sites, did not differ between lines at 24 hpi but was significantly reduced at 48 hpi in NILs with dQTL 6.01 ( $P \leq 0.05$ ; Table 4). Fungal growth in B73rhm1 was intermediate to that of B73 and the dQTL 6.01 NILs (NC292 and NC330) but not significantly different from that of any other lines ( $P \leq 0.05$ ). Consistent with its zero or small effect on overall juvenile disease severity observed previously (Fig. 9), dQTL 3.04 did not have an observable impact on fungal growth in this study at any timepoint.

Smith and Toth (32, 35) also reported on the histology of race O lesions in the *rhm1* line 1089Hm and the isogenic susceptible line 1089H. Our results confirm those of Smith and Toth for the timing of initial penetration events, at approximately 6 hpi (data not shown). Whereas the histological data presented here focuses on pathogen development, Smith and Toth examined host mesophyll cell deterioration within lesions via light and electron microscopy. Similar to our fungal staining results, they observed no difference between lesions in susceptible and resistant plants at 24 hpi. In contrast, while our staining results did show differences between lines at 48 hpi, Smith and Toth (32) did not detect any obvious reduction in symptoms at the cellular level until 96 hpi. This disparity in apparent onset of resistance may be due to the weaker precision of their more qualitative investigation - Toth and Smith (35) describe a high variance between lesions - or to a true earlier impact on fungal growth versus host necrosis by *rhm1* at the microscopic level.

*PR1* and *PR5* relative abundance were compared between lines at 15 and 24 hpi (Fig. 8 and Tables 5 and 6). Simmons et al (30) found using Western blots that PR1 protein levels at 16 and 24 hours post-inoculation were lower in infected *rhm1* plants compared with

infected wildtype controls. Yet, in our qPCR results, we found that *PR1* was upregulated in B73rhm1 compared to B73 at 15 and 24 hpi. Our results could be consistent with those of Simmons et al if either PR1 protein production is also regulated at the translation level or if B73rhm1 PR1 protein abundance is low enough at 0 hpi and in mock-inoculated plants to show a large enough relative increase in protein content, despite low absolute protein levels. As the Western blots of Simmons et al were absolute measures of PR1 while our qPCR *PR1*  $\Delta\Delta C_t$ 's were relative measures (relative to the two control groups), the results of the two methods are not completely comparable.

In further contrast to the *rhm1* results of Simmons et al (30), Morris et al (23) found *PR1* and *PR5* expression, as measured by Northern blots, to be similar or higher in resistant versus susceptible plants at 24 and 48 hpi for both infected and uninfected plants. When compared across all contrast and treatment combinations, our results also found the relationship between resistance and *PR* gene expression to be inconsistent. A large number of the contrasts testing for relationships between *PR* gene abundance and the presence of individual resistance loci were significant (Table 5), and displayed a trend of active defense gene upregulation with the dQTL 6.01 and *rhm1* resistance loci. However, the relative expression in individual lines undermines the contrasts results. There is no distinctive pattern in observed expression between lines with and without dQTLs 6.01 and 3.04 (Fig. 8 and Table 6). This lack of correspondence is even more distinct if *rhm1* and dQTL 6.01 represent the same resistance gene – not simply by the patterns in actual expression fold changes (Fig. 8 and Table 6), but by a high proportion of significant contrasts both for a difference between B73rhm1 and the dQTL 6.01 NILs in a addition to the contrast testing the effect of a shared *rhm1*/dQTL 6.01 gene (5 and 6 of 8, respectively; Table 5). It would appear then that the significance of any contrasts – along with the decrease or increase, respectively, in *PR* gene expression in resistant lines found by Simmons et al (30) or Morris et al (23) - are in fact due to the chance assortment of high- and low-expression lines between the resistant and susceptible genotypes selected in all experiments, not from a true difference in expression

due to the presence or absence of any resistance loci. These results do not exclude the possibility that *rhm1*, dQTL 6.01 (or *rhm1*/dQTL 6.01), or 3.04 uses active defense mechanisms to curb fungal growth. However, in light of this data, it seems highly unlikely that juvenile plant resistance due to the three resistance loci examined in these experiments involves active defenses related to *PR1* and *PR5* expression.

There are two caveats to the previous statement. First, dQTL 3.04 resistance has only been reported as significantly effective in adult plants (Fig. 8) (45); and, if measured, *PR* gene expression in infected adult plants could certainly demonstrate a relationship with *PR* gene-associated active defense. Likewise, *rhm1*/dQTL 6.01 resistance in adult plants may operate in a manner different to juvenile plants that does involve *PR* gene-associated active defense (juveniles were also used by Simmons et al (30)). Second, resistance due to dQTL 3.04 and *rhm1*/dQTL 6.01 may well involve an alternative active defense mechanism. In fact, while *PR* gene induction has been observed for almost every class of plant biotic or abiotic stress including biotrophs (15), necrotrophs (5), SA-dependent defense signaling pathways (40), JA/ET-mediated defenses (27), herbivory (19) and wounding (7), *PR* genes are far more commonly associated with plant defenses involving SA-dependent signaling pathways - defenses normally used to guard against biotrophic pathogens, not necrotrophs such as *C. heterostrophus*. In general, necrotrophic pathogens are suppressed via defenses that use JA and ET-dependent signaling pathways (33).

Although there are examples of JA and ET pathways inducing *PR* genes, JA and ET pathways are more consistently shown to induce expression of other pathogenesis response gene groups, such as the *OPR* genes (43). From semi-quantitative PCR experiments using cDNA from the same samples used for the *PR* gene qPCR in this study, we have found preliminary evidence that *OPR2* and *OPR6* RNA abundance correspond with juvenile plant resistance (data not shown).

It is also of note that the *PR* gene expression results indicate a relatively fast active response to infection by the host. The earliest post-inoculation sample timepoint, 15 hpi, is only nine hours after the time at which *C. heterostrophus* has been shown to start penetrating leaf epidermal cells in our work (data not shown) and that of Smith and Toth (32). While there is yet no conclusive evidence that resistance to SLB race O involves an active defense mechanism, it does appear that plants, resistant or susceptible, are actively “aware” of infection at a very early point in the disease cycle.

The processes which underlie SLB race O resistance and quantitative disease resistance as a whole are poorly understood. While these results did not identify a precise functional basis for the resistance loci *rhm1*, dQTL 6.01, and dQTL 3.04, they have established some important properties of the mechanism(s) at work. First, the timing of the effect: there was no inhibition of fungal development (germination, penetration, or growth) in resistant plants during the first 24 hours after inoculation, but fungal growth was reduced in *rhm1* and dQTL 6.01 lines at 48 hours. Second, the nature of the effect: quantitative differences were measured, but no qualitative differences were observed. Finally, associated defense systems: while we could not determine whether an active or a passive mechanism was inhibiting disease, we have ruled out the involvement of the active defense systems that induce *PR* gene expression. We are currently investigating the possibility of the JA/ET pathways playing a role in *rhm1*- and dQTLs 6.01- and 3.04-mediated resistance by quantifying *OPR* gene expression during infection. These improvements in the model for quantitative resistance systems will hopefully lead to increased success in the efforts towards identifying and deploying quantitative disease resistance genes in the field.

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**Table 2.1. Markers used to represent SLB dQTL in the *rhm1*-dQTL 6.01 genetic complementation test.**

Asterisks indicate markers that were included in the final genetic complementation test model. SSR marker data taken from Zwonitzer et al. (46).

<b>dQTL Bin</b>	<b>SSR Marker</b>
1.09	umc50b
	*bnl8.10a
	bnl7.25a
3.03	CPH1206
	*bnl8.35a
5.05-5.06	*SNPM18
	*umc51a
6.01	*bnl6.29a
	*php20527
9.02	*umc113a
	*php20052

**Table 2.2. Least-squares means of differences in disease severity between genotypic classes within markers from the B73rhm1 x NC250A *C. heterostrophus* resistance QTL mapping experiment of Zwonitzer et al. (46).**

Disease severity is on a 1-9 scale, with 1 being completely symptom-free and 9 being killed by disease. Although only the dQTL 6.01 markers (dQTL 6.01a and 6.01b) are of interest for the current research, the 3.03 (which is identical to the NC250P dQTL 3.04 resistance locus used in these experiments) and 9.02 loci were also included for comparison with the dQTL 6.01 results.

<sup>a</sup>, <sup>b</sup> Genotypic classes used to obtain estimates (each estimate is the difference in least-squares means between genotypic class 1 and 2). Classes are as follows: A=NC250A homozygous, B=B73rhm1 homozygous, H=NC250A/B73rhm1 heterozygous

<sup>c</sup> Because Fisher's protected LSD was applied for multiple comparisons, comparison tests between levels of effects non-significant in the overall ANOVA are not appropriate. Therefore, the p-values obtained from the bin 6.01 marker class comparisons (the Type III F-tests for both 6.01 markers were highly non-significant) should not be taken as accurate statistics but merely semi-quantitative measures of the lack of difference shown between 6.01 heterozygous and homozygous classes.

dQTL	Geno. Class 1 <sup>a</sup>	Geno. Class 2 <sup>b</sup>	Estimate	Standard Error	DF	t Value	Pr >  t
<b>3.03</b>	A	B	-0.6701	0.0881	342	-7.61	<0.001
	H	A	0.6531	0.0824	342	-7.93	<0.001
	H	B	-0.0170	0.0730	342	0.23	0.816
<b>6.01a</b>	A	B	-0.2232	0.2447	342	-0.91	0.362 <sup>c</sup>
	<b>H</b>	<b>A</b>	<b>0.0837</b>	<b>0.1746</b>	<b>342</b>	<b>-0.48</b>	<b>0.632<sup>c</sup></b>
	<b>H</b>	<b>B</b>	<b>-0.1395</b>	<b>0.1711</b>	<b>342</b>	<b>0.82</b>	<b>0.415<sup>c</sup></b>
<b>6.01b</b>	A	B	0.2492	0.2499	342	1.00	0.319 <sup>c</sup>
	<b>H</b>	<b>A</b>	<b>-0.2069</b>	<b>0.1788</b>	<b>342</b>	<b>1.16</b>	<b>0.248<sup>c</sup></b>
	<b>H</b>	<b>B</b>	<b>0.0423</b>	<b>0.1686</b>	<b>342</b>	<b>-0.25</b>	<b>0.802<sup>c</sup></b>
<b>9.02</b>	A	B	-0.5307	0.0978	342	-5.43	<0.001
	H	A	0.2065	0.0803	342	-2.57	0.011
	H	B	-0.3241	0.0785	342	4.13	<0.001

**Table 2.3. Germination and penetration efficiency estimates obtained by the PROC GLIMMIX models.**

The values displayed for germination efficiency are the least-squares means and associated errors at 14 hpi estimated by the model. Based on the inferred growth curve pattern of germination efficiency over time (Fig. 6A), 14 hpi was selected as the representative time at which to infer the endpoint germination efficiency of each line. The values listed for penetration efficiency are least-squares means and associated errors. Host genotype was not a significant effect for any of the germination or penetration efficiency models ( $P \geq 0.18$  for all Type III F-tests of host genotype effect). The first two columns indicate whether a given dQTL is present (+) or absent (-) in an inbred line.

dQTL 3.04	dQTL 6.01	NIL	Germination Efficiency	Penetration Efficiency	
			<u>14 hpi</u>	<u>24 hpi</u>	<u>48 hpi</u>
-	-	<b>B73</b>	0.9851 (0.0126)	0.8747 (0.0713)	0.9834 (0.0151)
-	+	<b>B73rhml1</b>	0.9897 (0.0088)	0.9027 (0.0573)	0.9339 (0.0303)
+	+	<b>NC292</b>	0.9709 (0.0231)	0.8693 (0.0716)	0.9697 (0.0207)
-	+	<b>NC330</b>	0.9846 (0.0127)	0.8139 (0.0946)	0.9250 (0.0382)

**Table 2.4. Hyphal growth estimates for each hyphal growth measurement class for all timepoint and host genotype (line) combinations, given as the natural log transformation.**

Standard errors are displayed in parentheses after each estimate. The original units for parallel length, perpendicular length, and estimated elliptical area were  $\mu\text{m}$ ,  $\mu\text{m}$ , and  $\mu\text{m}^2$ , respectively. Different superscript letters for estimates within the same column represent significant differences for those Lines within that Time period ( $\alpha=0.05$ ). F- and P-values for tests of effect slices are also given for tests of a Line simple effect by a Time slice of Time\*Line interaction effects for each measurement class at each timepoint. The first two columns indicate whether a given dQTL is present (+) or absent (-) in an inbred line.

<sup>a</sup>  $F_{3,1115}$  from test of effect slices (Line simple effect by Time slice of Time\*Line interaction effect; Winer, 1971)

<sup>b</sup> P-value from associated F-tests of effect slices

			<u>24hrs</u>			<u>48hrs</u>		
dQTL	dQTL	NIL	Parallel	Perpendicular	Ellipse	Parallel	Perpendicular	Ellipse
3.04	6.01							
-	-	<b>B73</b>	5.9476 <sup>A</sup> (0.1370)	5.6323 <sup>A</sup> (0.1101)	11.3376 <sup>A</sup> (0.2342)	6.9957 <sup>A</sup> (0.112)	6.4492 <sup>A</sup> (0.0900)	13.2052 <sup>A</sup> (0.1915)
-	+	<b>B73rhm1</b>	6.1909 <sup>A</sup> (0.1368)	5.6875 <sup>A</sup> (0.1099)	11.6383 <sup>A</sup> (0.2340)	6.6859 <sup>AB</sup> (0.1143)	6.1082 <sup>AB</sup> (0.0917)	12.5475 <sup>AB</sup> (0.1957)
+	+	<b>NC292</b>	5.9845 <sup>A</sup> (0.1356)	5.4671 <sup>A</sup> (0.1084)	11.2090 <sup>A</sup> (0.2320)	6.4935 <sup>B</sup> (0.1120)	5.9261 <sup>B</sup> (0.0901)	12.1788 <sup>B</sup> (0.1915)
-	+	<b>NC330</b>	5.9301 <sup>A</sup> (0.1495)	5.6161 <sup>A</sup> (0.1200)	11.3035 <sup>A</sup> (0.2558)	6.4133 <sup>B</sup> (0.1141)	5.9288 <sup>B</sup> (0.0913)	12.1028 <sup>B</sup> (0.1952)
<b>Time*Line sliced by Time</b>			F <sup>a</sup> =0.76 p <sup>b</sup> =0.519	F=0.75 p=0.524	F=0.63 p=0.597	F=5.29 p=0.001	F=7.43 p<0.001	F=6.85 p<0.001

**Table 2.5. Contrasts comparing PR gene relative abundance ( $\Delta\Delta C_t$ ) between different host resistance locus groups.**

<sup>a</sup>From contrast statements, indicates the two genotypic groups being compared with each statement and which of the two groups had positive versus negative coefficients.

<sup>b</sup>Indicates the control group used to obtain the  $\Delta\Delta C_t$  values for a given contrast (all contrasts used  $\Delta\Delta C_t$  values as the dependent variable). A “0 hpi” control group indicates  $\Delta C_t$  values for infected samples at 15 and/or 24 hpi normalized to  $\Delta C_t$  values for inoculated plants at 0 hpi. A “mock” control group indicates  $\Delta C_t$  values for infected samples at 15 and/or 24 hpi normalized to  $\Delta C_t$  values for mock-inoculated plants of the same genotype at the same timepoint.

Comparison <sup>a</sup>	Control Group <sup>b</sup>	Time (hpi)	Gene	Estimate <sup>c</sup>	Error	t Value	Pr >  t
dQTL 3.04 <sup>+</sup> - dQTL 3.04 <sup>-</sup>	0 hpi	15 & 24	<i>PR1</i>	0.919	0.237	3.87	<0.001
		15 & 24	<i>PR5</i>	-0.997	0.208	-4.79	<0.001
		15	<i>PR5</i>	-0.952	0.295	-3.23	0.004
		24	<i>PR5</i>	-1.043	0.295	-3.54	0.002
	mock	15 & 24	<i>PR1</i>	-0.112	0.244	-0.46	0.650
		15 & 24	<i>PR5</i>	-0.144	0.217	-0.66	0.514
		15	<i>PR1</i>	0.063	0.345	0.18	0.857
		24	<i>PR1</i>	-0.287	0.345	-0.83	0.413
dQTL 6.01 <sup>+</sup> - dQTL 6.01 <sup>-</sup>	0 hpi	15 & 24	<i>PR1</i>	1.382	0.237	5.82	<0.001
		15 & 24	<i>PR5</i>	0.206	0.208	0.99	0.333
		15	<i>PR5</i>	0.067	0.295	0.23	0.823
		24	<i>PR5</i>	0.346	0.295	1.18	0.254
	mock	15 & 24	<i>PR1</i>	0.763	0.244	3.13	0.005
		15 & 24	<i>PR5</i>	0.704	0.217	3.24	0.003
		15	<i>PR1</i>	0.489	0.345	1.42	0.169
		24	<i>PR1</i>	1.037	0.345	3.01	0.006
B73rhml - B73	0 hpi	15 & 24	<i>PR1</i>	2.583	0.368	7.02	<0.001
		15 & 24	<i>PR5</i>	-0.200	0.323	-0.62	0.542
		15	<i>PR5</i>	-1.022	0.456	-2.24	0.037
		24	<i>PR5</i>	0.622	0.456	1.36	0.188
	mock	15 & 24	<i>PR1</i>	2.458	0.377	6.51	<0.001
		15 & 24	<i>PR5</i>	3.072	0.336	9.14	<0.001
		15	<i>PR1</i>	2.650	0.534	4.96	<0.001
		24	<i>PR1</i>	2.267	0.534	4.25	<0.001

**Table 2.5. (continued)**

Comparison <sup>a</sup>	Control Group <sup>b</sup>	Time (hpi)	Gene	Estimate <sup>c</sup>	Error	t Value	Pr >  t
rhm1/dQTL 6.01 <sup>+</sup> - rhm1/dQTL 6.01 <sup>-</sup>	0 hpi	15 & 24	<i>PR1</i>	1.374	0.225	6.10	<0.001
		15 & 24	<i>PR5</i>	0.321	0.198	1.62	0.120
		15	<i>PR5</i>	-0.014	0.279	-0.05	0.961
		24	<i>PR5</i>	0.656	0.279	2.35	0.029
	mock	15 & 24	<i>PR1</i>	1.251	0.231	5.41	<0.001
		15 & 24	<i>PR5</i>	1.388	0.206	6.74	<0.001
		15	<i>PR1</i>	1.196	0.327	3.66	0.001
		24	<i>PR1</i>	1.306	0.327	3.99	<0.001
dQTL 6.01 - rhm1	0 hpi	15 & 24	<i>PR1</i>	0.035	0.300	0.12	0.908
		15 & 24	<i>PR5</i>	-0.457	0.263	-1.74	0.098
		15	<i>PR5</i>	0.322	0.373	0.86	0.397
		24	<i>PR5</i>	-1.237	0.373	-3.32	0.003
	mock	15 & 24	<i>PR1</i>	-1.951	0.308	-6.33	<0.001
		15 & 24	<i>PR5</i>	-2.735	0.274	-9.96	<0.001
		15	<i>PR1</i>	-2.828	0.436	-6.49	<0.001
		24	<i>PR1</i>	-1.074	0.436	-2.46	0.021

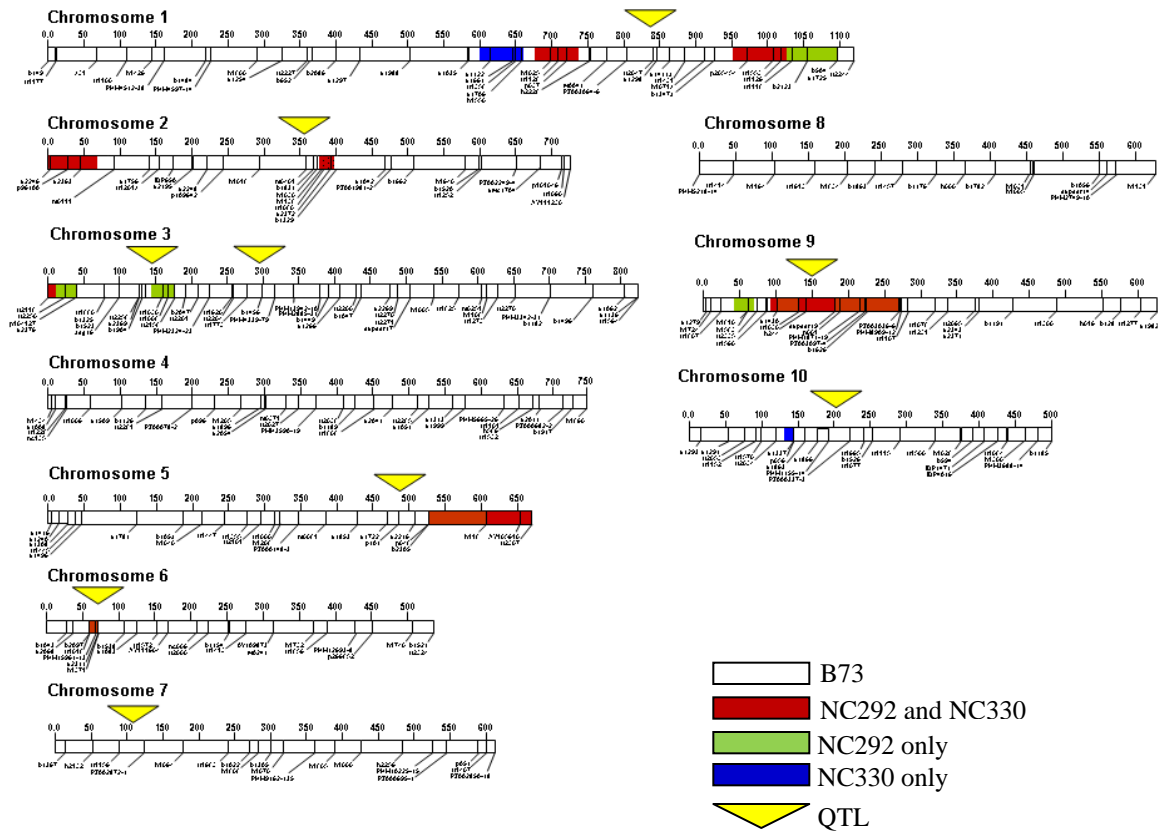
**Table 2.6. *PR* gene transcript fold change estimates (and standard errors in parentheses) derived based on the final models from PROC GLM.**

The first two columns indicate whether a given dQTL is present (+) or absent (-) in an inbred line.

<sup>a</sup>Indicates the control group used to obtain the  $\Delta\Delta C_t$  values presented above. A “0 hpi” control group indicates  $\Delta C_t$  values for infected samples at 15 and/or 24 hpi normalized to  $\Delta C_t$  values for inoculated plants at 0 hpi. A “mock” control group indicates  $\Delta C_t$  values for infected samples at 15 and/or 24 hpi normalized to  $\Delta C_t$  values for mock-inoculated plants of the same genotype at the same timepoint.

<sup>b</sup>The same *PR1*  $\Delta\Delta C_t$  value (0 hpi control group) estimates are present above for both 15 and 24 hpi. Neither time post-inoculation nor a time\*line interaction was a significant effect in the final model for the *PR1*-0 hpi control group variable ( $P>0.18$ ).

		Time	15 hpi				24 hpi			
		Control Group <sup>a</sup>	0 hpi		mock		0 hpi		mock	
		Gene	PR1 <sup>b</sup>	PR5	PR1	PR5	PR1 <sup>b</sup>	PR5	PR1	PR5
dQTL	dQTL	Line								
3.04	6.01									
-	-	<b>B73</b>	5.467 (0.260)	2.778 (0.323)	5.300 (0.378)	-2.999 (0.257)	5.467 (0.260)	2.822 (0.323)	6.600 (0.378)	3.868 (0.257)
-	+	<b>B73rhm1</b>	8.050 (0.260)	1.756 (0.323)	7.950 (0.378)	6.071 (0.257)	8.050 (0.260)	3.444 (0.323)	8.867 (0.378)	6.940 (0.257)
+	-	<b>JZ191</b>	7.939 (0.260)	1.244 (0.323)	3.967 (0.378)	2.266 (0.257)	7.939 (0.260)	0.900 (0.323)	6.911 (0.378)	3.134 (0.257)
+	+	<b>JZ196</b>	8.361 (0.260)	1.589 (0.323)	6.656 (0.378)	4.160 (0.257)	8.361 (0.260)	2.133 (0.323)	8.722 (0.378)	5.029 (0.257)
+	+	<b>NC292</b>	7.400 (0.260)	2.178 (0.323)	4.233 (0.378)	2.566 (0.257)	7.400 (0.260)	1.922 (0.323)	6.156 (0.378)	3.434 (0.257)
-	+	<b>NC330</b>	8.494 (0.260)	2.467 (0.323)	4.478 (0.378)	3.282 (0.257)	8.494 (0.260)	2.567 (0.323)	8.500 (0.378)	4.151 (0.257)



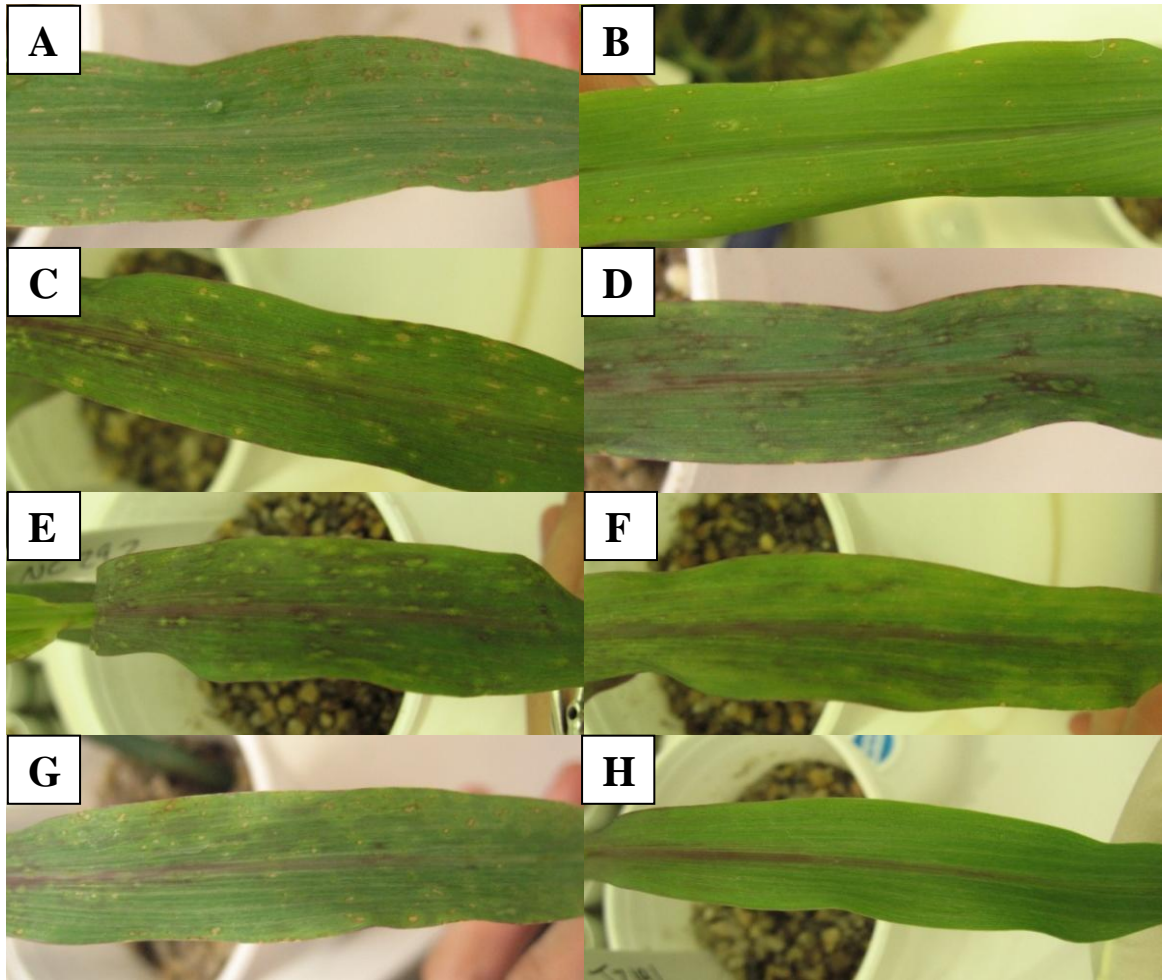
**Figure 2.1. Chromosome map showing genome-wide SSR marker analysis of NC250P introgressions.**

Labels above chromosomes are bins. Labels below chromosomes are SSR markers. QTL were mapped in a B73rhml x NC250A or a NC250A x B73 population. Taken from Zwonitzer (45).



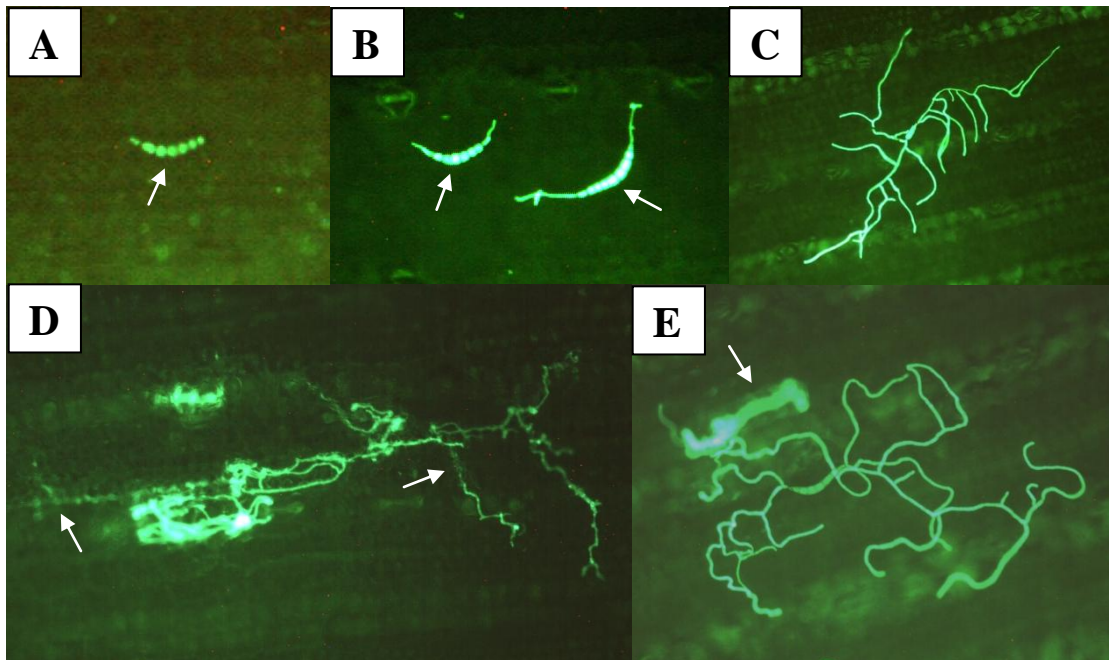
**Figure 2.2. Incubation bags (transparent autoclave bags) from the first hyphal growth experiment.**

The picture above was taken in one of the NCSU Phytotron growth chambers, in which all juvenile plant experiments were conducted (24).



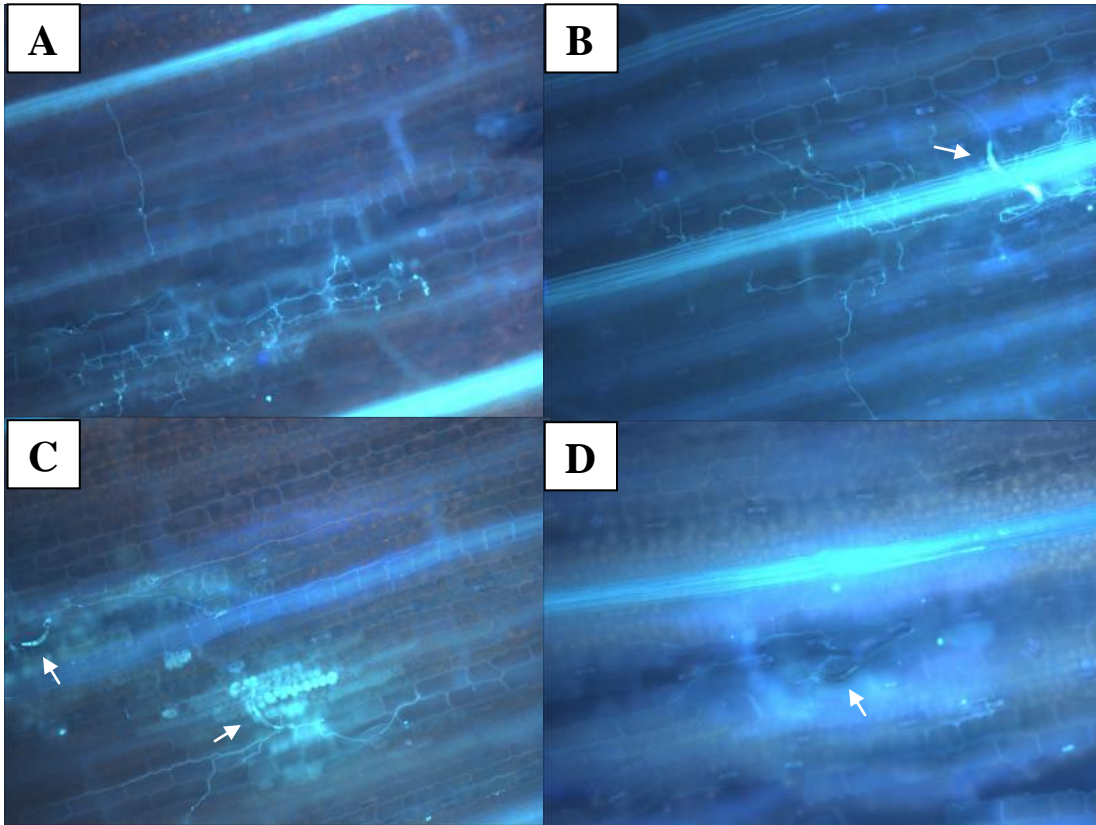
**Figure 2.3. Wildtype *C. heterostrophus*-infected leaf samples, a sample infected with a GFP-transformed *C. heterostrophus* strain, and a mock-inoculated sample (JZ191) at 48 hpi.**

All images were taken of the middle third of the second leaf each plant, the section samples were collected from in all juvenile plant experiments. The spore germination and penetration experiments used a GFP-transformed isolate (G, B73), while the hyphal growth and *PR* gene expression experiments used a wildtype isolate (A-F; A also B73). Although JZ191 (C), JZ196 (D), NC292 (E), and NC330 (F) have more visible anthocyanin production (purple pigment) than B73 (A and G), B73rhmm (B), and NC330 (F) in these images, such differences were not consistent in plants, whether infected or mock-inoculated (H; JZ191). JZ191 (C, H), JZ196 (D), and NC292 (E) all carry dQTL 3.04. dQTL 6.01 is found in JZ196 (D), NC292 (E), and NC330 (F), all of which are approximately equal in resistance as juveniles.



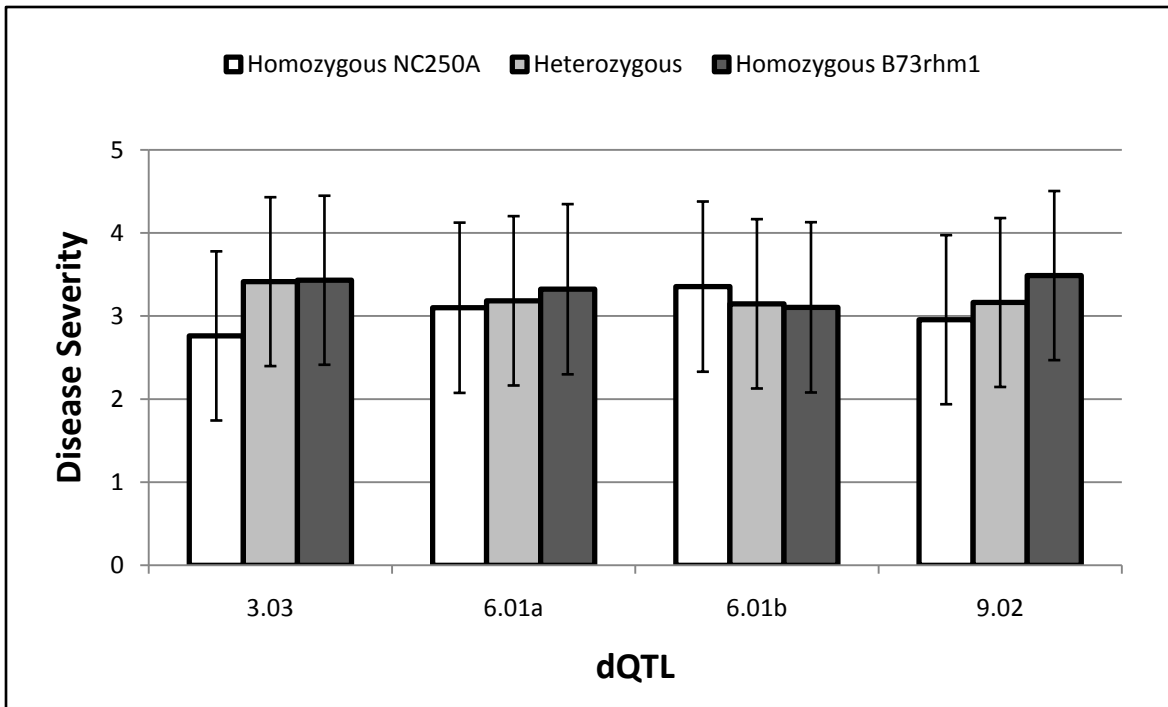
**Figure 2.4. GFP-transformed isolate of *C. heterostrophus* used to visualize spore germination and penetration efficiency.**

Spores (conidia; arrows in A and B) were considered germinated if any portion of a germ tube was seen extending from the spore (B versus the non-germinated spore in A). The fungus was assumed to have penetrated if a portion of the hyphae were obscured by (assumedly) the leaf tissue (arrows in D and E, versus non-penetrating fungus in C).



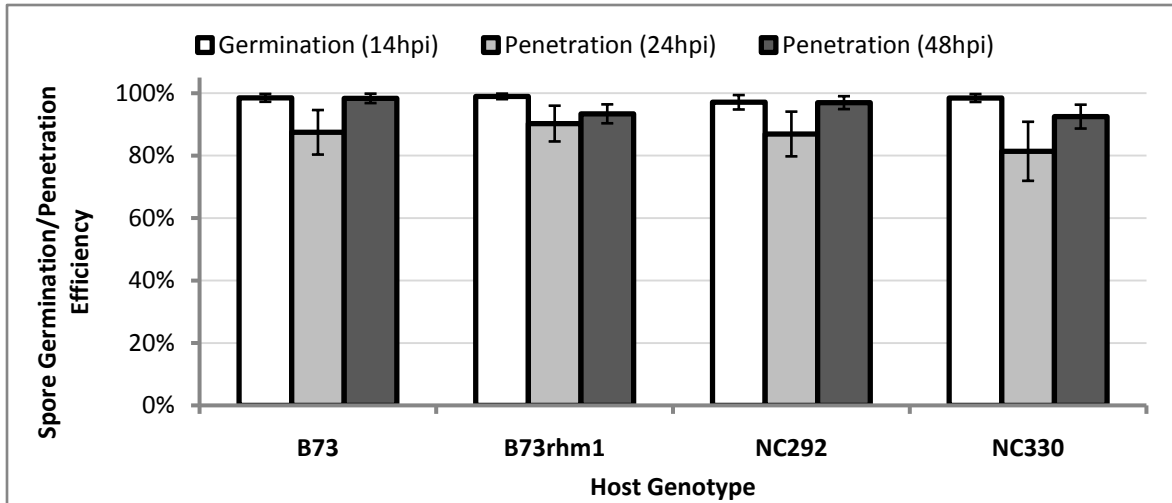
**Figure 2.5. Hyphal growth experiment staining pictures at 48 hpi for lines B73, B73rhm1, NC292, and NC330.**

Infection sites in B73 (A), B73rhm1 (B), NC292 (C), and NC330 (D). Samples were cleared, stained, and visualized by KOH aniline blue fluorescent microscopy using the methods described by Hood and Shew (14). Infection sites were defined by conidia (arrows) and the hyphae visibly attached to conidia.



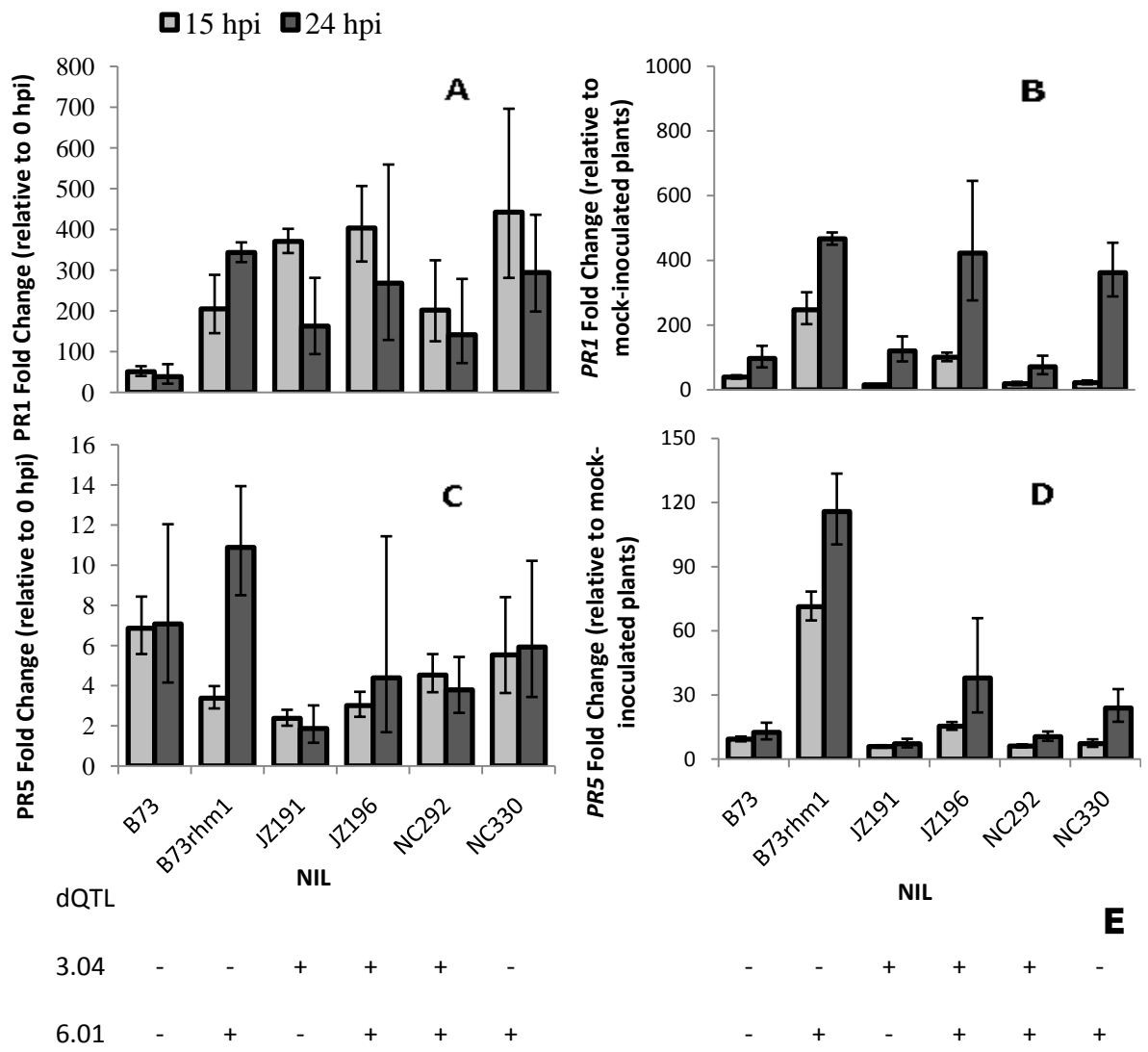
**Figure 2.6. Disease severity least-squares means of genotypic classes within markers from the B73rhm1 x NC250A *C. heterostrophus* resistance QTL mapping experiment of Zwonitzer et al. (46).**

Disease severity was rated as the average of disease scores for a line within each of three experiments. Scoring was on a 1-9 scale, with 1 being completely symptom-free and 9 being killed by disease. Although only the dQTL 6.01 markers (dQTL 6.01a and 6.01b) are of interest for the current research, the 3.03 (which is identical to the NC250P dQTL 3.04 resistance locus used in these experiments) and 9.02 loci were also included for comparison with the dQTL 6.01 results.



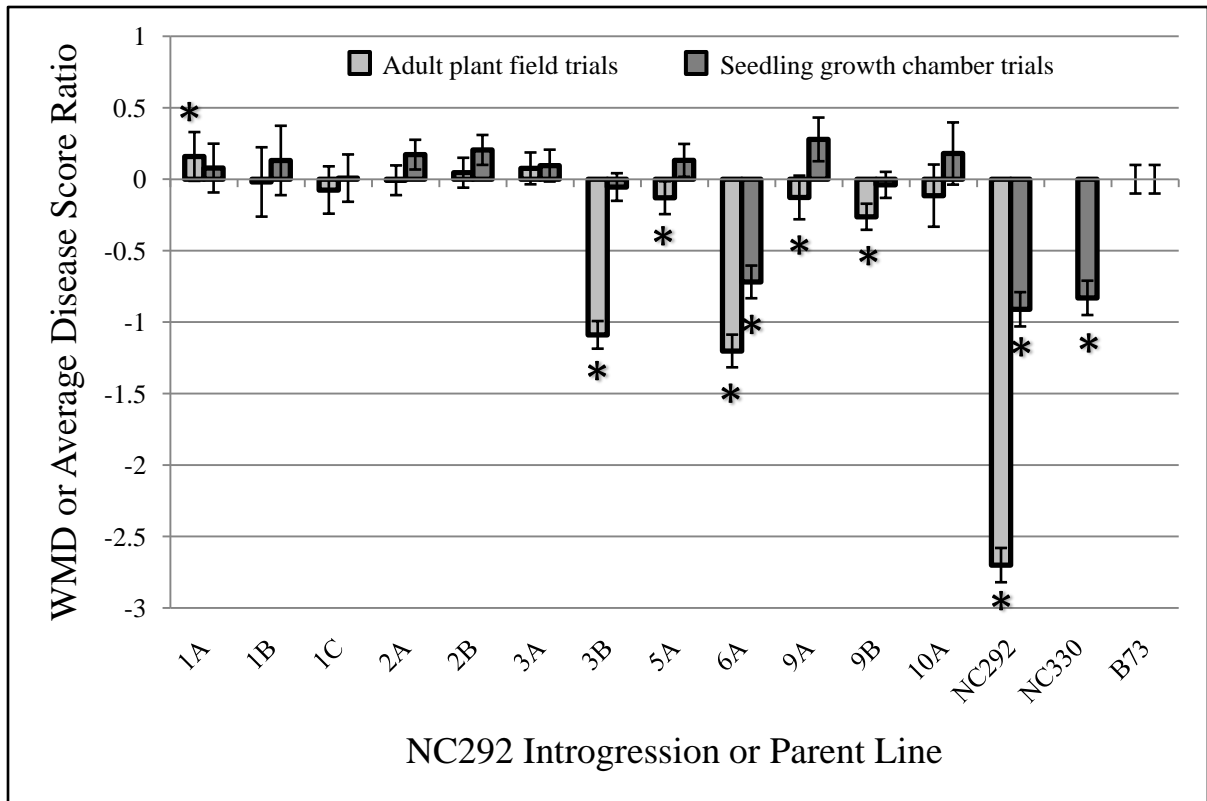
**Figure 2.7. Results of the three germination and penetration efficiency trials.**

Spore germination and penetration efficiency on leaves were measured over time in four host genotypes: B73, B73rhm1 (which contains the *rhm1* gene), NC292 (which has both dQTLs 3.04 and 6.01), and NC330 (contains dQTL 6.01 but not dQTL 3.04). A final binomial logistic-link model for germination efficiency was fitted in PROC GLIMMIX, and host genotype was not a significant effect in the model ( $P=0.18$ ). The germination efficiency for each genotype predicted by the model for 14 hpi is displayed above, along with the associated standard errors. Least-squares means and associated standard errors for the fungal penetration efficiency of each host line at 24 and 48 hpi were derived with PROC GLIMMIX using a reduced binomial logit-linked model with only host genotype as a fixed effect. Although included in the final models, host genotype was not a significant effect for penetration efficiency at 24 or 48 hpi ( $P=0.32$  and  $0.40$ , respectively).



**Figure 2.8.** *PR* gene expression (RNA abundance in infected plants relative to actin and a control group) means and standard errors for NILs B73, B73rhm, JZ191, JZ196, NC292, and NC330 at 15 and 24 hours post-inoculation.

*PR1* (A and B) and *PR5* (C and D) expression in *C. heterostrophus*-inoculated plants were measured using the  $\Delta\Delta C_i$  method: first relative to actin, or  $\Delta C_i$ , and then as fungus-inoculated plant  $\Delta C_i$ 's relative to mock-inoculated (A, C) or 0 hpi (B, D) plant  $\Delta C_i$ 's, or  $\Delta\Delta C_i$ . The tables below the graphs (E) indicate whether a given dQTL is present (+) or absent (-) in a NIL. Values in graph are from raw data, although only the errors - not the means - were changed between the raw data and the final PROC GLM model estimates (Table 5). Raw data was selected for display to allow comparison of relative experimental variance within each treatment combination.



**Figure 2.9. Estimates of effects in field and growth chamber studies for the 12 NC250P introgressions present in NC330, NC292, and the NILs.**

Introgression effects are expressed as the estimated mean difference from B73 of a single-introgression line homozygous for NC250P alleles within the genetic marker boundaries of a given introgression. Adult field trial scores are expressed as weighted mean disease (WMD) normalized to the scores for the parent lines NC292 and B73. Seedling growth chamber trial plants were scored as average percent diseased leaf area over the course of the trial normalized to the scores for the parent lines NC292 and B73 in each trial (the “ratio” of Average Disease Ratio is the ratio of entry average diseased leaf area to NC292 and B73 diseased leaf area). Error bars are for standard deviations estimated from least squares means generated by PROC MIXED in SAS. Taken from Zwonitzer (45).

**-CHAPTER III-**

**Multiple Disease Resistance QTL in Maize Near-Isogenic Lines Selected for Southern  
Leaf Blight Resistance**

**by**

Araby R. Belcher, John C. Zwonitzer, Consuelo Arellano, Peter J. Balint-Kurti

PLANNED SUBMISSION TO PHYTOPATHOLOGY

Araby R. Belcher, John C. Zwonitzer, Consuelo Arellano, Peter J. Balint-Kurti

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### 3.1. Abstract

The phenomenon of multiple disease resistance (MDR) could significantly increase plant breeding efficiency if it were better exploited. The objective of this research was to detect and quantify any MDR effects conferred by 12 small chromosomal regions (introgressions) originating in the MDR maize line NC250P that had been retained in NC292 and NC330, two inbred near-isogenic lines (NILs) derived by following an initial cross between NC250P and the multiple disease susceptible line B73 with three or four subsequent generations, respectively, of backcrosses to B73 and selection for resistance to southern corn leaf blight (SLB). Through B73x (NC292 x B73) and B73 x (NC330 x B73) crosses, we created a set of BC<sub>2</sub>F<sub>3</sub> NILs, each carrying 1-5 NC250P introgressions. 236 of these BC<sub>2</sub>F<sub>3</sub> NILs – along with B73, NC292, and NC330 – were rated in field trials for resistance to SLB, northern leaf blight, gray leaf spot, aflatoxin production by *Aspergillus flavus*, and Fusarium ear rot and fumonisin production by *Fusarium verticillioides*. Correlations for resistance to any given disease pair were low across the NIL population ( $-0.247 \leq r \leq 0.535$ ). However, a QTL in bin 3.04 had a significant effect on resistance for 4 diseases; and the QTLs in bins 2.06, 5.07-5.09, and 6.01 had significant effects on 3 diseases. The QTLs in bins 3.00-3.01,

9.01, 9.02-9.03, and 10.02 had significant effects on resistance for 2 diseases ( $P \leq 0.058$  for all significant effects). These findings both identify naturally occurring exploitable MDR loci and provide candidate genome regions for fine-mapping and cloning MDR genes.

### **3.2. Introduction**

Plant breeding represents a multi-billion dollar industry in the United States, and is likely to be increasingly important in the future with the increasing emphasis on the use of crops as energy sources (15). In areas of the world where the expense of high-input farming is not an option, plant breeding may be the only tool by which to prevent pests, harsh environmental conditions, and diseases from destroying the local food source (21). A potential means to improve breeding efficiency is the use of multiple disease resistance (MDR) genes, the phenomenon of a single gene conferring resistance to multiple pathogens. Few, if any, crops are affected by only one disease in a given agricultural region, but the introgression of multiple resistance genes or quantitative trait loci (QTL) to develop lines resistant to all of the pathogens threatening the yield of a crop can be a very lengthy, expensive process (16, 18). Furthermore, some studies suggest that each resistance gene added to a line comes with a decrease in fitness (7, 20, 28). This may be a disadvantage with the common strategy of pyramiding genes for resistance to multiple diseases. Both MDR genes and tightly linked clusters of single disease resistance genes conferring resistance to a set of different diseases (here termed MDR gene clusters) would facilitate the production of multiple disease resistant lines, and MDR genes would decrease the risk of trading disease resistance for subtle yield penalties.

Scattered examples exist of MDR genes, although most are indirect or not applicable to many breeding programs. The indirect examples include correlations between resistance ratings for multiple diseases across the lines of large populations (19) and co-localization of

resistance genes and QTL for different diseases (32, 33, 34). The correlation studies published are indirect because linkage disequilibrium (the degree to which genetic linkage across the genome has not been broken by recombination) limits their genetic resolution to large sections of a genome or even entire chromosomes. Obviously, unless resolution is at the single-gene level, resistance to multiple diseases cannot be attributed to a single gene with pleiotropic effects. Co-localization studies improve resolution to the level of much smaller chromosome fragments. They involve the consolidation of various resistance locus mapping studies to create a composite genetic map, allowing for identification of loci from resistance studies for different diseases that occupy – non-randomly – the same position on a chromosome. Regardless of their finer resolution, they are still indirect because they involve loci either not mapped in the same population (i.e., not in the same genetic background) or not tested by the same researchers. An unpublished association-mapping analysis has found correlations for MDR in a single mapping population with very low linkage disequilibrium - so low that mapping resolution with this population is estimated at the intra-gene level (Wisser, personal communication). Based on preliminary analyses, the results do indicate true single pleiotropic MDR genes. However, any genes identified in the final analysis would still not be direct examples of MDR. As in the co-localization studies, potential interference from other genes would need to be ruled out by creating and comparing near-isogenic lines (NILs) differing only by the suspected MDR genes (1).

The studies that do use NILs and show single-gene MDR in the same experiment almost all involve resistance mutations (6, 12, 14, 22). While these mutant studies have definitively identified pleiotropic MDR genes, there are three major obstacles to their usefulness to breeders. First, many of these mutants also have pleiotropic growth defects (22). For example, the increased resistance to both *Botrytis cinerea* and *Plectosphaerella cucumerina* conferred by the *Arabidopsis ocp3* mutant allele is coupled with deficiencies in growth and pigmentation (13). Second, along with growth defects, many mutations to

resistance against one disease increase susceptibility to other diseases (8, 22). Loss of function mutations in the Arabidopsis gene *ASI* lead to both increased resistance to *Botrytis cinerea* and increased susceptibility to *Pseudomonas syringae* pv. *tomato* and *Pseudomonas fluorescens* (22). Third, artificially induced mutations do not help us understand the mechanisms or identifiable features of naturally occurring MDR genes. Naturally occurring MDR genes would likely be more exploitable than artificially induced MDR mutations for a number of reasons, including: a) they would presumably be far more abundant than laboratory-generated mutations, thus being available in more crops, against more diseases, and as more variants capable of replacing defeated MDR genes and b) MDR genes identified in adapted germplasm should be far less likely to cause growth defects once deployed in commercial lines.

To our knowledge, there are very few published examples of naturally occurring MDR genes. The qualitative resistance gene *Mi-1* confers resistance to both aphids and nematodes in tomato (29). The quantitative but large effect resistance gene *Lr34/Yr18* confers resistance to leaf rust, stripe rust, powdery mildew and various other diseases of wheat (17). Another wheat stripe rust locus, *Lr46/Yr29*, also confers resistance to leaf rust (24). The wheat yellow rust gene *Yr30* has been linked to an undefined resistance locus for both leaf rust and yellow rust (31) and to the *Sr2* stem rust gene (4). However, *Sr2* and the leaf rust gene *Lr27* were also reported as linked, but later identified as separate genes (27). All of these genes have been widely exploited. We hypothesize that there exists a much larger number of MDR genes within naturally-occurring germplasm which can be exploited if they are identified and characterized.

An earlier project in our lab involved the creation of a set of 253 maize NILs derived by marker-assisted selection of F<sub>1</sub> progeny from a backcross between the recurrent parent B73 and two sister lines, NC292 and NC330 (35). NC292 and NC330 are themselves the products of recurrent backcrossing to B73 following an initial cross between B73 and the

MDR experimental line NC250P. NC250P is highly resistant to the necrotrophic fungal diseases southern corn leaf blight (SLB; causal agent *Cochliobolus heterostrophus*), northern leaf blight (NLB; causal agent *Exserohilum turcicum*), and gray leaf spot (GLS, causal agent *Cercospora zea-maydis*). Because NC250P has relatively poor overall agronomic traits, the F<sub>1</sub> progeny of each successive backcross to B73 (which has good agronomic traits) were selected for SLB resistance and general plant quality. NC292 and NC330 are inbred lines selfed from BC<sub>3</sub>F<sub>2</sub> and BC<sub>4</sub>F<sub>2</sub> plants, respectively (both lines share a BC<sub>3</sub>F<sub>1</sub> family) that were developed through the backcross program described. Therefore, although they are more than 90% genetically identical to the SLB-susceptible line B73, NC292 and NC330 are themselves highly resistant to SLB.

Zwonitzer et al (36) mapped the NC250P-derived chromosome segments (NC250P introgressions) to twelve total genomic regions (ten in each line), eight of which are at least in part shared between NC292 and NC330. Since these twelve NC250P introgressions represented the only genetic differences between NC292 or NC330 and B73, it was assumed that at least a portion of them must contain the genes conferring the difference in SLB resistance. To estimate the resistance effect (relative to B73) of each NC250P introgression, Zwonitzer (35) first backcrossed NC292 and NC330 to B73, next backcrossed the resulting BC<sub>1</sub>F<sub>1</sub> plants to B73, and then genotyped and selfed the resulting BC<sub>2</sub>F<sub>1</sub> plants. In the BC<sub>2</sub>F<sub>2</sub> generation, 2,246 lines were genotyped and selected for NC250P introgressions. From those 2,246 lines, 253 plants were selfed to produce a final collection of BC<sub>2</sub>F<sub>2:3</sub> NILs, each of which retains one to five of the twelve total NC250P introgressions present in NC292 and NC330. The NILs were tested in the field for SLB resistance, and the resistance or susceptibility effects of all twelve introgressions were estimated.

The objective of the current study was to use 236 of the original 253 NILs to investigate the MDR potential of the twelve NC250P introgressions. To do this, we rated subsets of the NILs for five necrotrophic fungal diseases of corn: SLB, NLB, and GLS (the

three diseases against which NC250P was rated as highly resistant), along with ear rot and aflatoxin production by *Aspergillus flavus* and ear rot and fumonisin production by *Fusarium verticillioides*. We report low correlations between diseases across the NILs, but significant MDR effects for the 3a, 3b, 6a, 9a, and 9b introgressions ( $P \leq 0.058$  for all significant effects).

### 3.3. Materials and Methods

#### 3.3.1. Plant materials

The 236 NILs used were planted as BC<sub>2</sub>F<sub>3</sub> or BC<sub>2</sub>F<sub>4</sub> seed for these experiments. They were derived by marker-assisted selection on the BC<sub>2</sub>F<sub>1</sub> and BC<sub>2</sub>F<sub>2</sub> progeny of B73x (NC292 x B73) and B73 x (NC330 x B73) crosses (36); also see Chapter 1, Fig. 3). B73 is an inbred line derived by recurrent selection of a randomly mated Iowa Stiff Stalk Synthetic line (25). It has good agronomic qualities, but is highly susceptible to a number of common maize diseases (2), including the five examined here. NC292 and NC330 are sister lines both derived by an initial NC250P (SLB resistant parent) x B73 cross followed by repeated backcrossing to B73 and selection for SLB resistance. NC292 was selfed from a BC<sub>3</sub>F<sub>2</sub> line. NC330 shared the same BC<sub>3</sub>F<sub>1</sub> family as NC292, but was selfed from a BC<sub>4</sub>F<sub>2</sub> line. NC250P is a highly SLB-resistant, yellow dent inbred line developed at NCSU from an initial (Nigeria Composite A-Rb x B37) x B37 cross. B37 is an inbred line from the same randomly mated Iowa Stiff Stalk Synthetic line as B73, although from an earlier selection cycle (Cycle 1 versus Cycle 5 for B73) (26). The average marker similarity between B37 and B73 is 0.176 (5).

#### 3.3.2. Field Trials

All field trials were planted as augmented alpha lattices designed using the software AlphaGen (Scottish Agricultural Services, Edinburgh, UK). Each complete replication for

the SLB, NLB and GLS trials consisted of 17 incomplete blocks with 15 entries each block (234 NILs used total), augmented with 1 plot per block of NC292 and B73. The SLB trial was planted over two summer field seasons (2007 and 2008) and two locations (Clayton, NC and Tifton, GA). In 2007, two replications of the experimental design were planted at each of the two SLB locations; one replication was planted in 2008, at the Clayton, NC location. Data from the 2007 replications was also incorporated in a previous research project (35). The NLB and GLS trials both included 2 replications at each of 2 locations (Clayton, NC and Aurora, NY for NLB and Andrews, NC and Blacksburg, VA for GLS). The fumonisin and aflatoxin trials were conducted in the same plots at a single location (Clayton, NC). They were planted as an augmented alpha design with 2 complete replications using 2 incomplete blocks with 20 entries per block (40 NILs used total) augmented with one plot each of NC292, NC330, B73, and the inbred line Mo17 (used as a supplemental standard) per block. One half of the plants in every plot were allocated to each toxin trial. The toxin trials shared the same field as the NLB trial Clayton, NC replications. The plants in the toxin trial were inoculated with NLB, although not scored for NLB symptoms.

Plots in the SLB, NLB, and ear rot trials were planted as single rows 2m long with 0.97m between rows and 0.6m alleys between ranges. GLS plots were planted to the same specifications as those in the other trials, but with 4m plot lengths. The rows were not thinned. Ten seeds were planted in each row for the SLB, NLB, and toxin trials. 15 were planted per row for the GLS trial. The Blacksburg, VA plots of the GLS trial were planted in a no-till, continuous corn cropping field, as is the majority of the field corn commercially grown in that area (30).

### 3.3.3. Fungal inoculation

The SLB and NLB trials were inoculated by placing ~15 grains of a sorghum culture of mixed *C. heterostrophus* or *E. turcicum* isolates into the leaf whorls of individual plants at

the 4-6 leaf stage. The Aurora, NY NLB plots were inoculated with a mixture of isolates. *E. turcicum* Race 1 constituted the majority of that mixture, but races 0 and 2,3 were all present in significant amounts. Preparation of cultures and inoculum for SLB and NLB inoculation were performed as published previously (9, 10, 11). Plants were inoculated with *F. verticillioides* by first injecting a suspension of fungal spores and mycelium down the silk channel of the top ear of each plant within 2 weeks after silking, simulating natural rain-borne inoculum. A week after the silk channel inoculation, the ears were puncture-injected with a similarly prepared inoculum solution, simulating natural insect-borne inoculum. *A. flavus* was applied to the ears by the same method as used for *F. verticillioides*, with the exception that the puncture injection immediately followed the silk channel injection on each ear. Preparation of cultures and spore suspensions were performed as published previously (23). Natural wind-borne inoculum was used to infect the GLS trials at both sites.

#### 3.3.4. Scoring

Values directly assigned or measured during the experiments for individual experimental units (plots, ears, etc.) are here referred to as "ratings", while those values calculated from ratings as individual NIL averages within and across diseases, locations, years, and replications (i.e., those values reported in the tables and figures) are referred to as "disease severity values". The ratings that were only taken once for each experimental unit (such as incubation period and toxin concentration) are therefore also disease severity values. All scores were based on whole-plot averages. Incubation period (IP), here taken as the number of days between inoculation and the appearance of necrotic spots within lesions on at least half the plants in a plot, was rated for the NLB trials. The date for most incubation periods fell between one and two weeks prior to anthesis. All other ratings were taken between anthesis and senescence. The NLB trials were also rated for diseased leaf area (DLA), the estimated percentage of necrotic area out of the total leaf area of a plant, at 5 dates in Clayton, NC and 3 in Aurora, NY. GLS and SLB were rated in increments of 0.5 on

a 1-9 semi-quantitative scale, with a 1 being a dead plant and a 9 being a plant free of obvious symptoms. SLB was rated on 5 dates in the summer, 2008 trial of this experiment. GLS was rated on 3 dates in Andrews, NC and 4 in Blacksburg, VA.

*F. verticillioides*- and *A. flavus*-inoculated ears were rated for toxin content as described previously (23). Each *F. verticillioides*-inoculated ear was also rated for ear rot, with 1-5 ears per plot. *A. flavus*-inoculated ears were not rated for ear rot in this experiment, as few ears had visible rot symptoms.

Days to anthesis (DTA) was also rated in the NLB and SLB plots, taken as the number of days post-planting at which at least half the plants in a plot were shedding pollen.

### 3.3.5. Statistical analyses

Disease severity was calculated as weighted mean disease (WMD) for all plots in the SLB, NLB, and GLS trials as described previously (3) and used as a dependent variable in the analyses of introgression effect estimates for these diseases. NIL least-squares means for each disease severity were calculated using SAS PROC MIXED (v.9.1; SAS Institute, Cary, NC) and compared across disease severity values with Pearson correlation coefficients obtained by PROC CORR. The full PROC MIXED models for the GLS and toxin trials included NIL identification number as a fixed effect and incomplete block as a random effect. The SLB and NLB models included NIL identification number and DTA as fixed effects along with location, incomplete block, and year (SLB only) as random effects.

The effect of an introgression was defined as the mean difference in disease expected with lines homozygous for NC250P alleles in that genomic region relative to lines homozygous for B73 alleles. ESTIMATE statements in PROC MIXED were used to obtain the “empirical” introgression effect estimates described by Zwonitzer (35).

The empirical effect of each introgression was obtained by either a) averaging the disease severity values (WMD or IP) for all single-introgression lines (single-introgression lines were available for introgressions 1c, 2a, 2b, 3a, 3b, 9a, and 10a) and subtracting the average disease severity value of B73 or b) when single-introgression lines were not available, averaging the differences of each class of double-introgression lines containing the introgression for which an effect was being estimated minus the single-introgression lines of the second introgression in said class. For example, the effect of introgression 6a would be estimated by first calculating the mean of all double-introgression lines in the 1c/6a class minus the mean of all 1c single-introgression lines, then averaging that value with the differences calculated for all other 6a double-introgression line classes.

The empirical estimates for the 12 NC250P introgressions were calculated using a maximum combined total of 39 single- and double-introgression NILs per disease. It is inherent to the alpha design used for these trials that every  $BC_2F_{3,4}$  NIL be represented only once per repetition of the experiment. This led to the issue of each empirical estimate being calculated from five or fewer observations per single- or double-introgression NIL used to obtain that estimate. For example, most of the aflatoxin and fumonisin empirical effect estimates are based on one or two observations. A second type of introgression effect analysis that included a large number of observations per estimate was used to corroborate the empirical analysis results: the “minimal-model” estimates. The empirical estimates for each disease were obtained using a single model that included NIL identification number as an independent variable. In contrast, the minimal-model estimates for each disease were obtained using a separate model for each introgression that directly incorporated an independent variable for that introgression with three class levels: NC250P allele-homozygous, heterozygous, or B73 allele-homozygous. Variables for up to two auxiliary introgressions were included in some models, but no additional variables accounting for plant genotype (e.g., NIL identification number) were used.

The number of introgressions that could be included in the minimal-models was limited by the number of missing values in the genetic marker data. Observations that include missing values for any of the variables contained in a MODEL or CLASS statement in PROC MIXED will be rejected from the analysis by SAS. The marker data used in these analyses contained missing values at one or more introgressions for 206 of the 236 NILs in the experiments. The combined missing values were sufficient to prevent, in general, more than three introgressions from being included as fixed-effect variables if a representative minimum number of observations were to be retained. For this effect estimation method, “representative number” refers to a quantity of observations large enough to account for variability due to both field effects and the effects of introgressions not included in a given model. The observations for the 28 NILs with complete marker data were too few to account for field effects. Therefore, it was necessary to include observations from NILs with incomplete marker data, which then required multiple models that each excluded variables for some introgressions. If the excluded introgressions were biased in their distribution throughout the NILs, they could then bias the data without the models being able to account for this skew when generating the analysis results.

For example: introgression 2a, although determined by Zwonitzer (35) to have no significant effect on SLB resistance, is found in both of the resistant parent NILs NC292 and NC330 and is, of course, absent in the susceptible NIL B73. Because NC292 and B73 are checks in the alpha lattice design, they represent 2 of the 17 NILs in each incomplete block (12% of the total possible observations). Even if, among the remaining NILs, all introgressions were distributed in a perfectly homogenous manner (which would include 2a being included in exactly 50% of the  $BC_2F_{3,4}$  NILs), then 2a could still be falsely estimated as conferring a significant SLB resistance effect due to the 12% of the 2a NILs represented by the highly resistant NC292 and the 12% of the 2a (-) NILs (those allelic for B73 in the 2a region) represented by the highly susceptible B73. Although, for this very reason, the parent

NIL observations were not included in the minimal-model analysis, this example nevertheless illustrates the potential for estimate inaccuracy due to biased introgression distributions.

However, as the number of observations used increases, the expected introgression distribution bias decreases. Essentially, the effects of the excluded introgressions create a large experimental error that necessitates a large sample size if an accurate estimate of the mean is desired (see Results section for further explanation). For the minimal-model analysis, a minimum of 75% of the original observations was generally imposed when estimating each introgression effect.

The genotype data incorporated into the analysis was taken from the same previous experiment as the summer 2007 SLB resistance data (35).

### **3.4. Results**

#### *3.4.1. Correlations between diseases for genetic effect of NILs*

NIL least-squares means for SLB severity from the 2008 data significantly correlated ( $r=0.686$ ,  $P<0.0001$ ) with those previously reported by Zwonitzer (35). Correlations between NIL least-squares means for all seven disease severity value classes (SLB, NLB-WMD, NLB-IP, GLS, aflatoxin, fumonisin, and fusarium ear rot) in 2008 were low (Table 1). The range of  $r$  was -0.247 to 0.535; the mean and median for  $r$  were 0.123 and 0.116, respectively. Only 8 of the 21 correlations coefficients given were significant ( $P\leq 0.10$ ), although all of the significant correlations denoted a positive relationship for resistance between diseases for each pair. Because disease resistance in these experiments is defined by lower WMD, rot, or toxin concentration but higher IP values, NLB-IP is expressed as “disease severity” (the opposite of the original IP values) in all results reported here.

### 3.4.2. Introgression effect estimates

DTA was found to be a significant source of variation and included as a random effect in the final models for SLB and NLB. With SLB and NLB, as with many necrotrophic pathogens, infection is more severe as the plants mature and approach senescence. DTA is a means of rating plant maturity, and is often used as a covariate to separate the effects of plant maturation rate from the host factors affecting pathogen success independently of other agronomic traits (3).

Of the twelve NC250P introgressions present in the NILs, eight were identified as MDR loci using the empirical estimation method (Fig. 1). Of the eight, only 9b had directionally consistent effects (i.e., conferred only resistance or only susceptibility against all of the diseases on which that introgression had an effect), conferring only resistance to both SLB and GLS. The NC250P alleles at 2b provided significant susceptibility to SLB but significant resistance to NLB-IP and fusarium ear rot. The NC250P alleles at both the 3b and 9a introgressions provided resistance to SLB but susceptibility to GLS, and 3b also provided resistance to aflatoxin production but susceptibility to fusarium ear rot. The NC250P alleles at introgression 3a provided resistance to SLB but susceptibility to NLB-WMD. The NC250P alleles at introgression 6a had an effect against all three foliar diseases - conferring resistance to both SLB and NLB-WMD but susceptibility to GLS – but did not have a significant effect on any of the ear infections. The NC250P alleles at introgression 5a provided significant resistance against both SLB and fusarium ear rot, but contributed to increased production of aflatoxin. Finally, the NC250P alleles at introgression 10a provided both one of the largest effects on resistance to GLS but also one of the largest effects on susceptibility to fusarium ear rot (Fig. 1).

Of the remaining four introgressions, two were effective against a single disease. The NC250P alleles at the 2b and 5a introgressions conferred resistance and susceptibility to

NLB-WMD, respectively (Fig. 1). Introgressions 1a and 2a were not found to contribute significantly to any of the five diseases (seven disease ratings). Empirical effect estimates could not be obtained for introgressions 1a and 1b regarding the ear rot diseases (aflatoxin production by *A. flavus* and fumonisin production and ear rot by *F. verticillioides*). Single-introgression lines were not available for 1a and 1b, nor were there appropriate combinations of single- and double-introgression lines available from which to estimate individual introgression effects (see materials and methods). Minimal-model effects estimates were obtained for both introgressions 1a and 1b against the three ear rot disease ratings, and none of these estimates was significant ( $P \geq 0.118$ ; Fig. 2).

The introgression effects estimated by the empirical and minimal-model analyses were significantly correlated when combined across all disease ratings ( $r=0.310$ ,  $P=0.006$ ; Table 2). When only significant effects were included ( $P \leq 0.10$ ), the Pearson correlation coefficient for the two methods was  $r=0.701$  ( $P < 0.001$ ). Within disease ratings,  $r$  ranged from -0.267 to 0.917, with a mean and median of 0.388 and 0.335, respectively. Only the estimates for SLB, NLB-WMD, and fusarium ear rot were significantly correlated ( $P \leq 0.034$  for all significant,  $P \geq 0.167$  for all non-significant).

Of the 23 empirically estimated significant introgression effects ( $P \leq 0.058$ ), 14 were corroborated in significance and direction by the minimal-model estimates (Fig. 2). The exceptions were the effects of 2b on NLB-IP, 3a on SLB, 3b on aflatoxin production and fusarium ear rot, 5a on SLB and aflatoxin production, 6a on GLS, 9a on GLS, and 10a on GLS. Of these nine non-corroborative minimal-model estimates, six were not significant: the effects listed for 2b, 3b, 5a (SLB only), 6a, and 10a. Of the six non-significant effects, only the effects of 2b on NLB-IP and 6a on GLS were opposite in direction for the minimal-model estimates compared to the empirical estimates, and the minimal-model estimates for both of these effects were quite small (-1.53%,  $P=0.248$  and -0.29%,  $P=0.811$ , respectively). The minimal-model effects estimated for 3a (SLB only), 5a (aflatoxin production only) and 9a

were significant but opposite in direction (minimal-model estimates of SLB susceptibility conferred by 3a, decrease in aflatoxin by 5a, and GLS resistance by 9a) from the empirical estimates. The empirical effect analysis detected a marginally significant decrease in aflatoxin production conferred by introgression 3a ( $P=0.097$ ; Fig. 1). The minimal-model analysis did estimate a reduction in aflatoxin due to 3a, although it was not significant ( $P=0.289$ ; Fig. 2).

The “effects” (least-squares mean disease relative to B73) of the three parental lines (B73, NC292, and NC330), the unrelated inbred line Mo17 (only as a standard line for the ear rot disease trials), and, for NC292 and NC330, the sum of all significant effects of the introgressions found in each inbred line (Figures 3 and 4). NC292 and NC330 were found to be significantly resistant in all of the foliar disease ratings ( $P\leq 0.001$ ; Figures 5, 6, and 7), and significantly susceptible to fusarium ear rot ( $P\leq 0.027$ ).

### **3.5. Discussion**

The low inter-disease correlations for the NILs themselves could be regarded as evidence against the loci identified as MDR in the introgression effects analysis. Except, when one considers the genetic structure across the population of the phenotypes measured, two alternative explanations become clear. First, the disease correlations are constructed from the means for lines, and line means are the combined effects of all introgressions within those lines. Even in the presence of strong MDR genes, multiple introgressions with resistance effects that vary in strength and direction for different diseases can lead to low correlations between lines. For example, a line may have hypothetical introgressions *X* and *Y*, where *X* confers resistance to both SLB and NLB and *Y* confers resistance to SLB but susceptibility to NLB. A line with both introgressions *X* and *Y* would then be relatively resistant to SLB but potentially have a neutral rating (equal to B73) for NLB. Therefore, while single-introgression lines for *X* and *Y* could have strong correlations between SLB and

NLB experiments, double-introgression lines with both *X* and *Y* would have weaker correlations or none at all, depending on the relative strengths of the individual effects. The estimated introgression effects from SLB, NLB, and GLS trials do indeed vary in strength and direction across diseases. This would suggest that low inter-disease correlations for the NILs could actually be the product of MDR loci versus evidence against them.

The second explanation for the low correlations between lines is that, while MDR loci may be present, so may single-disease resistance loci. If these single disease loci were present in greater strength or quantity, then their effects could overshadow those of the MDR loci and lead to low correlations between lines.

Two potentially sounder arguments against the presence of MDR introgressions in these lines would be 1) the disagreements between the empirical and minimal-model introgression effect estimates with the MDR loci 3a, 5a, 9b, and 10a and 2) the disparity between the actual resistance levels of NC292 and NC330 and the sum of the estimated effects for the introgressions that they contain. These arguments are not against the multiple disease nature per se of the six MDR introgressions identified, but against the accuracy of the methods used to estimate individual resistance effects. While having the validity of our analysis undermined would not negate the presence of MDR loci in these NILs, it would certainly negate our reported identification of them. However, for both of the apparent inconsistencies described there are likely explanations in support of the introgression effect estimates presented.

Either difference could be accounted for by two simple factors: scoring method and epistasis. Both scoring method and epistasis are associated with non-additivity, the failure of the combined effect of multiple genes present in the same plant to equal the sum of the effects of each gene when present individually. With regard to scoring method, while SLB and GLS scores using the 1-9 point scale are generally highly correlated with purely

quantitative disease scoring methods - generally, percent necrotic diseased leaf area, or DLA (unpublished results) - the 1-9 scale still remains a qualitative ordinal assessment of disease. In other words, the true quantitative difference in disease between a WMD=5 plant and a WMD=4 plant is not necessarily equal to the difference between a WMD=6 plant and a WMD=5 plant, although they are likely to be quite similar. Moreover, even while the quantitative difference in symptoms between a DLA=50% plant and a DLA=40% plant is indeed equal to the difference in symptoms between a DLA=60% plant and a DLA=50% plant, the differences in the biological effects of resistance that generated these symptomatic differences are not necessarily equal in quantity or even adaptable to quantitative definition by the additive models used for this research.

Other non-additive effects can be explained by epistasis. Epistasis is by definition any non-additivity caused by interactions between genes and gene pathways. It can be due to a number of factors. For instance, if hypothetical introgressions  $M$  and  $N$  each included a loss-of-function allele for genes necessary to pathway  $P$ , where  $P$  accounted for 20% of the average WMD for B73, then  $MN$  double-introgression line means would be equal to  $M$  and  $N$  single-introgression line means: 20% lower than B73. Epistasis can also be due to highly complex genetic background effects, either with regard to gene regulation or the biological factors that alter phenotype expression. Biological factors are especially relevant with disease. Just as the density of inoculum can influence how well a given resistance gene performs, so the inhibition of pathogen growth by one resistance introgression could modify the infection court and thus theoretically enable the effect of a second resistance introgression to be amplified, redundant, or even reversed in its effect (become a susceptibility introgression) to pathogen growth.

Both the empirical and minimal-model analyses assume no epistatic effects. The empirical estimates based solely on single-introgression lines would not be influenced by epistatic effects. The empirical estimates based on both single and double-introgression

effects could be influenced by epistasis. The minimal-model estimates, if epistasis is present, are all influenced by complex networks of epistatic effects.

The differences between the empirical and minimal-model estimates can also be explained by other inherent limitations of each method. With the empirical estimates models, the estimates for non-genotypic effects (DTA, location, field effects, etc) are based on observations from all of the NILs. However, the empirical estimates, although based on genotypic data without missing values (NIL identification number), were calculated using only the single- and double-introgression NILs (39 or 10 total for the foliar and ear rot diseases, respectively). This occasionally involved estimates based on a very limited number of observations. For example, only one 1c single-introgression line was available. The empirical effect estimates for 1c are therefore each based on five or fewer non-parental NIL observations.

While all minimal-model estimates are based on large sample sizes, their accuracy is restricted by the small portion of genotypic effects that they can account for. Because the minimal-models can only include variables for three or fewer introgressions during the calculation of each introgression effect estimate (see Materials and Methods), the accuracy of each estimate is directly related to the degree to which the significant introgressions excluded from that model were by chance balanced in influence among the observations (i.e., are homogeneously distributed between NILs). This assumption is impossible to meet by the introgressions not shared between NC292 and NC330. For example: both introgressions 3b and 9a were found to have a significant susceptibility effect for GLS, and neither introgression is found in NC330. Of the original 253 NILs, 101(40%) originated with NC330 x B73 crosses. Correspondingly, then, at least 40% of the NILs without the 3b introgression are derived from NC330, and therefore also do not have the GLS susceptibility introgression 9a. This non-random association between 3b and 9a would skew the calculated effect for 3b (which is defined in the minimal-model analysis as the difference between lines with and

without 3b) towards resistance. If the minimal-model for 3b does not include 9a, then that model cannot compensate for this skew. The effects of 3b and 9a are inherently linked because both introgressions are NC292-specific. However, any pair of the eight introgressions shared between NC292 and NC330 could have randomly become associated during the selection of the NILs. If two introgressions had a strong enough association and significant effects on resistance, then the minimal-model effect estimates for both introgressions would be skewed.

Whereas the differences between the empirical and minimal-model estimates are mostly trivial, the second apparent inconsistency within the results - the disparity between the actual resistance levels of NC292 and NC330 and the respective sums of the estimated effects for the introgressions that they contain - is quite drastic (Figures 3 and 4). Because the minimal-model and empirical analyses use divergent methods and groups of observations, the convergence of their results suggests that the effects estimated are both real and accurate. Given that the introgression effect estimates are approximately correct, the discrepancies mentioned above between NC292 and NC330 versus the summed effects of their respective introgressions would easily be explained by epistasis. If both accurate estimates and significant epistatic effects are assumed, then (because the minimal-model estimates are more vulnerable to bias from epistasis) the sums of the minimal-model estimates for the introgressions in NC292 and NC330 would be expected to more closely approximate the actual resistance levels in NC292 and NC330 than the sums of the empirical estimates. This is in fact the case (Figures 3 and 4). Therefore, it seems quite certain that the empirical introgression effect estimates reported have indeed accurately identified six new MDR loci.

The most important question that remains to be answered then is whether these MDR introgressions are the result of pleiotropic MDR genes or the product of multiple single-disease resistance genes for different diseases that are sharing introgressions. Two of the MDR loci - 3b and 6a - are currently the focus of fine-mapping and cloning projects in our

research group. The results of these projects will enable us to determine how closely linked the MDR traits are for these introgression. Before the remaining four introgressions – 2b, 3a, 9a, and 9b – can be considered for cloning, we will need to resolve possible single disease resistance gene clusters by either a) genotyping the NILs containing them at a higher marker density and reanalyzing the current phenotypic data or b) creating further B73-backcross progeny and testing them for segregation of MDR traits. It will also be of interest to further phenotype these alleles, possibly through histological and gene expression studies similar to those previously undertaken by our group for the 3b and 6a loci (see Chapter 2). Regardless, the identification of these loci will be of great value to improving our understanding and exploitation of the phenomenon of MDR.

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**Table 3.1. Correlations between NIL least-squares means estimates for each disease rating.**

<sup>a</sup> Pearson correlation coefficient

<sup>b</sup> 2-tailed significance

<sup>c</sup> NS, non-significant at  $\alpha=0.10$

Disease Rating	Statistic	Disease Rating						
		SLB	NLB-WMD	NLB-IP	GLS	Log (Aflatoxin)	Fumonisin	Fusarium ear rot
SLB	$r^a$	1.000	0.227	0.104	0.116	0.078	-0.247	-0.161
	$P^b$	...	<0.001	0.101	0.079	NS <sup>c</sup>	NS	NS
NLB-WMD	$r$	...	1.000	0.436	0.366	-0.075	0.098	-0.132
	$P$	...	...	<0.001	<0.001	NS	NS	NS
NLB-IP	$r$	...	...	1.000	0.303	-0.105	0.175	0.067
	$P$	...	...	...	<0.001	NS	NS	NS
GLS	$r$	...	...	...	1.000	-0.090	0.171	0.216
	$P$	...	...	...	...	NS	NS	NS
Log(Aflatoxin)	$r$	...	...	...	...	1.000	0.236	0.274
	$P$	...	...	...	...	...	NS	0.076
Fumonisin	$r$	...	...	...	...	...	1.000	0.535
	$P$	...	...	...	...	...	...	<0.001
Fusarium ear rot	$r$	...	...	...	...	...	...	1.000
	$P$	...	...	...	...	...	...	...

**Table 3.2. Correlations between NC250P introgression effect estimates obtained by empirical versus minimal-model analyses.**

<sup>a</sup>Correlation between empirical and minimal-model introgression effects estimates across all disease ratings

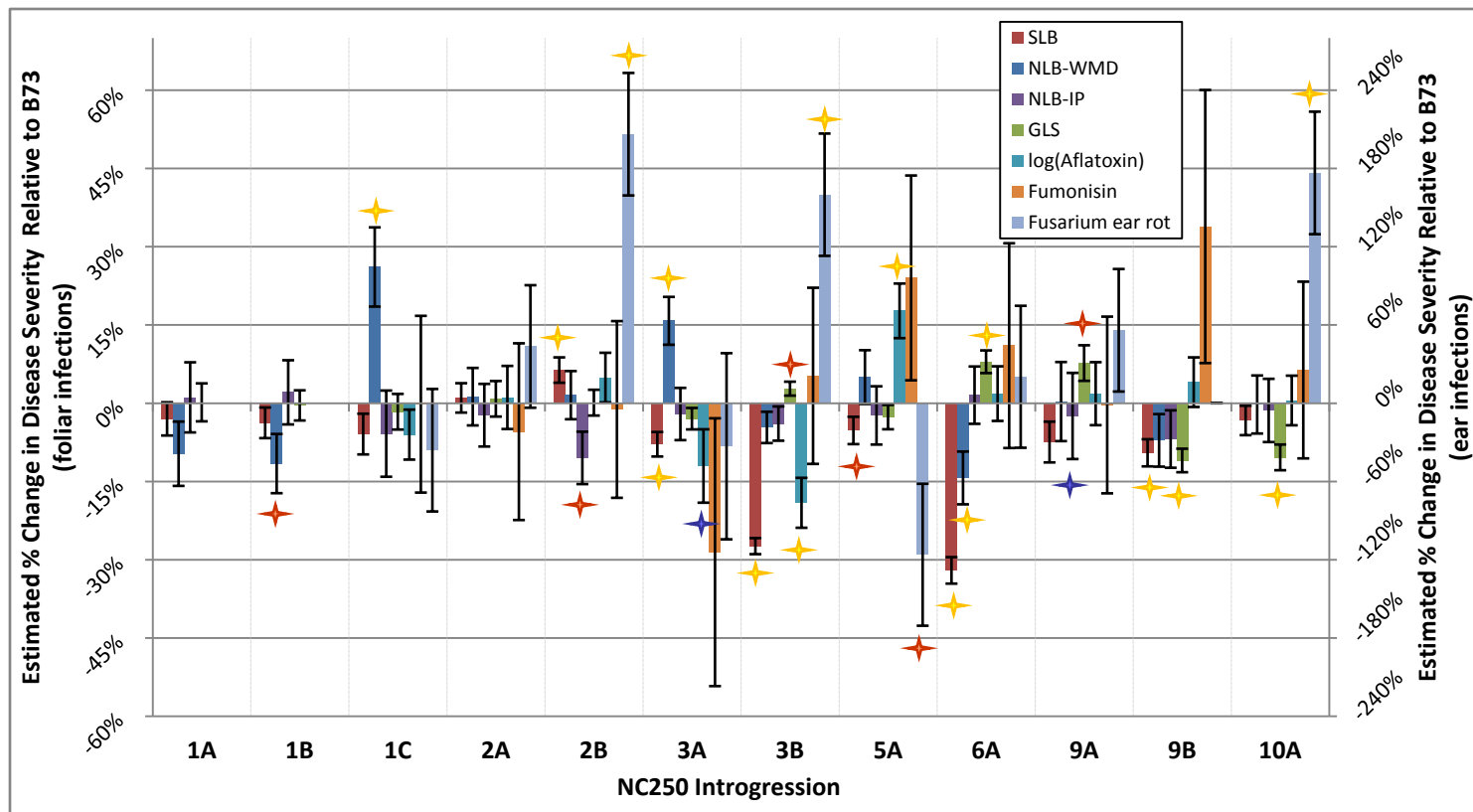
<sup>a</sup>Correlation between significant ( $P \leq 0.10$ ) empirical and minimal-model introgression effects estimates across all disease ratings

<sup>c</sup>Pearson correlation coefficient

<sup>d</sup>2-tailed significance

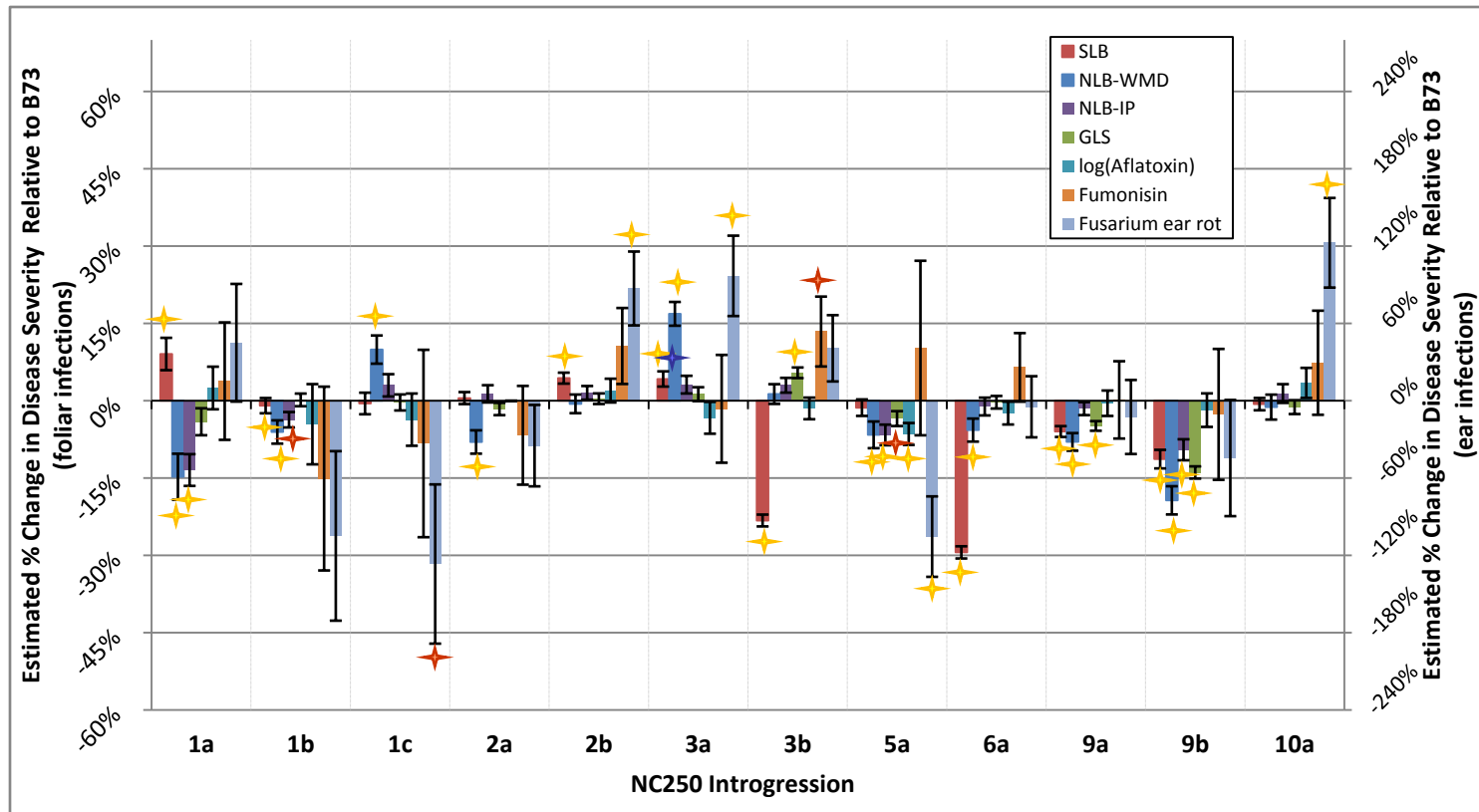
<sup>e</sup>NS, non-significant at  $\alpha=0.10$

Statistic	Disease Rating								Significant Combined <sup>b</sup>
	SLB	NLB-WMD	NLB-IP	GLS	Log(aflatoxin)	Fumonisin	Fusarium ear rot	Combined <sup>a</sup>	
<i>r<sup>c</sup></i>	0.917	0.730	-0.267	0.426	-0.036	0.244	0.705	0.310	0.701
<i>P<sup>d</sup></i>	<0.001	0.007	NS <sup>e</sup>	NS	NS	NS	0.034	0.006	<0.001



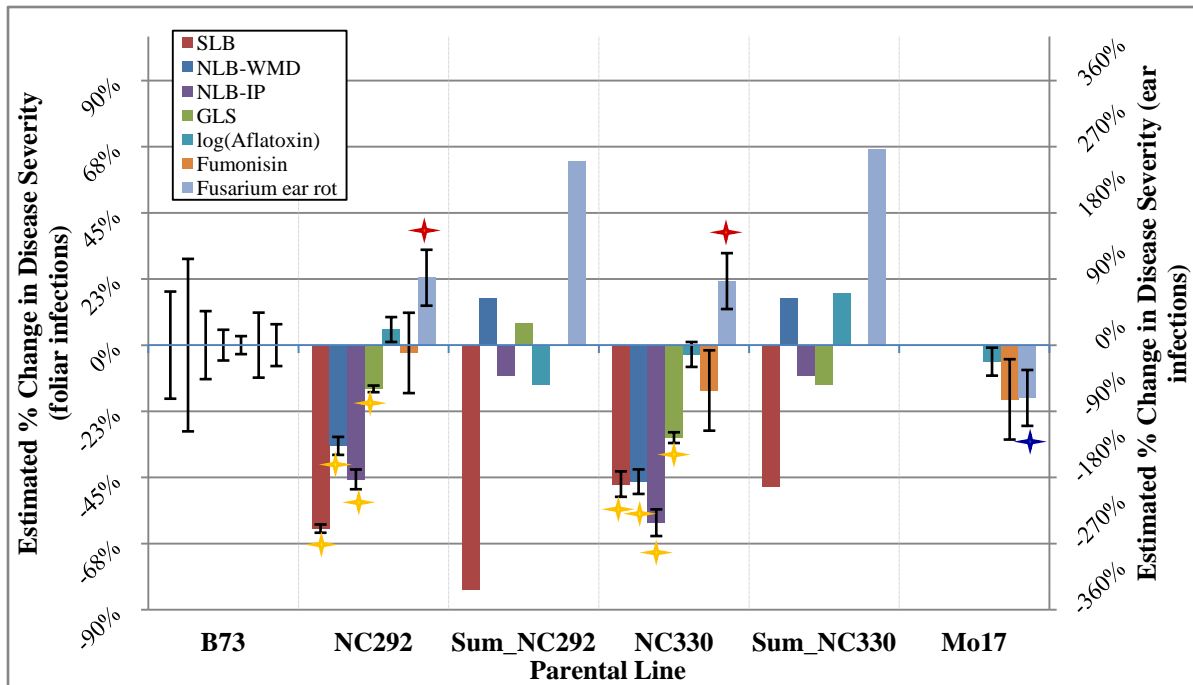
**Figure 3.1. “Empirical” estimates of introgression effects against all seven disease ratings investigated for the 12 NC250P introgressions that differentiate the NILs.**

The introgression effects for each disease were normalized as the percentage increase or decrease that each introgression conferred relative to the least-squares mean disease rating of the susceptible parent line B73. The left-hand vertical axis applies to estimates for effects against the four foliar disease ratings: SLB, NLB-WMD, NLB-IP, and GLS. NLB-IP refers to the incubation period, the number of days between inoculation and the first disease symptoms for NLB. All other foliar disease ratings were weighted mean disease (WMD) values. The right-hand vertical axis applies to effect estimates against the three ear rot disease ratings: log(Aflatoxin), Fumonisin, and fusarium ear rot. Colored stars above or below columns indicate statistically significant estimates. Blue stars indicate significance at  $\alpha=0.10$ , red stars at  $\alpha=0.05$ , and yellow stars at  $\alpha=0.01$ .



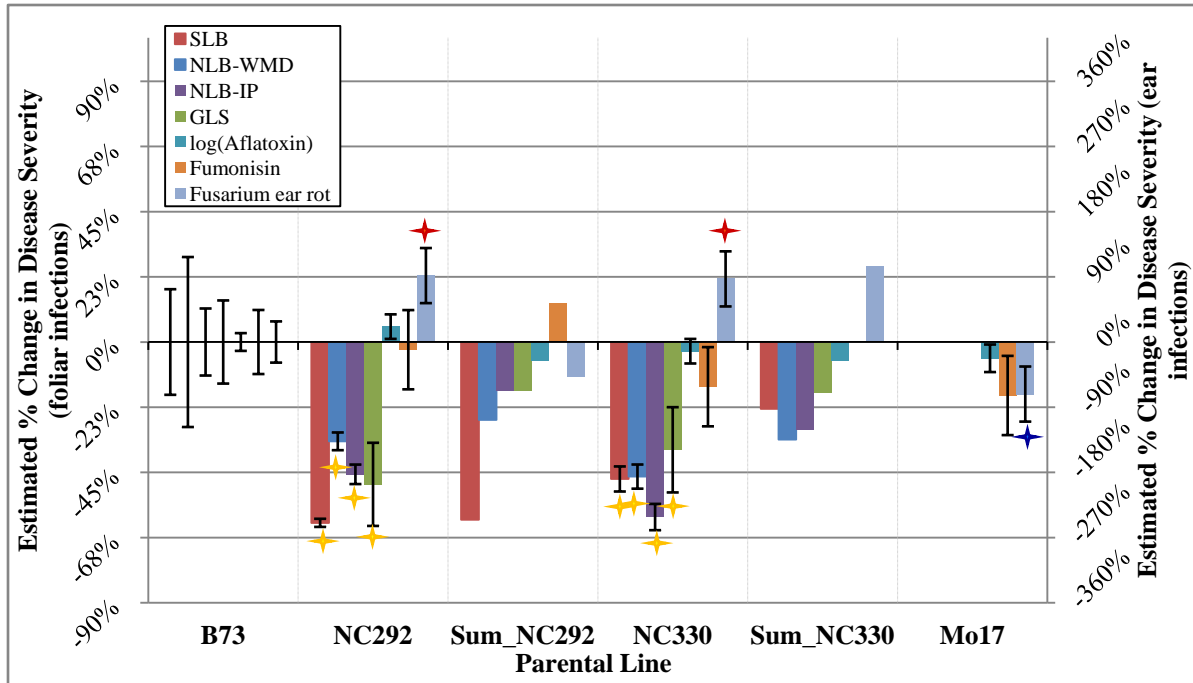
**Figure 3.2. “Minimal-model” estimates of introgression effects against all seven disease ratings investigated for the 12 NC250P introgressions that differentiate the NILs.**

The introgression effects for each disease were normalized as the percentage increase or decrease that each introgression conferred relative to the least-squares mean disease rating of the susceptible parent line B73. The left-hand vertical axis applies to estimates for effects against the four foliar disease ratings: SLB, NLB-WMD, NLB-IP, and GLS. NLB-IP refers to the incubation period, the number of days between inoculation and the first disease symptoms for NLB. All other foliar disease ratings were weighted mean disease (WMD) values. The right-hand vertical axis applies to effect estimates against the three ear rot disease ratings: log(Aflatoxin), Fumonisin, and fusarium ear rot. Colored stars above or below columns indicate statistically significant estimates. Blue stars indicate significance at  $\alpha=0.10$ , red stars at  $\alpha=0.05$ , and yellow stars at  $\alpha=0.01$ .



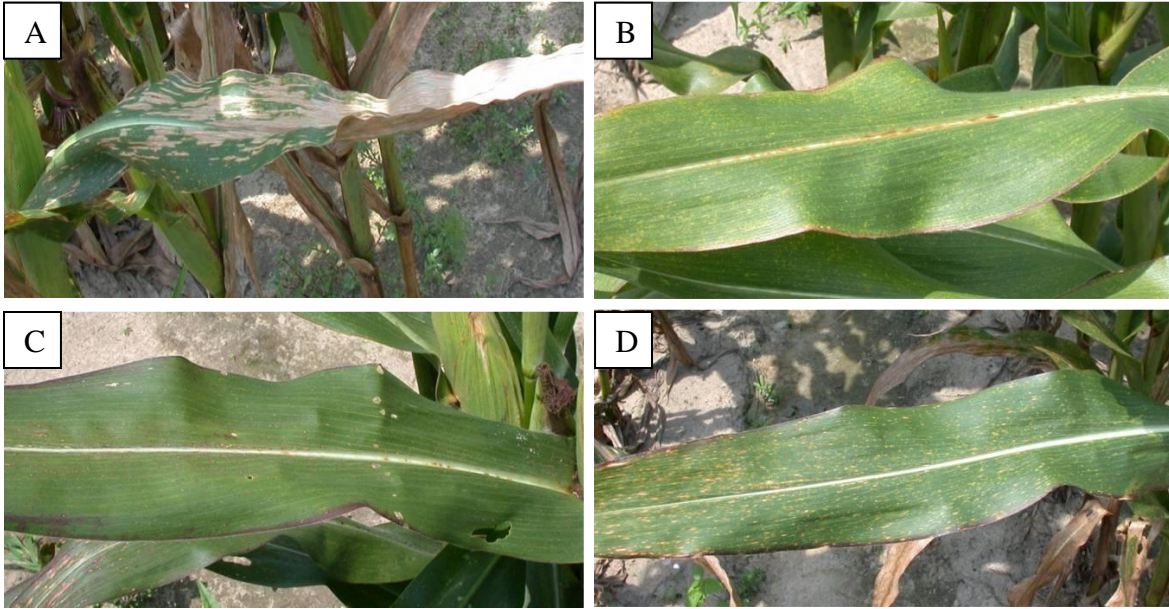
**Figure 3.3. “Empirical” estimates of inbred line effects against all seven disease ratings investigated for the NILs, the parent lines (B73, NC292, and NC330), and an unrelated ear rot standard inbred line (Mo17).**

“Sum\_NC292” and “Sum\_NC330” are the sums of all introgressions with significant effects present in NC292 and NC330, respectively. The introgression effects for each disease were normalized as the percentage increase or decrease that each introgression conferred relative to the least-squares mean disease rating of the susceptible parent line B73. The left-hand vertical axis applies to estimates for effects against the four foliar disease ratings: SLB, NLB-WMD, NLB-IP, and GLS. NLB-IP refers to the incubation period, the number of days between inoculation and the first disease symptoms for NLB. All other foliar disease ratings were weighted mean disease (WMD) values. The right-hand vertical axis applies to effect estimates against the three ear rot disease ratings: log(Aflatoxin), Fumonisin, and fusarium ear rot. Colored stars above or below columns indicate statistically significant estimates. Blue stars indicate significance at  $\alpha=0.10$ , red stars at  $\alpha=0.05$ , and yellow stars at  $\alpha=0.01$ .



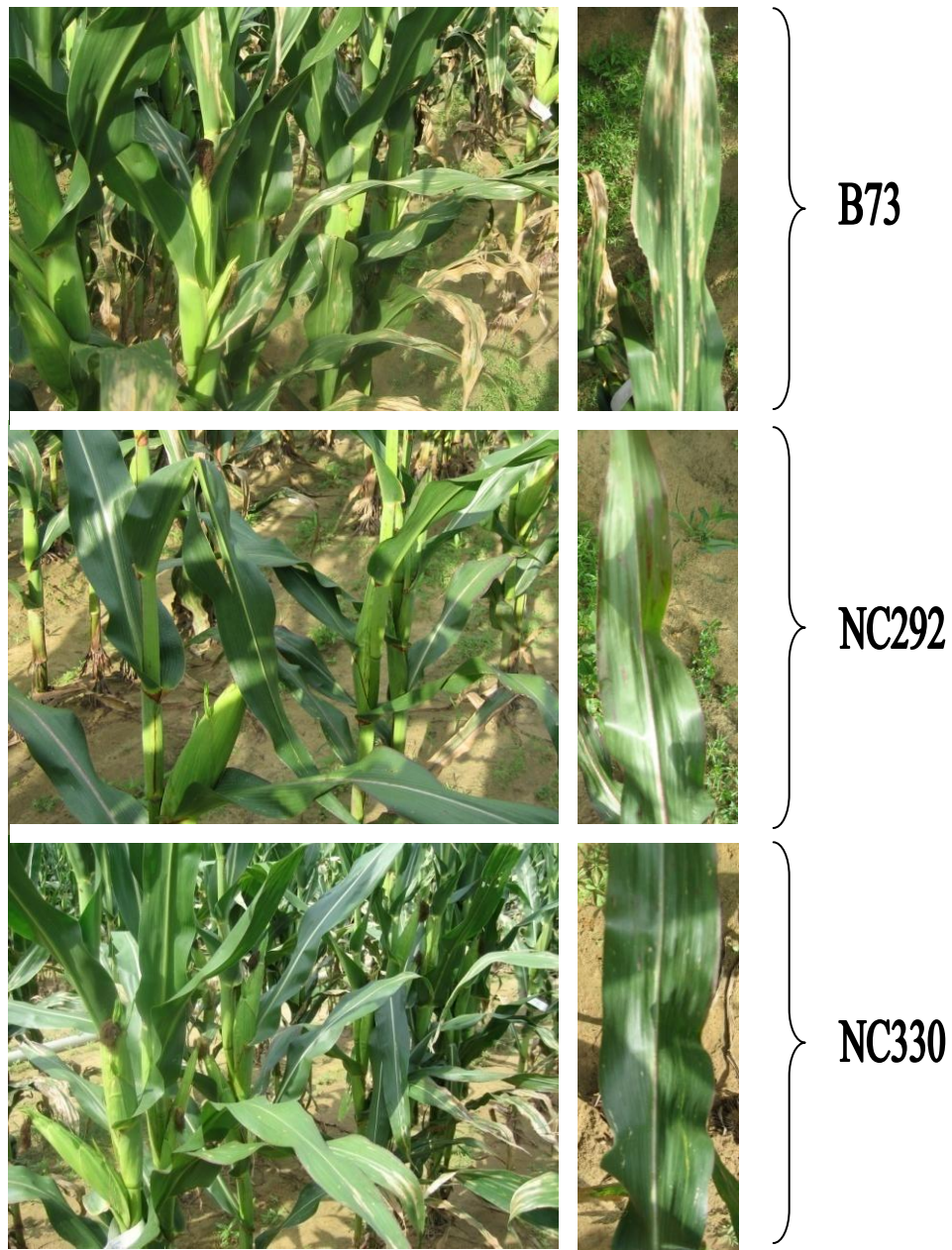
**Figure 3.4.** “Minimal-model” estimates of the “Sum\_NC292” and “Sum\_NC330” effects against all seven disease ratings investigated for the NILs, the parent lines (B73, NC292, and NC330), and an unrelated ear rot standard inbred line (Mo17).

Sum\_NC292 and Sum\_NC330 are the sums of all introgressions with significant effects present in NC292 and NC330, respectively. Because line name identity is not included as an effect in the minimal-model analysis, all effects listed for true inbred lines are actually empirical estimates (see Fig. 3). The left-hand vertical axis applies to estimates for effects against the four foliar disease ratings: SLB, NLB-WMD, NLB-IP, and GLS. NLB-IP refers to the incubation period, the number of days between inoculation and the first disease symptoms for NLB. All other foliar disease ratings were weighted mean disease (WMD) values. The right-hand vertical axis applies to effect estimates against the three ear rot disease ratings: log(Aflatoxin), Fumonisin, and fusarium ear rot. Colored stars above or below columns indicate statistically significant estimates. Blue stars indicate significance at  $\alpha=0.10$ , red stars at  $\alpha=0.05$ , and yellow stars at  $\alpha=0.01$ .



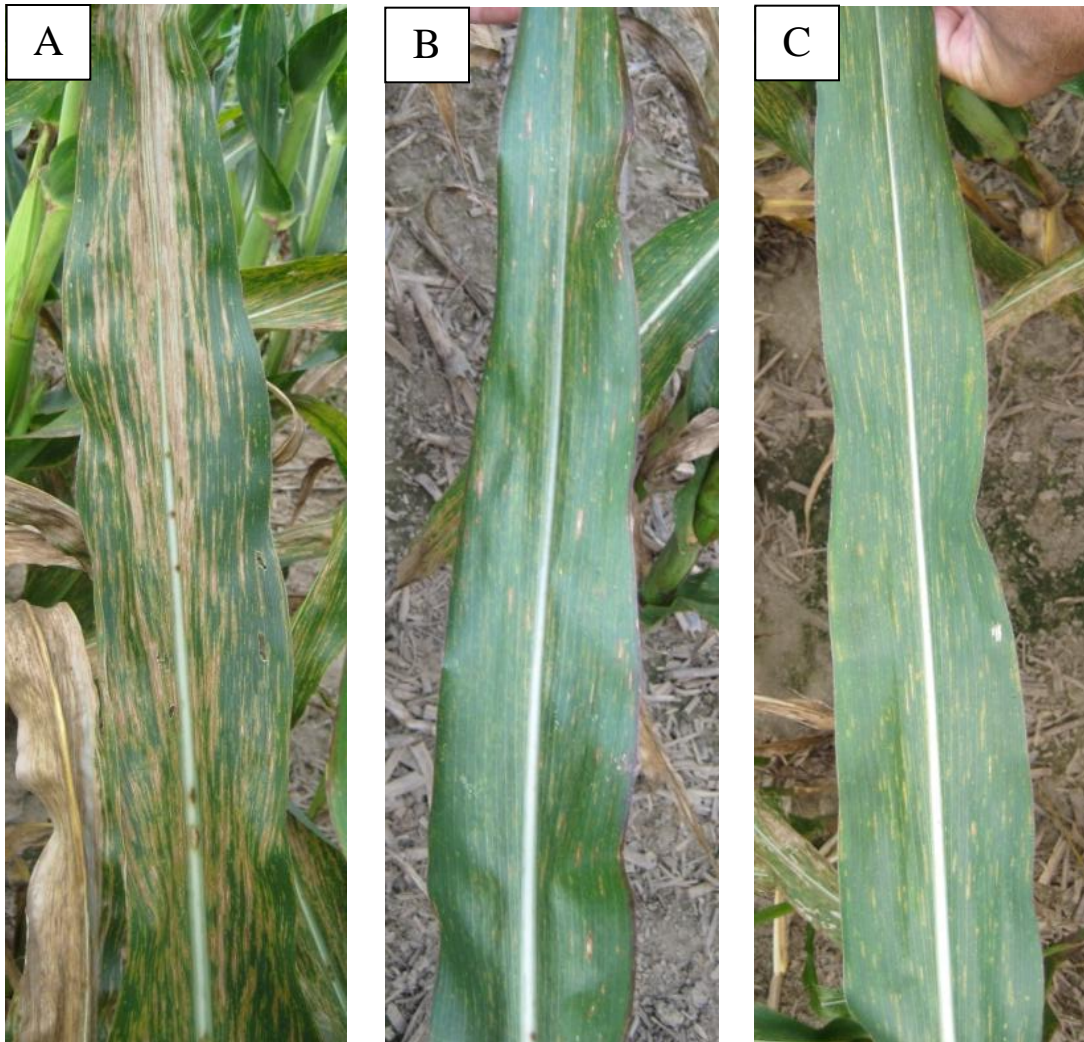
**Figure 3.5. SLB-infected ear leaves of the NIL inbred parent lines B73, NC292, and NC330 along with the inbred NIL NC250.**

NC250 (B) is derived from and almost completely allelic to NC250P, the line from which all of the introgressions investigated in the NILs were derived. NC292 (C) and NC330 (D) are the result of an initial NC250P x B73 (A) cross followed by recurrent backcrossing to B73 and selection for SLB resistance (presumably derived from resistant parent NC250P).



**Figure 3.6. NLB-infected stands and ear leaves of the NIL inbred parent lines B73, NC292, and NC330.**

The images above represent the three parent lines (B73, NC292, and NC330) and were taken at Clayton on July 25, 53 days after inoculation. The single-leaf photographs are of ear leaves. Of the three parents, B73 was significantly more susceptible than the SLB-resistant sister NILs NC292 and NC330 ( $p < 0.001$ ).



**Figure 1.7. GLS-infected ear leaves of the NIL inbred parent lines B73, NC292, and NC330.**

The photographs above represent ear leaves were taken at Andrews on August 7, approximately one week before plants began to senesce, from each of the three parent lines: B73 (A), NC292 (B), and NC330 (C). Of the three parents, B73 was significantly more susceptible than the SLB-resistant sister NILs NC292 and NC330 ( $p < 0.001$ ).