ABSTRACT

BASTOS MARTINS MARINO, LAIS. Validation of Multiple Disease Resistance QTL from Chromosome Segment Substitution Population in $F_{2:3}$ Family Populations. (Under the direction of Dr. James Holland).

Maize is a staple food in many countries. When not used directly in human consumption, is an essential component of animals' feed. Diseases are an important factor that can reduce yield or make crops not suitable for human or animal consumption, so managing them is a key aspect of modern agriculture. One way that presents less environmental risk and lower cost for the farmer is through genetic mechanisms for disease resistance. The first chapter of this thesis is a literature review that gives an overview of disease resistance, multiple disease resistance (MDR) concepts and explains quantitative trait loci (QTL) mapping tools. The chapter also characterizes the three foliar diseases of maize (*Zea mays* L. ssp. *mays*): southern leaf blight (SLB), northern leaf blight (NLB) and gray leaf spot (GLS).

The second chapter consists of a study that performed an independent test using a $F_{2:3}$ population of the putative MDR QTL previously found in a chromosome segment substitution line (CSSL) population. The goal was to have more precise estimates of allele additive effects and study dominance effects. Twelve $F_{2:3}$ populations were made from crosses between the CSSL that showed strongest resistance across the three diseases and their recurrent parent, maize line H100. The resulting populations were assessed for each of the three diseases in replicated trials and genotyped with the markers previously associated with disease resistance. The effects of 16 QTL out of 44 were validated; the study provides further evidence for the existence of MDR QTL and demonstrates the importance of independently evaluating QTL effects following their initial identification.

In the third chapter, a preliminary experiment was done to evaluate spore adhesion of the SLB pathogen *Cochliobolus heterostrophus* to different maize lines, as it could potentially be a mechanism of disease resistance. A novel spore adhesion protocol was developed, and a timeline of spore attachment was made using leaf samples from the maize lines B73 and Mo17. We evaluated the number of spores attached on a leaf sample of the lines Mo17, B73, P39, HP301, NC350 and Ki3. Although the number of spores attached to the leaf did not perfectly correlate with field disease resistance, we believe that in some cases maize host genetics affects spore adhesion to the leaf.

Validation of Multiple Disease Resistance QTL from Chromosome Segment Substitution Population in $F_{2:3}$ Family Populations

by
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DEDICATION

To Elvira, Gilberto, Priscilla and Cades, who have always loved and supported me.

BIOGRAPHY

Lais was born in Londrina, Brazil, a big city surrounded by farms. She grew up in an apartment and always had a book in her hands. She always liked to manage money and have leadership roles in her activities. In her high school junior year, she did a semester long exchange program and lived in Knoxville, TN with a lovely couple, whom she considers family. In this period, she grew great appreciation for the American culture and a family recipe of homemade cookies. When she went back to Brazil, she decided she would go to the university to major in agriculture. Her goal through her undergraduate studies was to find ways in which she could contribute to global food production. Her interests were broad, from science to business. In 2015, she came back to the US and worked two years as a research assistant at the NCSU/USDA Maize genetics lab to explore her scientist side. She found the research interesting and wanted to learn more, so in January 2017 she started her Master's in Crop and Soil Sciences investigating disease resistance in maize. After completing her Master's she will continue to her Ph.D. in the same department, but working with cover crop breeding.

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CHAPTER I

LITERATURE REVIEW

Maize is a staple food in many countries and when not used directly for human consumption is an essential component of animal feed. With the increasing demand for cheap animal protein, population growth, and limited agricultural lands, it is necessary to increase yield. Diseases are an important factor that can reduce yield or make crops not suitable for human or animal consumption, so managing them is a key aspect of modern agriculture. One approach that presents fewer environmental risks and lower cost for the farmer is through genetic mechanisms for disease resistance. This chapter will provide insight in disease resistance and three important foliar diseases in maize.

Disease resistance overview

In 1966, Van der Plank suggested that disease resistance could be classified in two categories, horizontal or vertical. If a plant variety showed some level resistance across many races of a pathogen, Van der plank called it horizontal resistance; if the variety had strong resistance against a few races of the pathogen it was called vertical resistance. In modern literature is possible to find the terms quantitative disease resistance (QDR) or partial resistance, referring to the phenomena Van der Plank would call horizontal resistance. On the other hand, the terms qualitative or complete resistance can be found to characterize what Van der Plank called vertical resistance (Agrios, 2005). Since there are more than one definition for those terms, it is always best to leave to the discretion of the writer to define the terms used in the research. Here, we will use QDR as synonymous to partial resistance; complete resistance will be synonymous to qualitative resistance.

QDR is usually controlled by many genes, each having relatively small contributions to resistance. Because the effect of the genes varies, and the same plant can have multiple genes involved in QDR, in a segregating population the symptoms of disease will vary across a phenotypic spectrum. QDR is usually effective against many pathogen races and even species. On the other hand, qualitative resistance is complete, and the phenotype is binary. In a population segregating for qualitative resistance, the disease symptoms will be either present or absent in each plant. In most (but not all) cases the genetics controlling qualitative resistance follow the gene-for-gene model stated by Harold Flor in 1946. In this model, the host has resistance genes (R genes) that encode proteins that will recognize avirulence (Avr) proteins encoded by pathogen Avr genes. When the Avr gene and R gene are both present, the result is resistance (Agrios, 2005).

Pathogen-associated molecular pattern (PAMPs) are well-conserved features throughout classes of microbes that are usually important for the basic life cycle of the pathogen but are not specifically involved in pathogenesis. An example of a PAMP is flagellin, a common protein in bacterial flagella (Felix et al., 1999). Flagellin is recognized by a transmembrane pattern recognition receptor (PRR) called FLS2 (Gomez-Gomez and Boller, 2000), which results in a low-level defense response called PAMP-triggered immunity (PTI). Other PAMPs such as chitin (a component of the fungal cell wall) and bacterial Elongation factor Tu (Ef-Tu) are recognized in similar ways and induce similar responses (Kunze, 2004; Wan et al., 2008).

Effectors are small molecules and proteins produced and secreted by the pathogen to facilitate pathogenesis. Many effectors suppress the PTI response. R genes in the host encode proteins that can recognize the effectors, and the effectors that can be recognized (a small proportion of the pathogen's effector repertoire) are encoded by Avr genes. When an R protein

and Avr protein “interact”, it results in effector triggered immunity (ETI). A common feature of ETI is the hypersensitive response (HR), a rapid, localized cell death on the attacked and surrounding cells, isolating the pathogen and usually conferring host resistance. Jones and Dangl (2006) designed a model to explain the plant immune system called a zig zag model and describes stages of the molecular interactions of plant and pathogens through the evolutionary timeline. It starts with PTI, the weaker form of defense. The pathogen will then deploy effectors to suppress PTI and will result in effector triggered susceptibility (ETS). Plants with R-genes that can interact with the pathogen AVR gene will recognize the effector, resulting in effector triggered immunity (ETI). Compared to PTI, ETI is a faster and stronger response. Once the pathogen population loses the function of the Avr gene or employs an effector that can suppress ETI, the pathogen can again colonize the host, causing ETS for a second time. Natural selection will then favor plants that have new R-genes that can recognize Avr genes and ETI is reestablished. The model keeps going from ETI to ETS, hence the name zigzag (Jones and Dangl, 2006).

The zig-zag model has been widely used to frame plant pathology studies. Since its publication more than 10 years ago, more discoveries have been made about the molecular interactions involved in plant defense responses. It has been proposed that the model might lack some important features and the elements might not be as distinct as once thought. For example, the zig-zag model describes the interaction between a host and pathogen that feeds on live tissue (biotrophic), but it does not account for a pathogen that feeds on dead tissue (necrotrophic). Necrotrophic pathogens can benefit from HR because they can get nutrients from the dead tissue that results from HR. When a pathogen attacks, host or pathogen structure can be compromised and molecules can be released, signaling the attack; those molecules are called damage-

associated molecular patterns (DAMPs) (Lotze et al., 2007). For example, upon the attack of a necrotrophic pathogen, the cell wall will be degraded and oligogalacturonides, which are part of the cell wall structure, may be released. Plant sensors can recognize oligogalacturonides and trigger PTI (Ferrari, 2013; Malinovsky et al., 2014). DAMPs are not accounted for in the zig-zag model (Pritchard and Birch, 2014). In the model proposed by Jones and Dangl (2006), effectors and PAMPs are very distinct, but some more recent studies show that the difference might not be that clear when we look at different plant-pathogen systems (Thomma et al., 2011). One example is the fungus *Cladosporium fulvum*, a pathogen of tomato that triggers PTI and release chitin, a PAMP. To suppress PTI, the fungus employs the effector Ecp6 that binds to chitin and prevents recognition by the host and can also trigger HR (Jonge et al., 2010). The majority of effectors are secreted inside the cell, but *C. fulvum* effectors, including Ecp6, are deployed in the apoplast like PAMPs. Conserved orthologues of Ecp6 can be easily found, suggesting that they are essential for the colonization of the hosts, a characteristic of PAMP molecules and not effectors (Bolton et al., 2008; De Jonge and Thomma, 2009). This is one example that illustrates that the distinction between PAMPs and effectors might not be so clear. Thomma, Nürnberger, and Joosten (2011) give an extensive review of how PTI and ETI are not always distinct.

A more recent model that attempts to summarize plant defense response to pathogens is the Invasion Model (Cook et al., 2015). It has broader view of pathogen-host interaction and can incorporate pathogens with different life styles. In the invasion model, the invasion pattern(s) receptors (IPRs) are host receptors that can detect invasion pattern(s) (IP). IP can be any molecule, external or self-modified ligand that signals plant invasion by a pathogen. Compared to the zigzag model (Jones and Dangl, 2006), the IP could be DAMPs, PAMPS or effectors. The next steps are IP-triggered responses (IPTRs), defined from the perspective of the invader. If the

pathogen fails to suppress IPTR, the symbiosis stops. If the microorganism suppresses IPTR, the symbiosis continues. A third outcome, not portrayed in the zigzag model, is to have the microorganism utilize the IPTR for its advantage and continue symbiosis. The invaders can use effectors to modulate the outcome of symbiosis in their favor, which can result in IPs and a new cycle in the model. For example, a necrotrophic pathogen can use HR in their favor to have more dead tissue to feed from (Cook et al., 2015).

Disease resistance is one of the goals when breeding more resilient crops. It can be achieved by selection on the available germplasm in the program by traditional selection methods, screening for presence of R genes or disease resistance quantitative trait loci (dQTL) or genomic selection. If the resistance is not present in the germplasm, introgression of resistance genes found in wild relatives could be done (Tanksley and McCouch, 1997; Sharma et al., 2013; Liu et al., 2015; Lennon et al., 2016, 2017) or it could potentially be incorporated by gene-editing in a near future (de Toledo Thomazella et al., 2016; Chandrasekaran et al., 2016).

Multiple Disease Resistance (MDR)

A quantitative trait locus (QTL) is a property of a mapping population and is a physical place on the genome at which allelic variation in the population is associated with a quantitative phenotype. Finding disease resistance QTL (dQTL) has been the focus of many studies (Balint-Kurti et al., 2010; Poland et al., 2011; Benson et al., 2015; Liu et al., 2016). Multiple disease resistance (MDR) can be defined as “resistance to two or more diseases” (Nene, 1988). MDR can be approached at a plant level or at a locus level. MDR at a plant level has been noted since at least 1902, when resistance to wilt and root-knot was documented in cowpea (Orton and Webber, 1902). In a breeding program, MDR is commonly approached at the whole plant level and is due to combined effects of multiple genes at multiple loci. For example, one study

screened 211 chickpea germplasm accessions for resistance to four diseases, and 16 lines showed resistance to more than one disease (Pande et al., 2006). In potato, wild relative species are a potential source for MDR for breeding programs (Jansky and Rouse, 2002). MDR at a whole plant level may not be common even for similar pathogens. In one study screening for resistance to three major leaf spot diseases in wheat, out of 164 genotypes only four were resistant to all three diseases (Ali et al., 2008) and in another study in wheat that screened a more comprehensive panel, 11% of the accessions showed resistance to two diseases (Gurung et al., 2009). On the other hand, MDR at a locus level is when the interest lies in the genes/QTL and their interactions to understand the genetic basis of the MDR. At a locus level, MDR is due to linked genes or an individual gene that gives resistance to more than one disease (pleiotropy). If two alleles that give resistance to two diseases are linked in coupling, the plant will show MDR; if they are linked in repulsion, resistance to one disease won't co-occur with resistance to the other. It is important to note that pleiotropy is not common, even when pathogens are similar. One example of pleiotropic genes is *Lr34* in wheat. It confers resistance to leaf rust, stripe rust and powdery mildew (Dyck, 1987; MCINTOSH, 1992; Lagudah et al., 2009). *Lr34* is not naturally found in corn, but through transformation a maize line expressing *Lr34* showed increased resistance to common rust and northern corn leaf blight (Sucher et al., 2017).

Quantitative Trait Loci (QTL) Mapping and Validation for Disease Resistance

The purpose of a QTL study is to find an allelic variation associated with a desired trait. If a dQTL is found, it means that the parents of the population have alleles at that specific physical place with contrasting effects, susceptibility and resistance to the pathogen. A QTL study needs four elements: a population that has phenotypic variability for the heritable desired

trait, genotypic data, a consistent phenotyping method that allows reproducible trait measurements, and statistical models to detect and locate the QTL (Cavanagh et al., 2008). One way of mapping a QTL is using a structured population derived from biparental mating: F_2, backcross (BC), recombinant inbred lines (RILs), advanced intercross lines (AIL), near isogenic lines (NIL) or chromosome segment substitution lines (CSSL). Mapping from biparental population is called linkage-based mapping. Limitations of this method are that it uses only two parents to make the population, limiting the genetic diversity that is assessed; furthermore, the resolution is relatively low due to the few numbers of recombination events that are captured during the development of the population. From the populations mentioned above, BC populations have the least number of recombinant events when compared to the other bi-parental populations.

NILs and RILs have the advantage of being "immortal" populations; since the lines are homozygous, the seed can be indefinitely increased, allowing many replications for phenotypic evaluations and different studies of the same population. NILs or CSSLs, are populations in which each line's genome is derived largely from a recurrent parent with a small part of the genome from a donor parent. The donor parent will have the trait of interest and the recurrent parent will have a contrasting phenotype. NILs are generally derived from an initial cross between the donor and recurrent parent followed by repeated backcrossing to the recurrent parent and several rounds of selfing to obtain the desired level of homozygosis. The goal is often to have the genome from the donor parent fully represented in small introgressions amongst all the lines in the population, resulting in a CSSL population. Across the NIL population, for each locus, most lines will have the allele from the recurrent parent and a few lines will have the allele from the donor parent. Unlike NILs, development of RILs does not involve the backcrossing

stage. RILs are made from a cross of lines with contrasting phenotypes, and subsequent self-pollination cycles starting in the F_2 generation via single seed descent until the lines reach the desired level of homozygosity. RILs have a random assortment of alleles from both parents, with each allele being represented on average in half the lines in a population. Each RIL has roughly half of the genome from each parent, which results in potentially more genetic interactions between loci (epistasis) from the donor parent than in NILs. Since most of the alleles in NILs are from the recurrent parent, NILs allow the observation of epistasis of donor alleles with the background of interest; resulting in a more accurate perception of the allele effect expected in a breeding program. The number of recombination events in RILs is higher than in NILs, resulting in potentially smaller introgressions which may allow the use of smaller mapping populations than NILs and provide a more precise localization of QTL. On the other hand, in NILs there is only a small difference in the genome among the lines and the phenotypic difference from a NIL and the recurrent parent is probably due to the allelic difference from the introgressed loci. Since each introgression is represented only a few times in a NIL population and tested against most of the lines having the allele from the recurrent parent, the power to detect QTL is lower than in a RIL population and effect estimate might be imprecise. Also, seeing that lines are homozygous, it is not possible to determine dominance. To have a more precise estimate of allele effect and to test for dominance an independent study can be done. $F_{2:3}$ or $F_{3:4}$ family populations derived from a cross of between the NIL of interest and recurrent parent are ideal for the validation study. In a locus of interest, in an $F_{2:3}$ population half of the families will be segregating, a quarter of the families will be homozygous lines for the donor parent allele and a quarter of the families will be homozygous lines for the recurrent parent allele. Having partially heterozygous lines allow the study of dominance effects, and having an equal representation of both alleles present in the

population can lead to better effect estimate (Keurentjes et al., 2007; Szalma et al., 2007; Jamann et al., 2015; Lennon et al., 2016, 2017).

Another mapping method is by using a random mating population or diversity panel and it is called a genome-wide association study (GWAS) (MacKay et al., 2009). Since GWAS can assess thousands of sequence variants, the mapping is not limited to the alleles that segregate in two lines as in a bi-parental population, but it is always necessary to account for population structure. In contrast to QTL mapping, GWAS has the advantages of allowing the use of already existing lines and genetic stocks and avoiding the step of having to generate new bi-parental populations. GWAS can access all the recombinant events that happened in the evolution of the lines in the population, which are potentially more recombinant events than in a bi-parental population, and can result in more precise mapping Also, because of the higher diversity in the parents than that of a bi-parental population, GWAS can incorporate more genetic diversity (Zhu et al., 2008; Myles et al., 2009; Jamann et al., 2015). One drawback of GWAS is the low power to detect rare variants and small effect size variants (Asimit and Zeggini, 2010; Gibson, 2012).

Linkage and association analysis can be used to validate each other (Navara and Smith, 2014; Sonah et al., 2015) with a GWAS conducted first to identify putative QTL, followed by bi-parental population and linkage mapping to validate those QTL. They can also be used to increase the precision of QTL mapping with a linkage analysis followed by a GWAS to fine map the region (Mammadov et al., 2015). Both methods can be used in parallel populations to find QTL (Mammadov et al., 2015). Finally, they can be used together in the same population to complement each other, as in the maize nested association mapping (NAM) study (Yu et al., 2008). The NAM population consists of 5,000 recombinant inbred lines (RIL) that represent some of maize diversity. Twenty-five maize lines were crossed to line B73, a well-characterized

line that has a public genetic map, and through single seed descent 200 lines of each family were made. This population was fine mapped and made available for public use. The high number of recombinant events in the RIL population paired with fine genetic mapping allow high power and high resolution to map traits of interest (McMullen et al., 2009).

In general, dQTL studies are most commonly done for a single disease (Poland et al., 2011; Benson et al., 2015; Liu et al., 2016) and colocalization of dQTL for single diseases in the same population can suggest evidence of an MDR loci. It is possible to analyze the information of dQTL associated with a single disease resistance in the same plant species through a meta-analysis (Wisser et al., 2006; SHI et al., 2007; Kou and Wang, 2011; Wiesner-Hanks and Nelson, 2016) to map to a finer scale resolution and identify QTL that have consistent effects (Corwin and Kliebenstein, 2017).

Mapping QTLs for MDR has challenges related to the genetic architecture of the traits. Linkage can cause positive or negative correlations between resistances. If one gene associated with resistance to one pathogen is linked in coupling to a gene that associated with a second pathogen, resistance to both diseases would be positively correlated. If the mapping resolution is not precise enough to separate different dQTL associated with resistance to different pathogens, the result could be dQTL overlap. If resistance alleles are linked in repulsion, resistance to one disease could be negatively correlated to resistance to a second disease. Uneven pleiotropy can also affect MDR QTL mapping, as an allele that has a major effect for one disease might have a minor effect for another (Balint-Kurti et al., 2010; Wiesner-Hanks and Nelson, 2016).

After the discovery of a QTL it is sometimes advisable to do a second study to validate any findings before deploying the alleles in a breeding program or conducting fine scale mapping. There are multiple ways of validating a QTL but they all involve the assessment of an

independently-derived population in which the same alleles found in the mapping population are segregating. To test the QTLs found in a GWAS study, one can create a bi-parental population or near isogenic lines (Navara and Smith, 2014; Sonah et al., 2015; Hindu et al., 2018).

Overview of Selected Diseases

Southern Leaf Blight

Southern leaf blight (SLB) is a foliar disease in maize caused by the necrotrophic fungus *Bipolaris maydis* (teleomorph phase: *Cochilobolus heterostrophus*) that belongs to the Ascomycota phylum. Favorable conditions for pathogen development are temperatures between 22-30^{o}C and high humidity. Symptoms are leaf lesions that are oblong, parallel-sided and tan to grayish color; lesions may develop a brown border. If the disease is present early and it is severe, it can predispose the plants to stalk rots. Primary inoculum comes from overwintering mycelium and spores are spread by wind or water splashing in growing plants. After the fungus infects and colonizes the plant, the primary lesions will serve as a source of secondary inoculum. The disease cycle can repeat every seven days if conditions are favorable. It is very important to control the disease from 14 days before tasseling to 21 days after tasseling, because this is the period when SLB can cause more damage to yield (University of Illinois Extension, 1997). Although SLB is not one of the most economically important disease in the U.S nowadays (Mueller et al., 2016), the disease became very well-known from the 1970-71 epidemics, when North America loss 254 million hectoliters of grain and more than $1.0 billion at that time, more than $6 billion by today standards. Back then, maize breeding programs largely deployed the use of Texas male sterile lines (cms-T) to avoid the labor-consuming task of detasseling the plants in seed production fields. The mitochondrial gene responsible for male sterility also gave

susceptibility to T-toxin produced by race T of *C. heterostrophus*. Unfortunately, in 1970-71 the environmental conditions were favorable for disease development and extensive area was planted with maize containing cms-T cytoplasm, allowing a major epidemic. After that year, cms-T lines were not used and the epidemics brought awareness about the need of genetic diversity in our crops system (Arnold Bruns, 2017).

Northern Leaf Blight

Northern leaf blight (NLB) is a foliar disease in maize caused by the necrotrophic fungus *Setosphaeria turcica* (anamorph phase: *Exserohilum turcicum*) that belongs to the Ascomycota phylum. Ideal conditions for the development of the fungus are temperatures ranging from 17-27 ^{o}C, wet and humid weather. The symptoms are tan lesions, parallel to leaf margins, in long and oblong form ("cigar shaped") ranging from 1 to 7 inches. *Setosphaeria turcica* survives on infected corn residue through the winter and produce new spores as the temperature rise. New spores will be dispersed by wind and water splashes (Wise, 2011).

NLB was the major cause of leaf-disease related loss in corn in the US and Canada during the years 2012-2015 (Mueller et al., 2016), resulting in a total of more than 1.1 billion bushels loss. To control the disease different practices can be implemented. Tillage and one- or two-year rotation will decrease the residue and overwinter spores. Fungicides can be effective against the fungus, although they increase the cost of production (Wise, 2011). Incorporation of genetic disease resistance is a good management strategy, it has been broadly studied and R-genes and QTL have been found and are currently being used in breeding programs (Galiano-Carneiro and Miedaner, 2017).

Gray Leaf Spot

Gray Leaf Spot (GLS) is a leaf disease in corn caused by the necrotrophic fungus *Cercospora zeae-maydis* and *Cercospora zeina* that belong to the Ascomycota phylum (Crous et al. 2006). The primary inoculum comes from debris of previous years infected plants and a no-till regimen with conductive environment can lead to high disease pressure. High relative humidity and dew are the prime factors for the disease development. Warm temperatures can also contribute to better fungal growth. The symptoms in mature leaves are lesions parallel to the major veins, which start as small tan spots with distinctive borders and develop to rectangle shapes. When sporulation occurs, it produces a grayish cast. The lesions are 1-6 cm long and 2-4 mm wide. In comparison to SLB an NLB, the latent period of GLS is long and it can take from 14 to 28 days after infection for lesions to sporulate (Beckman and Payne, 1982; Latterell, 1983; Ward et al., 1999).

One of the current most yield damaging foliar disease, GLS was the second most damaging foliar disease in the US and Canada from 2012 to 2015 (Mueller et al., 2016). Many studies found QTLs associated with GLS resistance based on RIL populations, GWAS, Nested Association Mapping or wild relatives introgressions (Berger et al., 2014; Benson et al., 2015; Mammadov et al., 2015; Lennon et al., 2016; Kuki et al., 2018). An MDR gene that has a small effect in SLB and GLS has been characterized (Yang et al., 2017). No R genes have been found for GLS.

In the next chapter we describe a study that aimed to validate MDR QTL in maize found in a CSSL population using $F_{2:3}$ families. The third and last chapter is a preliminary study to investigate variation of spore adhesion to leaves in different maize genotypes.

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CHAPTER II

VALIDATION OF MULTIPLE DISEASE RESISTANCE QTL FROM CHROMOSOME SEGMENT SUBSTITUTION POPULATION IN $F_{2:3}$ FAMILIES

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Abstract

Southern Leaf Blight (SLB), Northern Leaf Blight (NLB), and Gray Leaf Spot (GLS) are foliar diseases of maize caused by ascomycete fungi and are among the most important foliar diseases of maize worldwide. Previously, disease resistance quantitative trait loci (dQTL) were found in a connected set of chromosome segment substitution line (CSSL) populations (Lopez Zuniga et al., 2016). Some QTL co-localized, suggesting the presence of multiple disease resistance (MDR) QTL. The goal of this study was to do an independent test of the putative MDR QTL to have a more precise estimate of allele additive effect and study allele dominance effects. Twelve $F_{2:3}$ populations were made from crosses between the CSSL that showed strongest resistance across the three diseases and their recurrent parent, H100. The resulting populations were assessed for each of the three diseases in replicated trials and genotyped with the markers previously associated with disease resistance. Pairwise phenotypic correlations across all the populations for resistance to the three diseases ranged from 0.2 to 0.3 and were all significant ($P = 0.01$). The effects of 16 QTL out of 44 were validated and new QTL/disease associations were found. Only two alleles had dominance effects. Two distinct MDR QTL were validated and associated with resistance to all three diseases. In both cases, the resistance alleles derived from line NC304. This study reinforces the existence of MDR QTL and demonstrates the importance of independently evaluating QTL effects following their initial identification before deploying them in a breeding program.

Introduction

Maize is a staple food in many countries and when not used directly for human consumption it is an essential component of animals' feed. With population growth, limited agricultural lands, and the increasing demand for cheap animal protein, it is necessary to increase corn yield. Diseases are an important factor that can reduce yield or make crops not suitable for human or animal consumption, so managing them is a key aspect of modern agriculture. One way that presents less environmental risk and lower cost for the farmer is through genetic mechanisms for disease resistance.

Qualitative disease resistance is controlled by one or few genes with major effects and disease symptoms will be either present or absent. A genotype with quantitative resistance may be susceptible to one race of the pathogen and resistant to another race, this is known as race specific resistance. On the other hand, quantitative disease resistance (QDR) can be characterized as a partial reduction in disease symptoms. This trait is usually controlled by multiple genes which confer relatively small effects and it is often non-race specific. QDR can be more durable than qualitative resistance because it relies on the mechanisms of many different genes instead of just one gene and mechanism (Agrios, 2005). Multiple disease resistance (MDR) can be defined as "host-plant resistance to two or more diseases" (Nene, 1988). MDR can be due to different genes, each associated with resistance to one disease, or to an individual gene that gives resistance to more than one disease (pleiotropy) (Wiesner-Hanks & Nelson, 2016). One example of pleiotropic genes is *Lr34* in wheat. It confers resistance to leaf rust, stripe rust and powdery mildew (Dyck, 1987; Lagudah et al., 2009; MCINTOSH, 1992). *Lr34* it is not naturally found in corn, but through transformation, a maize line expressing *Lr34* showed increased resistance to common rust and northern corn leaf blight (Sucher et al., 2017). A quantitative trait locus (QTL)

is a property of a mapping population and is a physical place on the genome at which allelic variation in the population is associated with a quantitative phenotype. Finding disease resistance QTL (dQTL) have been the focus of many studies (Balint-Kurti et al., 2010; Benson et al., 2015; Liu et al., 2016; Poland et al., 2011). Colocalization of dQTL for single diseases in the same population can suggest evidence of an MDR locus. In tobacco, two QTL associated with resistance to *Phytophtora nicotianae* were also found to have large effect on resistance to *Ralstonia solanacearum* (Drake-Stowe et al., 2017). A study evaluating resistance to three foliar diseases in corn found QTL colocalization to three and two diseases (Zwonitzer et al., 2009).

Southern leaf blight (SLB) is caused by the fungus *Cochliobolus heterostrophus;* northern leaf blight (NLB) is caused by the fungus *Setosphaeria turcica* and gray leaf spot (GLS) is caused by the fungi *Cercospora zeae-maydis* and *Cercospora zeina*. SLB, GLS and NLB are among the most important foliar diseases of maize worldwide. NLB alone caused a total of more than 1.1 billion bushels loss in the US and Canada during the years 2012-2015 (Mueller et al., 2016). The pathogens are ascomycete fungi that share the common characteristic of penetrating young leaf but acquiring nutrients from dead tissue. Since they share some pathogenesis characteristics, it is possible that a plant could have mechanisms of resistance that affected all three pathogens.

Wisser et al. (2011) conducted a multivariate analysis in a maize panel of 253 maize inbred lines to test the hypothesis that allelic variation could result in MDR for NLB, SLB and GLS. After accounting for maturity, kinship and population structure, they found high positive genetic correlations among all diseases. The population had low linkage disequilibrium, so the correlation of disease resistance was probably due to pleiotropy and not to linked genes in segregating blocks.

Lopez Zuniga et al. (2016) developed a set of chromosome segment substitution line (CSSL) populations in which segments from an MDR donor line were introgressed in a multiple disease susceptible (MDS) background. To develop the population, four lines identified as MDR in the Wisser et al. (2011) panel were used as donor parents and crossed to MDS lines that were used as recurrent parents to develop $BC_3F_{4:5}$ CSSL. The study's goal was to conduct MDR QTL mapping for SLB, NLB and GLS. Many dQTL were found and some QTL associated with two or three diseases were also reported, indicating potential MDR QTL. An analysis using a composite score for the three diseases was also performed and the QTL found were considered MDR QTL.

Using CSSL as a mapping population has the benefit of studying the introgressed regions in a background like what would be in a released cultivar. Therefore, the epistasis events that could occur in a breeding program are more accurately represented. Also, follow up studies concerning specific introgressions of interest can be done with the development of an independent segregating population that uses as parents the line with the desired introgression and the original recurrent parent. Since the donor allele is present in only a few lines in a CSSL population, effect estimates can have grater inaccuracy than in other bi-parental population with the alleles from each parent present in equal number of lines across the population. Also, since the lines are nearly completely homozygous, it is not possible to study dominance in CSSL populations (Jamann et al., 2015; Kaeppler, 1997; Keurentjes et al., 2007).

The goal of this study was to do an independent test of the putative QTL associated with MDR by Lopez Zuniga et al. (2016) to confirm their existence, have a more accurate additive effect estimate and to test dominance effects. Twelve $F_{2:3}$ populations were made from crosses between the CSSL that showed strongest resistance across the three diseases and their recurrent

parent, H100. The resulting populations were assessed for each of the three diseases in replicated trials.

Materials and Methods

Populations

As part of a previous study (Lopez Zuniga et al., 2016), four chromosome segment substitution populations, $BC_3F_{4:5}$, were created by crossing four multiple disease resistance maize (*Zea mays*) lines, Ki3, NC262, NC304, and NC344 as donors, with the disease susceptible line H100, as male. After the first cross, the F_1 was backcrossed three times to H100 and four consecutive generations of self-pollination were performed via single seed descent. The final $BC_3F_{4:5}$ lines were increased by sib-mating (Figure 2.1). The final populations were genotyped using Pioneer Illumina publicplex platform with 765 SNP markers (Jones et al., 2009) and after quality control between 245 and 271 SNPs were informative within each population. All the populations were evaluated for GLS, SLB and NLB in replicated trials in two locations. 30 QTL were associated with variation in resistance to a single disease, 17 to two diseases, and four to all three diseases.

For this study, the twelve DRIL lines that showed the strongest resistance across all three diseases in Lopez Zuniga et al. 2016 were chosen to make $F_{2:3}$ families (Figure 2.2, Table 2.1). Four lines belonged to the Ki3 introgression population, three lines belonged to the NC626 introgression population, three lines belonged to the NC304 introgression population and two lines belonged to the NC344 introgression population. Each line was crossed with H100 to generate F_1 plants; those were self-pollinated to create F_2 populations that were then selfed again to generate $F_{2:3}$ families (Figure 2.2).

Experimental Design

Twelve populations, varying from 49 to 101 $F_{2:3}$ families in each of them (Table 2.1) were tested in a replicated trial for each of the diseases: SLB, NLB and GLS. Each experiment

had an augmented compete block design, with population as a block. The recurrent susceptible parent H100 was included at a random location within a block of 21 plots, and each MDR parent was planted once in its respective population.

During the summer of 2017, two full complete replications of 1029 plots each were planted at the Central Crops Research Station (CCRS, Clayton NC) for the evaluation of SLB; two complete replications were planted at the College Farm Research Station (Blacksburg VA), for the evaluation of GLS; and one incomplete replication consisting of 700 plots, was planted in Andrews NC for the evaluation of GLS. During summer 2018, two complete replications were planted in CCRS for the evaluation of NLB.

Inoculation Preparation and Inoculum Procedure

To prepare the inoculum sorghum kernels were soaked in water for 3 to 4 days, placed in 1L flasks, and autoclaved for one hour (834266 Pa and 121^{o}C). Autoclaved grain was inoculated with either *C. heterostrophus, S. turcica,* or *C. zeae-maydis*. Inoculum for the experiments was produced by allowing the fungus to grow at room temperature (23-25^{o}C) for at least 10 days until the sorghum grains were colonized by the fungus. The fungus-infested sorghum was air-dried and stored at 4^{o}C. 30-40 day old maize plants were inoculated by adding 6 to 10 infested sorghum kernels into the whorl of each maize plant.

Phenotypic Evaluation of SLB

The experiment was planted in a single field, with 8 seeds per plot, in 1.8-m single rows with 0.9-m row width. Inoculations of SLB were performed 40 days after planting, using *C. heterostrophus* isolates, including 2-16Bm, Hm540 (Carson, 1998), and an unnamed isolate

provided by Syngenta. Visual scores were taken three times at 10 days interval, starting 77 days after planting, when the plants were in the developmental stage R2. The scoring used a scale of 1 to 9, where 1 is equal to leaf area completely covered by the pathogen and 9 is equal to no disease. Each plot had one score given at each evaluation. If the plot was segregating it was given a score that represented the average disease severity of the plot. For each plot in both replications, days to anthesis (DTA) was recorded when half the plants in a plot were shedding pollen.

Phenotypic Evaluation of NLB

All field evaluations of NLB were performed during summer growing season of 2018, with two replicates grown at CCRS in Clayton, NC. The experiment was planted in the same fashion as the SLB trial. Inoculations of NLB were performed 26 days after planting, using several *Exserohilum turicum* isolates (ET238A, ET471A-1, ET30A, ET3A, ET28A, ET257A, Cairo05, 235A, race 0 from Syngenta, Race1 from Syngenta, ET252A, ET28A, ET30A, ET222A). Visual scores were taken three times at six to eleven days interval, starting on July 6th and days to anthesis was also recorded. NLB was scored using percentage of diseased leaf area, from 0 to 100. Each plot had one score given at each evaluation. If the plot was segregating it was given a score that represented the average disease severity of the plot. The disease scores were converted to fit the same scale of GLS and SLB (1 to 9). For each plot in both replications, days to anthesis (DTA) was recorded when half the plants in a plot were shedding pollen.

Phenotypic Evaluation of GLS

All field evaluations of GLS were performed during the summer growing season of 2017, with two replicates at College Farm Research Station in Blacksburg, VA and an incomplete

replicate in Andrews, NC. Trials were planted in 4-m single rows with a 1-m row width using 15 seeds per plot, in both locations. Inoculation was performed in the field in Virginia 30 days after planting, using several *Cercospora maydis* inoculum isolates (3-3; 7-1,3,4; 13-1a,3,4,5a,10,16 from 2015; 2.2;7.4, 5.3 from 2014, and a Syngenta inoculum from 2011,2015). No artificial inoculation was done in Andrews, since the field contained infected plant debris from previous years that provided enough inoculum for high disease pressure. Visual scores were taken twice at each location using a scale of 1 to 9, where 1 is equal to leaf area completely covered by pathogen and 9 is equal to no disease. Each plot had one score given at each evaluation. If the plot was segregating it was given a score that represented the average disease severity of the plot.

Genotyping

Tips of leaves were collected from 5 adult plants per $F_{2:3}$ family, bulked, and lyophilized. The samples were sent to Agriplex Genomics for DNA extraction and genotyping. Markers that were associated with resistance to one or more disease in Lopez Zuniga et al (2016) mapping population were selected for analysis. Primers were designed based on context sequence of the SNPs reported by Lopez Zuniga et al. (2016) using the B73 reference genome version 3. (Appendix 1).

Statistical Analysis

Phenotypic Data

Exploratory phenotypic data analysis was performed to better understand all data using Statistical Analysis System (SAS) v9.4 software (SAS Institute Inc., Cary, NC), Tableau v9.1 and R (R core development team, 2015). Heat maps were produced to visualize any disease severity spatial pattern in the field and box plots were created to check the severity of disease in

the $F_{2:3}$ lines in comparison to the susceptible and resistant checks. Correlation coefficients between disease scores of lines measured in different replications or on different diseases were calculated.

The standardized area under disease progress curve (sAUDPC) was calculated for each genotype within each disease, using two to three disease scores collected at different time points on the same plot. For each case, the sAUDPC was calculated as the sum of averages between data points divided by two, multiplied by the number of days between data points and then divided by the total numbers of days between the first and the last data point. As an example, 3 data points were collected, D1, D2 and D3, with 8 days between the first two and 10 days between the last two; then sAUDPC would be calculated as (((D1+D2)/2)*8) + ((D2+D3)/2)*10))/18.

Statistical analysis was performed using the MIXED procedure in SAS. To adjust the Least-square means (LSMeans) for field effects, range (horizontal line) and column (vertical line) were added to the model as random effects. The mixed linear model used to analyze data from the SLB trials was

$$y_{ijkmn} = \mu + L_i + D_j + R_k + G_m + C_n + \varepsilon_{ijknm}$$

where y is the response variable SLB AUDPC, L is the estimated effect of line, D is the estimated effect of days to anthesis (DTA), R is the random effect of replicate, G is the random effect of range, C is the random effect of column. A similar model was used to analyze data from the NLB trial:

$$y_{ijkmn} = \mu + L_i + D_j + R_k + G(R)_{m(k)} + C(R)_{n(k)} + \varepsilon_{ijknm}$$

the response variable y was NLB AUDPC, L is the estimated effect of line, D is the estimated effect of days to anthesis (DTA), R is the random effect of replicate, G(R) is the random effect of

range nested in replicate, C(R) is the random effect of column nested in replicate. To analyze the data from the GLS field trial, the model used was

$$y_{ijkmn} = \mu + L_i + O_j + R(O)_{k(j)} + G(O)_{m(j)} + C(O)_{n(j)} + \varepsilon_{ijknm}$$

where y is the response variable GLS AUDPC, L is the estimated effect of line, O is the random effect of location, R(O) is the random effect of replicate nested in location, G(O) is the random effect of range nested in location, C(O) is the random effect of column nested in location. LSMeans for each line and disease were calculated from the model.

Heritability on an entry mean and family mean-basis for each disease were calculated as described by Holland (Holland et al., 2003) using the variance components described above and a harmonic mean for the number of replicates per entry. We used combined data from all populations to estimate heritability.

Genotypic Data

A Chi square test was performed for each marker within each population to assess if the segregation was significantly different from the expected 1:2:1.

Single marker analysis of variance was performed using $F_{2:3}$ family LSMeans as the response variable and marker genotype at a single marker as an independent variable using SAS PROC MIXED. Additive and dominance effects were estimated from linear combinations of the genotype effects: $\hat{a} = \frac{M_{11} - M_{22}}{2}$; $\hat{d} = 2[M_{12} - (\frac{M_{11} + M_{22}}{2})]$, where M_{11}, M_{22}, and are the mean values of lines fixed as homozygotes for alleles 1 and 2, respectively, and M_{12} is the mean value of the segregating lines derived from heterozygous F_2 plants. The estimate of dominance effects includes a coefficient of 2 because only half of the individuals in the segregating lines are expected to be heterozygous. Significance at the type III test was used to validate the marker

effect; *P* value significance of α=0.05. If more than one marker was significant in the same bin and less than 20 cM apart, the marker that had the smallest P-value, or had a significant effect for the greatest number of diseases, was chosen to represent the effect of that QTL (Table 2.2). In cases where the population was segregating for more than one unlinked marker, marker interactions were tested by fitting the two markers and their interaction in the model using PROC GLM to verify epistasis. If there was no interaction, a third model was run with PROC GLM, fitting the two markers as fixed effects and the disease LSMeans as the response to obtain an accurate additive estimate effect.

Results and Discussion

Twelve $F_{2:3}$ populations made from crosses between 12 MDR CSSL identified by Lopez Zuniga et al. (2016) and the recurrent susceptible parent H100 were created (Figure 2.2) and evaluated in replicated trials for SLB, NLB and GLS resistance. Pairwise phenotypic correlations across all the populations for resistance to the three diseases were all significant at the 0.01 *P*-value level, ranging from 0.2 to 0.3. The pairwise disease resistance correlations within individual populations were often but not always significant (Table 2.3). Heritabilities on a single plot-basis were 0.45, 0.29, and 0.36, and heritabilities on an entry mean-basis were 0.57, 0.49 and 0.52 for SLB, GLS and NLB, respectively. Correlation and heritability estimates were relatively moderate due partly to the nature of these populations. Each population was only segregating at a small number of loci and therefore represented less genetic variation than typically seen in RIL and association populations, for which the entire genome is segregating.

When analyzing phenotypic data, the fixed effects of line and DTA covariate (when DTA data was available) were significant for all diseases. For all field trials range and column were significant and replicate was not (Table 2.4 to 2.6). The only disease that had field trials in different locations was GLS and the effect of location was not significant.

Each of the 12 DRILs that were selected as the parents of the 12 $F_{2:3}$ populations carried multiple introgressions. Of these, between one and four introgressions had been previously associated with resistance to at least one disease or the MDR composite score in our previous analyses (Lopez Zuniga et al., 2016). The segregation of all these previously-associated introgressions was genotyped for all subsequent analyses, but the segregation of any introgressions that were not associated with disease resistance in the original analysis was not followed.

The segregation of 28 markers (Appendix 1) was scored to characterize the effects of the segregation of 24 introgressions across the twelve $F_{2:3}$ family populations (Table 2.2). Each population was segregating for between one and five of these markers/introgressions. We assessed the association of each segregating marker with resistance to each disease regardless of which disease(s) it had been associated with in the previous study (Table 2.2). In several cases, more than one marker was used to follow the segregation of a single introgression. In these cases, we selected the marker that was most strongly associated with disease resistance as the marker to represent the QTL in that introgression (Table 2.2). The QTL was considered validated if it was associated with any of the diseases for which it had been previously identified (Lopez Zuniga et al., 2016), with the effect going in the same direction. In cases where two introgressions were both significant for a specific disease/population combination, the markers representing each introgression were incorporated in a final model to calculate effect sizes to account for any potential epistatic effects. After selecting only one marker for each introgression, we were able to test 44 previously identified QTL, 20 associated with the MDR composite score and 24 with single disease resistance (Table 2.2). Many of these QTL were previously associated with MDR and single disease resistance so the number of 44 includes a number of QTL counted multiple times (Table 2.2).

The effects of 16 QTL out of 44 were validated. Table 2.7 summarizes the significant marker/disease resistance associations that were detected. An MDR QTL that was detected with the composite score was validated when it was significant for more than one disease (Table 2.7). In some cases, the same dQTL was validated for MDR and a single disease resistance (Table 2.7).

In some cases a disease/marker association was identified in this study that was not identified in Lopez Zuniga et al. (2016). For example, marker PHM4586-12 in population H100_DRIL72.232 was previously associated with GLS and in the current study it was significant for NLB only. Marker PHM4495-14 in population H100_DRIL32.191 was previously associated with SLB and in the present study it was significant for NLB and not SLB (Table 2.7). In population H100_DRIL62.078, the marker PHM9635-30 was previously associated only with GLS resistance, but in the current study it was significant for GLS and NLB resistance, it was validated only for GLS (Table 2.7). New marker/disease association could happen because in the mapping population the marker effect was not big enough to be significant and in the $F_{2:3}$ populations, with a more accurate estimate, the marker has a significant effect.

In some cases, a non-significant phenotypic correlation coefficient between two diseases was reported but there was QTL colocalization. For example, the population H100_DRIL52.055 correlation coefficient between SLB and NLB was not significant (Table 2.3), but a marker on chromosome 9 at 114.49 cM (Table 2.7) was correlated with resistance to both SLB and NLB. This could be explained by the small effect of the QTL (R^2 <0.1).

Dominance was observed only in two cases out of the 26 instances where we observed a significant marker-trait association: marker PHM13420-11 in population H100_DRIL62.156 and marker PHM14412-4 in population H100_DRIL72.061 (Table 2.7). In both cases disease resistance was dominant. Since allele effects were calculated from $F_{2:3}$ families, the dominance estimated value is roughly half of the real value as half the families are fixed for each allele. In three cases alleles from the donor MDR parent were associated with disease susceptibility. NC262 conferred a susceptibility allele for SLB in population H100_DRIL52.055 at bin 2.07 (marker PZA03577-1) and in population H100_DRIL52.268 at bin 8.03(marker PHM4757-14).

NC344 conferred a susceptibility allele for NLB in population H100_DRIL72.232 at 2.05 (marker PHM4586-12) (Table 2.7).

There are several possible reasons why fewer than half the dQTL were validated. Concerning the experimental design, there are inherent issues with the use of a NIL population to map QTL. Each allele being sampled only a handful of times in each population and potential phenotyping errors in the CSSL mapping population might lead to a false QTL detection. Also, each line had more than one introgression and the genotyping of the mapping population was not dense, which can result in introgressions that affect disease resistance not being detected. If the introgression was not detected in the mapping population it was not accounted for in the validation study, and the undetected introgression could impact the validation rate. Furthermore, the number of $F_{2:3}$ families used in each population was less than 102 and varied between experiments (Table 2.2 and table 2.7), which can affect power of QTL detection (Melchinger et al., 1998; Vales et al., 2005; Wang et al., 2012).

Significance of QTL effects on MDR were tested by a composite score in the previous study by Lopez Zuniga et al. (2016). Significance for MDR effects in that study could have been achieved by a dQTL that had a large effect on one disease and only small effects on the other diseases. In this study, we tested for QTL effects on individual diseases and possibly would validate a previously-defined MDR QTL's effect on a single disease but not on MDR. For example, marker PHM14412-4 on population H100_DRIL72.061 was associated with GLS and MDR previously and it was validated only for GLS (Table 2.7). The same happened in population H100_DRIL62.030 and marker PHM13420-11. A similar example is marker PHM4757-14 in population H100_DRIL32.134, it was previously associated with GLS, NLB and MDR and in was validated only for NLB. It is important to point that even though the p-

value for the marker association with GLS was not significant (p-value=0.064) the associated additive effect of the marker was 0.14, a value similar to the effect of some validated markers (Table 2.7).

In some cases, the same marker was significant for the same disease in different populations. Marker PHM13420-11 was validated for GLS in population H100_DRIL62.030 and for GLS and MDR in population H100_DRIL62.156 (Table 2.7). The original donor for both populations was NC304 (Table 2.2). The fact that it was validated in distinct $F_{2:3}$ populations increase our confidence in the association of this marker and GLS resistance. Marker PHM4757-14 was segregating in three populations: H100_DRIL32.090, H100_DRIL32.134 and H100_DRIL52.268 (Table 2.2). Populations H100_DRIL32.090 and H100_DRIL32.134 had the same allele from donor Ki3 and the marker significant for NLB in both populations in the single marker analysis (Table 2.2). In population H100_DRIL52.268 the donor allele came from the line NC262 and the marker was significant for GLS (Table 2.2).

There were instances where the same allele was segregating in two populations but the results from single marker analysis differed (Table 2.2). This can happen if the marker is not tightly linked to the causal gene and does not always co-segregate with it. Marker PHM3457-6 was significant for all three diseases in H100_DRIL32.090 and only for GLS in H100_DRIL32.134 although the NLB p-value in this case was 0.058, only slightly above the threshold of 0.05. PZA00485-2 and PHM3457-6 in bin 2.05 were both segregating in populations H100_DRIL32.090 and H100_DRIL32.134. In population H100_DRIL32.090, PHM3457-6was significant for all three diseases, and marker PZA00485-2 was significant for GLS and NLB and had a p-value of 0.067 for SLB. In population H100_DRIL32.134 both markers were significant for GLS, as in population H100_DRIL32.090, but they were not

significant for SLB and NLB. Some of the p-values were slightly above the p-value threshold of 0.05. It is possible that the populations could have other distinct introgressions that affect MDR and that they are not being accounted for. Other examples of markers that showed different results in the single disease analysis are: marker PHM13420-11 was significant for all three diseases in the population H100_DRIL62.156 but only significant for GLS in population H100_DRIL62.030; marker PZA01886-1 was significant for SLB in population H100_DRIL32.095 but not in population H100_DRIL32.191; and marker PZA03577-1 was significant for SLB in population H100_DRIL52.055 and for GLS in population H100_DRIL52.268 (Table 2.2).

There were two distinct MDR QTLs that were validated and associated with resistance to all three diseases this study, in both cases the resistance alleles derived from NC304. These MDR QTL were identified in population H100_DRIL62.078 at bin 5.04 and in population H100_DRIL62.156 at bin 3.04. Both bins have been previously reported in disease resistance studies for all three diseases (Table 2.7). Wisser et al. (2006) made a consensus map with QTL information from previously published studies about 11 different disease resistance in different maize germplasms. They identified bin 3.04 and 3.05 as clusters of disease resistance genes. McMullen and Simcox (1995) also found bin 3.04 to be a cluster for disease resistance genes.

This study demonstrates the importance of independently evaluating QTL effects following their initial identification and before deploying them in a breeding program. In addition, the results of this study further confirm the existence of dQTL associated with multiple diseases that could be exploit in breeding programs. Future studies could fine map and investigate genes behind the MDR QTL to better understand the mechanisms that plants use for

resistance against pathogens. The most promising MDR QTL were donated from line NC304, in bin 3.04 and bin 5.04; they were validated for all three diseases.

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Foliar Diseases in a Maize Recombinant Inbred Line Population — Evidence for Multiple Disease Resistance ?

Table 2.1. $F_{2:3}$ Populations derived from crosses between a common multiple disease susceptible recurrent parent (Recurrent parent) and various disease-resistant near-isogenic lines (DRIL parent) evaluated in this study. The original donor parent, number of $F_{2:3}$ families created, and number of QTLs previously associated with disease resistance segregating in each population are also presented.

$F_{2:3}$ Population	**Recurrent parent**	**DRIL parent**	**Original donor parent**	**Number of $F_{2:3}$ families created**	**Number of QTL assessed**
H100_DRIL_32.090	H100	DRIL_32.090	Ki3	70	2
H100_DRIL_32.095	H100	DRIL_32.095	Ki3	86	2
H100_DRIL_32.134	H100	DRIL_32.134	Ki3	63	2
H100_DRIL_32.191	H100	DRIL_32.191	Ki3	86	1
H100_DRIL_52.055	H100	DRIL_52.055	NC262	68	3
H100_DRIL_52.157	H100	DRIL_52.157	NC262	101	1
H100_DRIL_52.268	H100	DRIL_52.268	NC262	77	3
H100_DRIL_62.030	H100	DRIL_62.030	NC304	49	1
H100_DRIL_62.078	H100	DRIL_62.078	NC304	87	2
H100_DRIL_62.156	H100	DRIL_62.156	NC304	63	2
H100_DRIL_72.061	H100	DRIL_72.061	NC344	79	2
H100_DRIL_72.232	H100	DRIL_72.232	NC344	63	1

Table 2.2. Results of analysis of all single marker and single disease resistance associations. Reporting only markers that were previously associated with disease resistance in each population (Lopez Zuniga et al. 2016). Population, marker, marker location (bin), traits previously associated with the marker (Lopez Zuniga et al. 2016), marker that were selected to represent the introgression in cases where more than one marker was used (Selected), number of distinctive associations in the introgression (Distinctive association), marker p-value for GLS (GLS p-value), marker p-value for SLB (SLB p-value), marker p-value for NLB (NLB p-value).

Population	Marker	Bin	Previous	Selected	Distinctive association	GLS p-value	SLB p-value	NLB p-value
H100_DRIL32.090	PHM3457-6	2.05	MDR	X	2	0.0148	0.048	0.0014
	PZA00485-2					0.047	0.0677	0.0003
	PZA00637-4		SLB			0.944	0.2505	0.3225
	PZA00379-2	8.03	MDR, SLB	X	4	0.1161	0.0418	0.0021
	PHM4757-14		MDR, GLS, NLB			0.0841	0.1566	0.005
H100_DRIL32.095	PHM1745-16	3.04	MDR		1	0.0759	0.3306	0.4362
	PZA01886-1	9.04	MDR, SLB		2	0.1608	0.0021	0.2098
H100_DRIL32.134	PHM3457-6	2.05	MDR			0.0067	0.1091	0.0586
	PZA00485-2			X	1	0.0002	0.0569	0.0727
	PHM4757-14	8.03	MDR, GLS, NLB		3	0.064	0.7353	<.0001
H100_DRIL32.191	PHM4495-14	9.03	MDR, SLB		2	0.7377	0.1658	0.0472
	PZA01886-1	9.04	MDR, SLB		2	0.0984	0.4386	0.6653
H100_DRIL52.055	PZA03577-1	2.07	MDR		1	0.8099	0.0199	0.2669
	PHM4720-12	9.03	SLB		1	0.8869	0.2741	0.6136
	PZA00060-2	9.04	MDR		1	0.465	0.0224	0.02
	PZA02519-7	10.04	MDR, SLB		3	0.977	0.1484	0.6371
	PZA02969-9		GLS			0.4391	0.3221	0.8482
H100_DRIL52.157	PHM4185-13	1.05	MDR, SLB, NLB		3	0.6451	0.4918	0.7396
	PHM5480-17		SLB			0.1304	0.9009	0.8308

Table 2.2. continued

Population	Marker	Bin	Previous	Selected	Distinctive association	GLS p-value	SLB p-value	NLB p-value
H100_DRIL52.268	PZA03577-1	2.07	MDR		1	0.0088	0.1047	0.6988
	PHM14618-11	4.05	NLB		1	0.8279	0.1815	0.5258
	PHM4757-14	8.03	GLS		1	0.0459	0.6461	0.6885
H100_DRIL62.030	PHM13420-11	3.04	MDR, GLS		2	0.0069	0.9588	0.1513
H100_DRIL62.078	PZA01926-1	4.05	MDR, SLB			<.0001	0.3494	0.1194
	PZA00492-26					0.0001	0.1552	0.0148
	PHM9635-30		GLS	X	3	0.0005	0.0765	0.0045
	PZA02209-2	5.04	MDR		1	0.0388	0.0102	0.0049
H100_DRIL62.156	PHM13420-11	3.04	MDR, GLS	X	3	0.0002	<.0001	0.0005
	PZA00667-1		SLB			0.0013	<.0001	0.0002
	PHM4677-11	8.03	GLS		1	0.4629	0.3404	0.9757
H100_DRIL72.061	PHM4586-12	2.05	GLS			0.8148	0.7092	0.6987
	PHM14412-4		MDR, GLS	X	2	<.0001	0.2839	0.4154
	PHM9914-11	3.04	GLS			0.8668	0.0006	0.0071
	PZA00667-1		MDR	X	2	0.388	0.0052	0.0015
H100_DRIL72.232	PHM4586-12	2.05	GLS		1	0.2727	0.0609	0.0065

Table 2.3. Pairwise correlations between diseases by population. Diseases: Gray Leaf Spot (GLS), Southern Leaf Blight (SLB), Northern Leaf Blight (NLB). Correlation coefficient and sample number (n).

Population	**H100_DRIL32.090**		**H100_DRIL32.095**		**H100_DRIL32.134**		**H100_DRIL32.191**	
Disease	SLB	GLS	SLB	GLS	SLB	GLS	SLB	GLS
NLB	0.62** (n=58)	0.33** (n=63)	0.26* (n=77)	0.28* (n=84)	0.32* (n=57)	0.32* (n=60)	0.31** (n=78)	0.18 (n=82)
GLS	0.44** (n=65)		0.25* (n=77)		0.53** (n=58)		0.37** (n=82)	
Population	**H100_DRIL52.055**		**H100_DRIL52.157**		**H100_DRIL52.268**		**H100_DRIL62.030**	
Disease	SLB	GLS	SLB	GLS	SLB	GLS	SLB	GLS
NLB	0.26* (n=59)	0.003 (n=61)	0.08 (n=90)	-0.04 (n=96)	0.2 (n=69)	-0.09 (n=72)	0.32** (n=68)	0.19 (n=76)
GLS	0.27* (n=62)		0.14 (n=93)		0.29* (n=73)		0.03 (n=74)	
Population	**H100_DRIL62.078**		**H100_DRIL62.156**		**H100_DRIL72.061**		**H100_DRIL72.232**	
Disease	SLB	GLS	SLB	GLS	SLB	GLS	SLB	GLS
NLB	0.31** (n=68)	0.19 (n=76)	0.38** (n=54)	0.5** (n=58)	0.13 (n=72)	0.13 (n=75)	0.01 (n=53)	0.17 (n=55)
GLS	0.03 (n=74)		0.21 (n=56)		0.19 (n=74)		0 (n=56)	

** Significant at the *P*=0.001 level

* Significant at the *P*=0.05 level

Table 2.4. GLS model parameters (variance components for random effects and F-value for fixed effects), estimates and their respective standard errors, and the fixed effect F-test for standardized area under disease progress curve (sAUDPC)

Cov Parm	Estimate	Standard Error	Pr > Z
loc	0.010	0.021	0.314
rep(loc)	0.003	0.007	0.356
range(loc)	0.037	0.009	<.0001
column(loc)	0.023	0.005	<.0001
Residual	0.170	0.006	<.0001
Fixed Effect	**F Value**	**Pr > F**	
line	2.76	<.0001	

Table 2.5. NLB model parameters (variance components for random effects and F-value for fixed effects), estimates and their respective standard errors, and the fixed effect F test for standardized area under disease progress curve (sAUDPC)

Cov Parm	Estimate	Standard Error	Pr > Z
rep	89.8561	127.27	0.2401
range(rep)	1.4984	0.3864	<.0001
column(rep)	4.3262	0.8025	<.0001
Residual	4.1511	0.2091	<.0001
Fixed Effect	**F Value**	**Pr > F**	
DTA	66.88	<.0001	
line	2.59	<.0001	

Table 2.6. SLB model parameters (variance components for random effects and F-value for fixed effects), estimates and their respective standard errors, and the fixed effects F test for standardized area under disease progress curve (sAUDPC)

Cov parameter	Estimate	Standard Error	Pr > Z
rep	0.127	0.182	0.243
range	0.043	0.012	0.0002
column	0.061	0.015	<.0001
Residual	0.169	0.009	<.0001
Fixed Effect	**F Value**	**Pr > F**	
DTA	35.57	<.0001	
line	2.64	<.0001	

Table 2.7. Results from selected markers to represent each introgression. Multiple disease resistant donor line (Donor), population name (Pop), number of F 2:3 families assessed (N), marker, chromosome (Chr), position (cM) on IBM4 genetic map, bin, trait previously associated with the marker (Lopez Zuniga et al. 2016) (Previous trait), additive effect previously associated with the marker (Prev. add) (Lopez Zuniga et al. 2016), disease that was associated with in this study (disease), additive effect (a), dominance effect (d), trait that was validated by this study (validated). All markers are significant at P=0.05.

Donor	Pop	N	Marker	Chr	cM	Bin	Previous trait	Prev. add	Disease	a	d	Validated
KI3	H100_DRIL32.090	70	PHM3457-6	2	96.4	2.05	MDR	0.39	GLS	0.18	NS	MDR
		63							NLB	0.07	NS	
KI3	H100_DRIL32.090	63	PZA00379-2	8	59.45	8.03	MDR, SLB	0.27, 0.28	SLB	0.16	NS	MDR, SLB
		63							NLB	0.10	NS	
KI3	H100_DRIL32.095	77	PZA01886-1	9	114.68	9.04	MDR, SLB	0.50, 0.29	SLB	0.30	NS	SLB
KI3	H100_DRIL32.134	61	PZA00485-2	2	99.07				GLS	0.28	NS	
KI3	H100_DRIL32.134	61	PHM4757-14	8	89.8	8.03	MDR, GLS, NLB	0.24, 0.16, -2.73	NLB	0.13	NS	NLB
KI3	H100_DRIL32.191	82	PHM4495-14	9	59.01	9.03	MDR, SLB	0.36, 0.25	NLB	0.05	NS	
NC262	H100_DRIL52.055	62	PZA03577-1	2	195.72	2.07	MDR	0.36	SLB	-0.19	NS	
NC262	H100_DRIL52.055	62	PZA00060-2	9	114.49	9.04	MDR	0.33	SLB	0.18	NS	MDR
		63							NLB	0.03	NS	
NC262	H100_DRIL52.268	76	PZA03577-1	2	195.72	2.07	MDR	0.36	GLS	0.13	NS	
NC262	H100_DRIL52.268	76	PHM4757-14	8	98.8	8.03	GLS	0.35	GLS	-0.11	NS	
NC304	H100_DRIL62.030	32	PHM13420-11	3	92.11	3.04	MDR, GLS	0.54, 0.17	GLS	0.32	NS	GLS
NC304	H100_DRIL62.078	82	PHM9635-30	4	96.04	4.05	GLS	0.17	GLS	0.26	NS	GLS
		76							NLB	0.05	NS	
NC304	H100_DRIL62.078	82	PZA02209-2	5	124.04	5.04	MDR	0.25	GLS	0.15	NS	MDR
		74							SLB	0.14	NS	
		76							NLB	0.06	NS	

Table 2.7. (Continued)

Donor	Pop	N	Marker	Chr	cM	Bin	Previous trait	Prev. add	Disease	a	d	Validated
NC304	H100_DRIL62.156	61	PHM13420-11	3	92.11	3.04	MDR, GLS	0.54, 0.17	GLS	0.27	NS	MDR, GLS
		56							SLB	0.43	NS	
		58							NLB	0.08	0.14	
NC344	H100_DRIL72.061	78	PHM14412-4	2	127.4	2.05	MDR, GLS	0.30, 0.16	GLS	0.27	0.54	GLS
NC344	H100_DRIL72.061	75	PZA00667-1	3	96.67	3.04	MDR	0.35	SLB	0.21	NS	MDR
		75							NLB	0.07	NS	
NC344	H100_DRIL72.232	56	PHM4586-12	2	79.29	2.05	GLS	0.14	NLB	-0.07	NS	

Table 2.8. Summary of QTL for SLB, NLB and GLS identified in previous studies on bins 3.03 and 5.04. Table includes BIN, marker, chromosome (Chr), population type (pop), disease (Dis), mapping method (Method) and reference.

BIN	Marker	Chr	Pop	Dis	Method	Reference	cM
3.03/3.04	asg48-phi036	3	B73*Mo17 (RILs)	SLB	CIM	(Carson et al. 2004)	50.9
3.04	UMC10	3	F2:3, ADENT*B73rhm	GLS	SIM	(Bubeck et al. 1993)	31.7
3.04	PIO200508	3	F2:3, ADENT*B73rhm	GLS	SIM	(Bubeck et al. 1993)	37.7
3.04	BNL10.24	3	F2:3, ADENT*B73rhm	GLS	SIM	(Bubeck et al. 1993)	53.6
3.04	us41	3	Propietary F2	GLS	CIM	(Lehmensiek et al. 2001)	.
3.04	PHM4621.57	3	NAM	NLB	GWAS	(Poland et al. 2011)	76.5
3.04	phi036-bnlg602	3	NC300*B104 (RILs)	SLB	MIM	(P J Balint-Kurti and Carson 2006)	19.8
3.04	PZA02077	3	NILs, Teosinte*B73	SLB	Jlinkage	(Lennon et al. 2014)	50.2
3.04	PZA00828	3	NILs, Teosinte*B73	SLB	Jlinkage	(Lennon et al. 2014)	70.6
3.04	PHM4145_18	3	B73*CML254, B97*CML254/Ki14 (RILs)	SLB	Joint Analysis	(Negeri et al. 2011)	71.8
3.04	umc2275-umc2008	3	T14*T4 F2:3	SLB	CIM	(P. Liu 2011)	107.83
3.04	umc2275-umc2008	3	T14*T4 F2:3	SLB	CIM	(P. Liu 2011)	109.83
3.04	npi446-umc2000	3	B73*Mo17 (AIRIL)	SLB	CIM	(P J Balint-Kurti et al. 2007)	164-166
3.04	mmp69-umc1920	3	B73*Mo17 (AIRIL)	SLB	CIM	(P J Balint-Kurti et al. 2007)	217-258
3.04	PHM4204.69-PHM2343.25	3	NAM	SLB	GWAS	(Kump et al. 2011)	49.2-53.4
3.04	asg48-phi036	3	B73*Mo17 (RILs)	SLB	CIM	(P J Balint-Kurti and Carson 2006)	50.9-52.50
3.04/3.05	umc010-umc 389b	3	F2:3 Lo951*CML202	NLB	CIM	(Welz, Schechert, et al. 1999)	95-140
5.03/5.04	umc001-bnl5.40	5	F2:3 Lo951*CML202	NLB	CIM	(Welz, Schechert, et al. 1999)	40-100
5.03-5.04	umc1171-bnlg1046	5	BC1F4, Y32*Q11	GLS	Linkage analysis	(Zhang et al. 2012)	112.8-151.9

Table 2.8. (Continued)

BIN	Marker	Chr	Pop	Dis	Method	Reference	cM
5.04	bnlg150	5	Propietary F2	GLS	CIM	(Lehmensiek et al. 2001)	80.5
5.04	UMC43 UMC40	5	BC1S5, FR1141*O61	GLS	CIM	(Clements et al. 2000)	82
5.04	UMC40 BNL7.71	5	BC1S5, FR1141*O61	GLS	CIM	(Clements et al. 2000)	84
5.04	ASG71 CSU440	5	BC1S5, FR1141*O61	GLS	CIM	(Clements et al. 2000)	140
5.04	BNL6.22-UMC51	5	HighLand*LowLand	NLB	CIM	(Jiang et al. 1999)	96.8
5.04	PHM532.23	5	NAM	NLB	GWAS	(Poland et al. 2011)	101.2
5.04	csu36a−bnl7.71	5	F3 D32*D145	NLB	CIM	(Welz, Xia, et al. 1999)	112
5.04	BNL5.7I-UMC51	5	B52*Mo17 F2:3	NLB	SIM	(Freymark et al. 1993)	115.4
5.04	bnl5.40-npi461	5	F2:3 Lo951*CML202	NLB	CIM	(Welz, Schechert, et al. 1999)	120-150
5.04	PZA03049.24-PZB01017.1	5	NAM	SLB	GWAS	(Kump et al. 2011)	69.5-73.5
5.04/5.05	umc068-bnl5.24	5	F2:3 Lo951*CML202	NLB	CIM	(Welz, Schechert, et al. 1999)	160-200

Acronyms: CIM: composite interval mapping; GWAS: genome wide association study; JA: joint analysis; JL: joint-linkage analysis; LA: Linkage analysis; MIM: multiple interval mapping; SIM: simple interval mapping.
Note: Adapted from (Lopez Zuniga et al. 2016)

Proportion of Donor Background	Homozygosity of introgression
50%	0%
25%	0%
12.5%	0%
6.25%	0%
6.25%	33%
6.25%	60%
6.25%	78%
6.25%	78%

Figure 2.1. Scheme used to represent the production of chromosome segment substitution lines population in previous study. Reprinted from Lopez Zuniga et al. (2016)

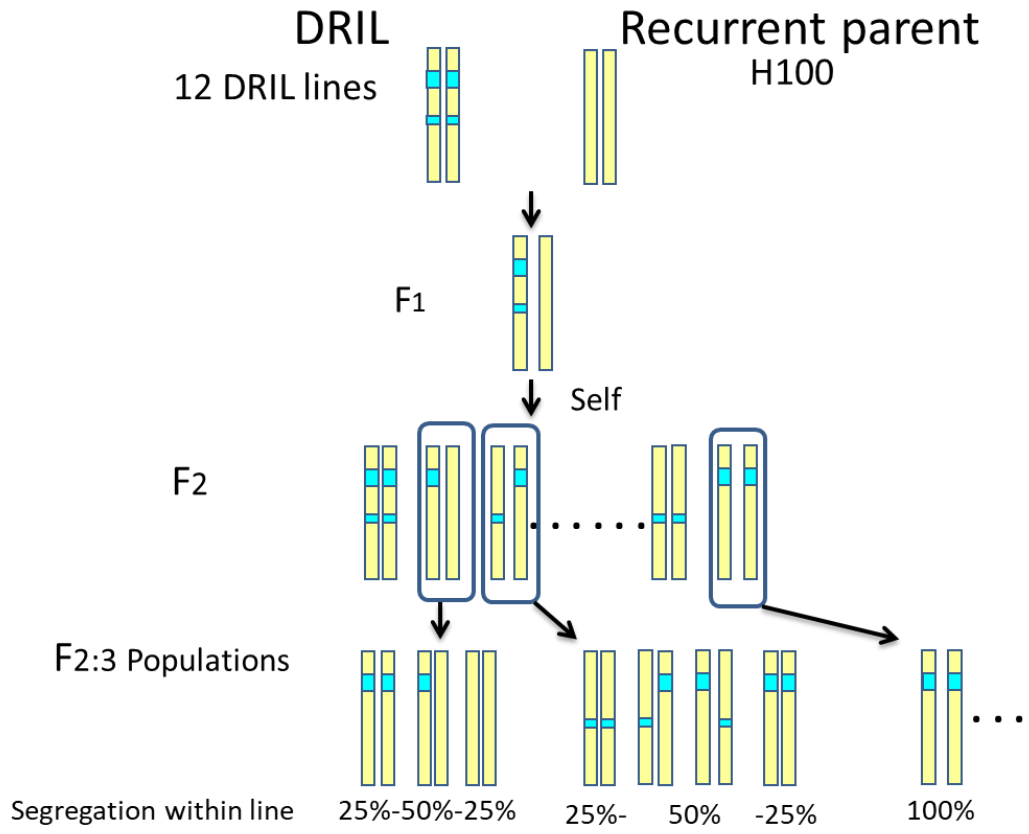

Figure 2.2. Scheme used to produce populations of $F_{2:3}$ families and the QTL segregation within line.

CHAPTER III

CONIDIA ADHESION IN DIFFERENT MAIZE GENOTYPES

Abstract

Southern Leaf Blight (SLB) caused by the Ascomycete *Cochliobolus heterostrophus* is one of the main foliar diseases of maize. The first step for a fungus to penetrate the host is to become attached to the host surface with extra-cellular adhesive material. A previous study investigated the attachment process of *C. heterostrophus* to different surfaces and developed an adhesion timeline. In this study, we explored whether host's genotype affects the efficiency of *C. heterostrophus* conidia adhesion and if that is correlated in apparent field disease resistance. To do so, we used a novel protocol aimed to develop a timeline of conidia adhesion using juvenile and adult leaf samples of lines Mo17 and B73, which have contrasting disease resistance phenotypes. We also evaluated the number of conidia attached on a juvenile leaf sample of the lines Mo17, B73, P39, HP301, NC350 and Ki3. The number of conidia attached and previously reported field disease resistance had a correlation of -0.55, indicating that more resistance lines have a tendency to have less conidia attached. We believe that conidia adhesion could be one aspect of SLB resistance, but more studies should be conducted.

Introduction

Some plant pathogens, such as virus, bacteria and protozoa use vectors to be transported directly inside plant cells. In contrast, most fungi, bacteria, and parasitic higher plants first have contact with the external surface of their host plants and must find ways of entering the organism. The first step to penetrate the host is to become attached to the host surface with extra-cellular adhesive material (ECM) that can vary in composition and environmental factors necessary to become adhesive. This step will enable the spores to germinate without being washed from the leaf surface. Before pathogen inoculation, it is possible to disrupt adhesion by the use of film forming compounds, such as mineral oils, antitranspirant polymers, surfactants and kaolin-based particle films (Agrios, 2005; Braun & Howard, 1994).

Cochliobolus heterostrophus is an Ascomycete that causes Southern Leaf Blight (SLB) in maize and is one of the main foliar diseases of the crop. *C. heterostrophus* produces both conidia (asexual spores) and ascopores (sexual spores). Braun and Howard (1994) detailed the adhesion of *C. heterostrophus* conidia to different surfaces. They concluded that the adhesion was non-specific and surface hydrophobicity of the surface did not affect adhesion. The spores were able to adhere to glass, Teflon, polystyrene, cellophane and leaves. A timeline of attachment to glass was performed; the conidia started to adhere after 20 minutes of hydration and reached ninety percent of adhesion at 50 minutes of hydration. With light microscopy they were able to show that adhesion occurred with the appearance of ECM at the tip of the conidia before germination started. Based on staining reactions, they determined that the ECM is likely made of proteins and mycopolyssaccharides. In the presence of cycloheximide, a protein synthesis inhibitor, no matrix was formed and there was no spore attachment, proving that matrix secretion was dependent on active metabolism (Braun & Howard, 1994).

Maize has two quite distinct leaf morphologies- adult and juvenile leaves. Adult maize leaves are wider than juvenile leaves and have thicker cuticle, epidermal hairs and bulliform cells which juvenile leaves lack. Unlike adult leaves, juvenile leaves have epicuticular wax and wavy cell walls (Poethig, 1990; Sylvester et al., 2001). Juvenile leaves appear duller on the growing plant and adult leaves appear shinier. The juvenile to adult transition occurs at about leaf 7-8 in most maize lines. It is important to note that the term 'young leaves' is distinct from 'juvenile leaves'. Young leaves are simply the most recently emerged leaves and can be adult or juvenile. A juvenile leaf will never be an adult leaf, but a young leaf will become an old leaf.

In this study we aim to develop a protocol to investigate adhesion of *C. heterostrophus* to maize leaf surface in the juvenile and adult phase. We wanted to explore whether host's genotype affects the efficiency of adhesion and if that is correlated in apparent disease resistance in field conditions.

Materials and methods

Plants

We wanted to test if there was a difference in conidia adhesion in the juvenile and adult leaves from the vegetative phase. The juvenile leaves harvested were the second fully expanded leaf from plants at V3. The adult leaves harvested were the seventh adult leaf (11^{th} plant node). For the experiment, plants were grown in the Method Road Greenhouses facilities at North Carolina State University during spring, summer and fall of 2018.

Spore Adhesion Protocol

We designed a protocol that would allow the analysis of the conidia attachment to the leaf and that would control for other plant architecture traits that could interfere in conidia adhesion, such as leaf angle and canopy. A green fluorescent strain of *C. heterostrophus* (Maor et al., 1998), was propagated on PDA plates, in a Percival growth chamber with a 11/13 hour light /dark cycle at a constant 27^{o}C. Conidia for all experiments were obtained from 15- to 17-day-old cultures. For the assays, a conidial suspension of $4x10^{9}$ conidia/mL was prepared in 500 µL/L Tween and .05g/L agar. Fresh conidia were harvested for each experiment. Whole leaves were cut from the plant and transported in a cooler container to the laboratory where the experiment was done. Only the middle third of the leaf's length was used, and the mid-rib was discarded. Using a hole-punch with a diameter of 5 mm, three leaf discs with the abaxial side up were fixed with clear polish to a glass microscope slide (Figure 3.1). 50 mL Propylene conical tubes were filled up to 50mL with the conidia suspension and the slides were placed inside the tubes. The tubes were put in a horizontal position in a rack in a shaker at speed of 50 rpm for inoculation. After the designated time prescribed by each experiment, the samples were removed

from the shaker, and the slides were individually placed in a petri-dish. Each dish was carefully flooded with water, without aiming the water directly on top of the slide, and then the water from the dish was discarded. The dishes were flooded twice in order to wash off the conidia that were not firmly attached.

Experimental Design

Three experiments were conducted, once each, using a completely randomized design. The first experiment developed a conidia adhesion timeline using juvenile leaves of two maize lines that contrast for multiple disease resistance (MDR); Mo17, an MDR line, and B73, a multiple disease susceptible (MDS) line. For logistical reasons, the maximum number of experimental units that could be run in each experiment was 24. In a preliminary experiment we observed conidia germination starting to occur at 60 minutes of incubation. As we wanted to measure the adhesion before germination, 60 minutes became the latest time point. To develop a timeline of spore adhesion, the time-points were 20, 30, 40, 50, 60 minutes of incubation with two replicates for each time point and genotype, Mo17 and B73. The second experiment aimed to develop a conidia adhesion timeline in adult leaves of the lines Mo17 and B73 and it used the same time points as experiment one and two replicates for each time and genotype combination. The third and last experiment evaluated conidia adhesion using juvenile leaves of six different maize lines: B73, Mo17, P39, NC350, Ki3 and HP301. The lines are a subset of the founder lines of the Nested Association Mapping (NAM) population (McMullen et al., 2009), they represent a small part of maize diversity and have been evaluated for SLB resistance in the field (Kump et al., 2011). To test if there was difference in conidia adhesion in the six different maize lines, samples were incubated for 50 minutes and four replicates of each genotype were used.

Imaging and Processing

Pictures of each sub-sample were taken with an Olympus MVX10 Microscope under a GPF filter. The images were processed using a macro (Appendix A) written for ImageJ software (Schneider, C. A.; Rasband, W. S. & Eliceiri, K. W., 2012) to determine the percentage of green pixels and number of conidia. The percentage coverage area was determined by selecting a rectangular shape, that eliminated any border effect and represented the sample, and then calculating the ratio of green to white pixels within the shape (Image 3.2). To determine approximate conidia count, the number of pixels constituting a conidium was averaged and used to divide the total number of green pixels within the shape. To control for software and picture variation, the samples were run through the macro twice and the results were averaged.

Data Analysis

Conidia count of the three leaf punches in a slide were averaged to constitute a replicate. Data were analyzed using the PROC GLM procedure in SAS (version 9.4). For the experiments 1 and 2, which developed a timeline of conidia adhesion, the response variable was number of spores adhered and fixed effects were genotype, minutes and their interaction. Means for the genotype and minutes of incubation effect were calculated using the LSMeans statement. For experiment 3, to test if the six maize lines differed in the number of conidia adhered, a model with genotype as fixed effect was used. Means for each genotype were calculated using the LSMeans statement. Person correlation was calculated to compare the result of experiment 3 and field resistance to SLB.

Results and Discussion

In experiment 1, we tested *C. heterostrophus* conidia adhesion to juvenile leaves of the maize lines Mo17 and B73 at 20, 30, 40, 50 and 60 minutes, and the main effects of genotype and time and their interaction were significant at p-value of 0.05 (Table 3.1). From 20 minutes of incubation to 40 minutes of incubation, there was no significant difference in the genotypes (Table 3.2, Figure 3.3). At 50 and 60 minutes of incubation, B73 had a significantly higher number of conidia adhered to the leaf (Table 3.2). At 50 minutes, B73 had more than double the number of conidia adhered than Mo17 (Figure 3.3). The adhesion timeline differs from that of Braun and Howard (1994). In their results the steepest part of the curve was between 20 and 40 minutes, while in this experiment it was after 40 minutes. However, this study used leaf surfaces while Braun and Howard (1994) used a glass surface, the difference in the surface could result in a different adhesion progression.

In experiment 2 the timeline essay was performed in the adult plant leaves, and virtually no conidia adhered, so no data analysis was performed. Over 15 years of field evaluations of SLB, we observed reduced disease symptoms in the vegetative adult plant than in the reproductive adult plant. If spores are less likely to attach at this growth stage, it could explain why there are less disease symptoms. We also have observed that inoculations performed in the vegetative adult plant in the greenhouse are rarely successful; whereas inoculations of juvenile plants or reproductive adult plants regularly result in development of the pathogen. Therefore, the results from the current experiment may provide some explanation for our previous observations.

For experiment 3, our hypothesis was that the variation in conidia attachment efficiency on different maize lines might be a component of apparent disease resistance. Juvenile leaves

from six lines that differed for SLB resistance were compared in the conidia attachment assay. Since we were able to observe some conidia starting to germinate in some samples at 60 minutes of incubation and we wanted to study adhesion before germination, we chose to assess the conidia adhesion differences in a broader set of maize genotypes at 50 minutes of incubation. Both in the development of the timeline (Table 3.2) and when comparing a set of six NAM founders the lines (Table 3.4) Mo17 and B73 behaved as we expected, with fewer conidia attaching to the MDR line Mo17 than to the MDS line B73, although not a significant difference (Table 3.5).

To verify if less conidia adhesion correlates to less disease in the field, we compared the mean number of spore adhered to a genotype and the Best Linear Unbiased Prediction value (BLUPs) of field score reported by Kump et al (2011) (Table 3.4, Figure 3.5). The rating scale for SLB resistance in the field ranges from 1-9, being 1 very susceptible and 9 resistant. Based on field resistance scores for SLB, three lines are considered resistant (Ki3, Mo17 and NC350) and three lines are considered susceptible (B73, P39 and HP301). We expected that lines that had higher resistance in the field (higher score in the rating scale), would have less conidia adhered do them, and a negative correlation would be observed between field resistance and number of conidia adhered to the leaf. Lines Ki3 and B73 showed opposite results that we expected. Ki is SLB resistant in the field and had large number of conidia attached and B73 if field susceptible and had a low number of conidia attached. We obtained a correlation of -.55, indicating that spore adhesion could be a component of field disease resistance.

There are many reasons why conidia adhesion would not perfectly correlate with field resistance. The most important reason is that conidia adhesion would be only one among many causes of resistance. Not all lines would necessarily deploy less adhesion as a resistance factor

and we only tested in 6 different maize lines, a very small sample of the maize diversity. Furthermore, disease severity in the field is measured in adult plants in the reproductive stage and conidia adhesion was measured in juvenile leaves at vegetative stage. In this study we showed that the plant genotype has an effect on spore adhesion. Future studies could broaden the number of different lines being tested for conidia adhesion; a different protocol for conidia adhesion could be made in order to validate the results and conidia adhesion could be tested in leaves from adult plants in the reproductive stage.

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Table 3.1. Analysis of variance for *C. heterostrophus* conidia attachment in maize lines Mo17 and B73 after five different inoculation times. Results from experiment 1.

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Genotype	1	40460.4	40460.4	8.74	0.0144
Minutes	4	515496.2	128874.0	27.85	<.0001
G*M	4	129372.6	32343.2	6.99	0.0059
Error	10	46270.9	4627.1		

Table 3.2. Differences in the least squares means of number of *C. heterostrophus* conidia attached to a 5-mm leaf sample in lines B73 and Mo17 after different time of incubation (Minutes). Results from experiment 1.

Minutes	Count difference	P
20	2	0.976
30	-102	0.179
40	-15	0.836
50	271	0.004
60	293	0.002

Table 3.3. Analysis of variance table for *C. heterostrophus* conidia attachment in six different maize lines. Results from experiment 3.

Source	DF	Sum of Squares	Mean Square	Error DF	F Value	Pr > F
Genotype	5	182042	36408	11	2.31	0.1144
Residual	11	173075	15734			

Table 3.4. Comparison of number of *C. heterostrophus* conidia that adhered to maize leaves (Adhesion LSMean) and maize resistance to *C. heterostrophus* in the field (Field Disease resistance scores) in six different maize lines (Genotype). Best Linear Unbiased Prediction value (BLUPs) of field score for SLB resistance reported by Kump et al (2011). Lower BLUP values are associated with less field disease resistance.

Genotype	Adhesion LSMean	Field Disease resistance scores (BLUPs)
Mo17	185.95	8.04
NC350	302.62	7.90
B73	304.69	5.56
P39	431.83	5.15
Ki3	465.36	7.52
HP301	528.47	5.41

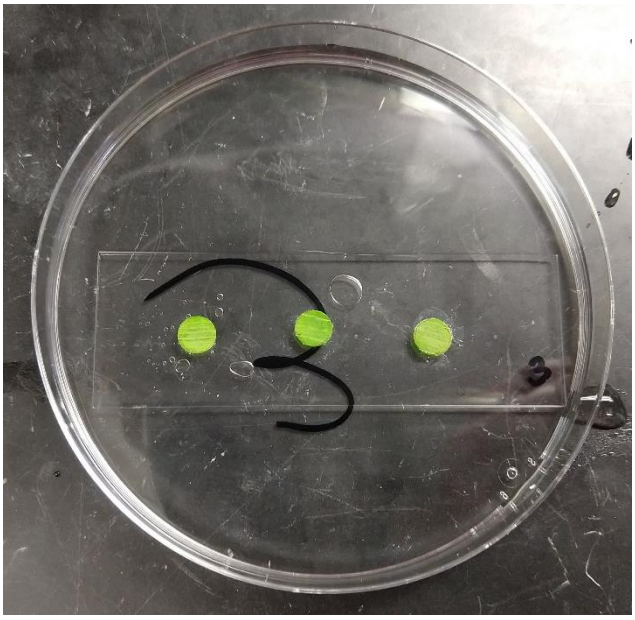

Figure 3.1. Leaf samples of 0.5cm in diameter attached to a glass slide

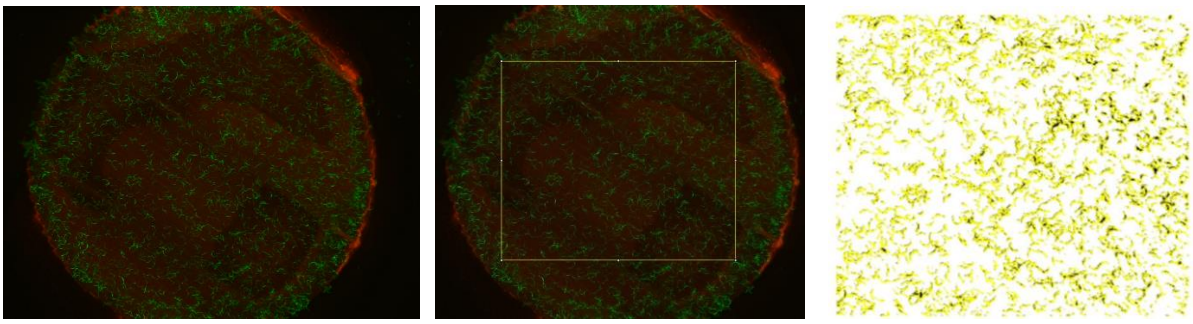

Figure 3.2. Image analysis steps. Picture of the leaf punch using a microscope with GFP filter (conidia in green), rectangle draw to select the area to have the conidia counted, distinction of the pixels that represent the conidia (yellow).

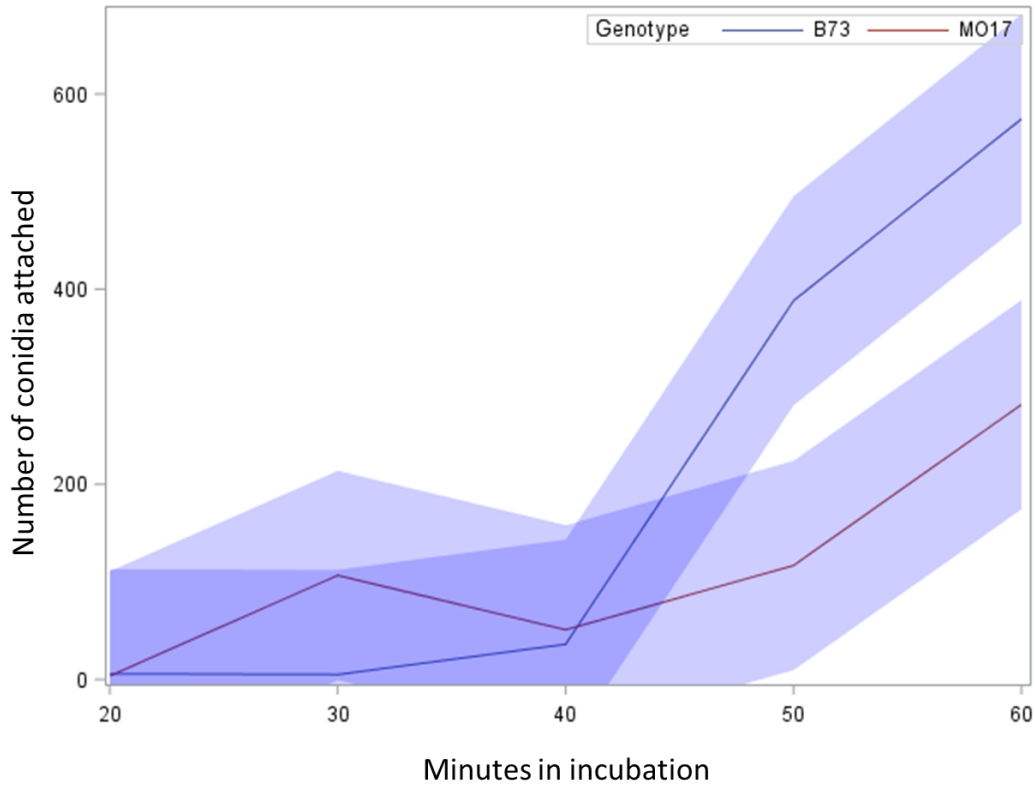

Figure 3.3. Timeline of *C. heterostrophus* conidia adhesion in the abaxial surface of a 5mm leaf sample of maize lines Mo17 and B73. Shaded area delimits the confidence interval. Results from experiment 1.

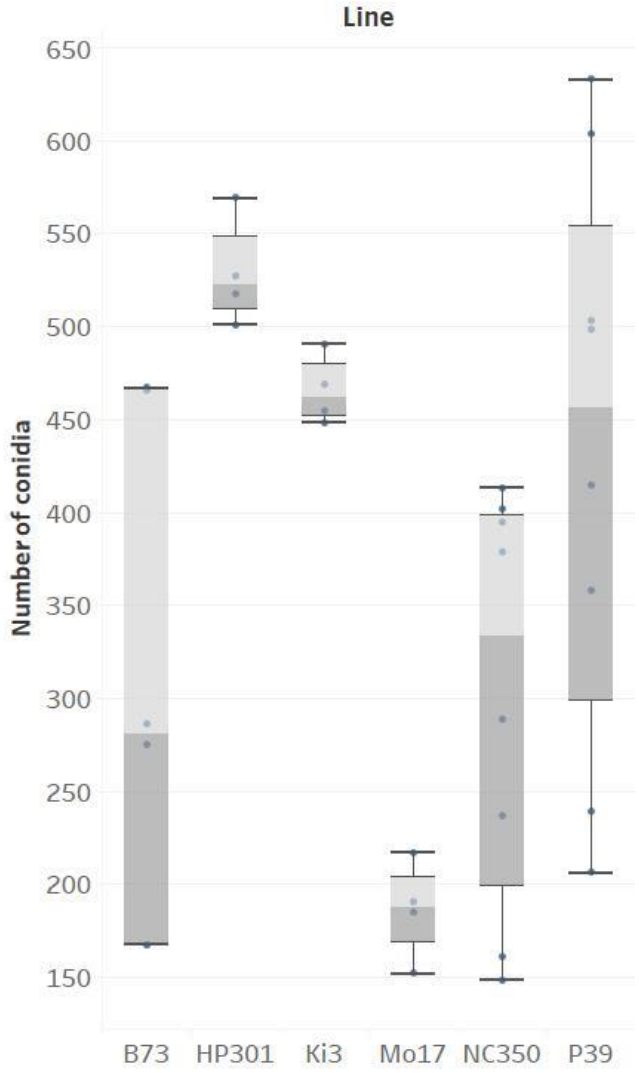

Figure 3.4. Boxplot graph of *C. heterostrophus* conidia adhered in the abaxial surface of a 5mm leaf sample of six different maize lines. Results from experiment 3.

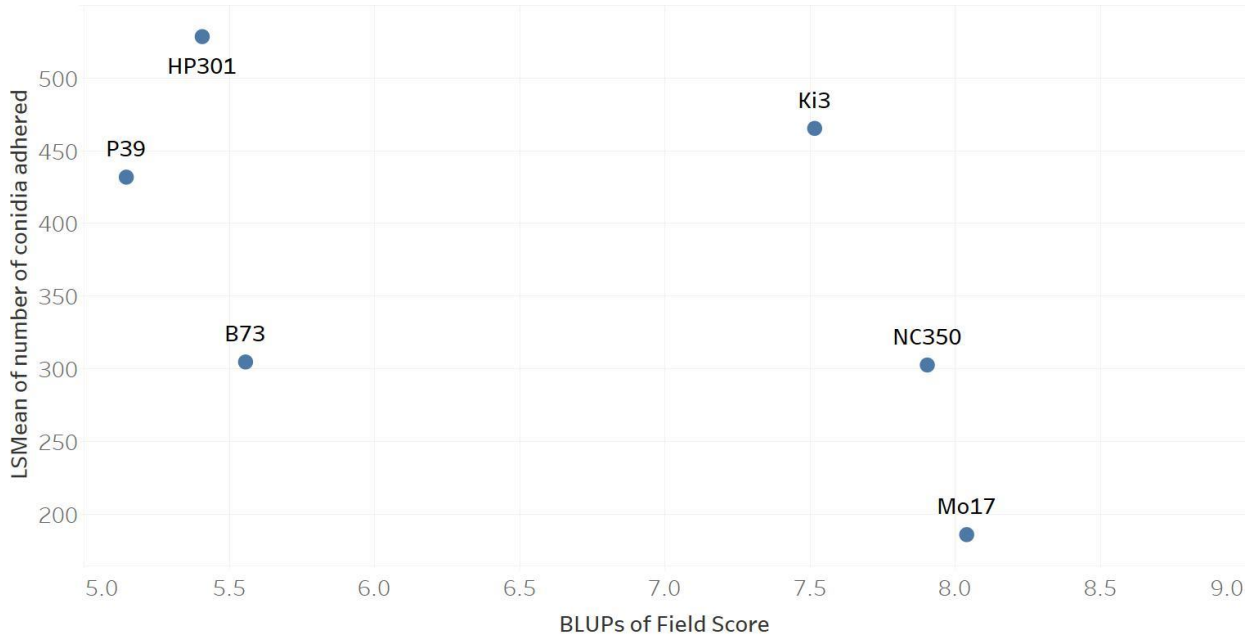

Figure 3.5. Scatterplot of the LSMeans of number of *C. heterostrophus* conidia adhered to a 5mm sample of the juvenile leaf and the Best Linear Unbiased Prediction value (BLUPs) of field score for SLB resistance reported by Kump et al (2011). Lower BLUP values are associated with less field disease resistance. Each dot represents a different maize line.

APPENDICES

Appendix 1. Marker, Chr (chromosome), chromosome position (cM) in IBM 4 map (IBM4_cM) and physical position based on B73 version 3 map (PublicV3_POS)

Marker	Chr	IBM4_cM	PublicV3_POS
PHM13360-13	2	98.62	111456119
PHM13420-11	3	92.11	158556508
PHM14412-4	2	127.4	203610640
PHM14618-11	4	117	181259942
PHM1745-16	3	85.92	143928532
PHM3457-6	2	96.4	63555891
PHM4185-13	1	113.04	83790877
PHM4495-14	9	59.01	26832690
PHM4586-12	2	79.29	30307725
PHM4677-11	8	90.36	153401883
PHM4720-12	9	44.98	17007768
PHM4757-14	8	89.8	152292467
PHM4880-179	2	98.5	107819754
PHM5480-17	1	169.08	204802481
PHM5805-19	8	71	121753452
PHM9635-30	4	96.04	165703732
PHM9914-11	3	95.07	161300790
PZA00060-2	9	114.49	129272199
PZA00379-2	8	59.45	66897042
PZA00485-2	2	99.07	124688681
PZA00492-26	4	91.78	161102050
PZA00637-4	2	106.37	173044019
PZA00667-1	3	96.67	162786508
PZA01886-1	9	114.68	134140227
PZA01926-1	4	90.25	159099990
PZA02209-2	5	124.04	181262867
PZA02519-7	10	91.95	141609305
PZA02731-1	2	125.76	200742661
PZA02969-9	10	97.93	143487977
PZA03577-1	2	195.72	237178781

Appendix 2. Macro used in the ImageJ software to analyze images from the leaf samples containing spores

```
// Create coordinate array
Xcoor = newArray(1);
Ycoor = newArray(1);
wS = newArray(1);
hS = newArray(1);
// Calibrate Color Threshold
path = File.openDialog("Select Image to Calibrate for Analysis");
open(path);
Threshold = getTitle();
run("Color Threshold...");
waitForUser("Adjust Threshold Color to Calibrate Image: KEEP WINDOW OPEN, Press OK");
makeRectangle(356, 200, 865, 640);
waitForUser("Adjust Rectangle Size for Analysis of All Images");
getSelectionBounds(Xcoor[0],Ycoor[0],wS[0],hS[0]);
// Save coordinate array to variable
xc = Xcoor[0];
yc = Ycoor[0];
wt = wS[0];
ht = hS[0];
selectWindow(Threshold);
run("Close");
// Select Directory for Image Analysis
dir = getDirectory("Choose Source Directory for the Analysis");
list = getFileList(dir);
// Choose Image to Start
// If Function Aborts Half Way Through, Select Next Image in Folder
Dialog.create("Start Image");
Dialog.addNumber("Input Starting Image Number for ANALYSIS:", 1);
Dialog.show();
imageStart = Dialog.getNumber() - 1;
// Loop Through All Images in Folder for
(i=imageStart; i<list.length; i++)
{
open(dir + list[i]);
image = getTitle();
//setTool("rectangle");
makeRectangle(xc, yc, wt, ht);
```

```
waitForUser("Adjust Shape Position, PRESS OK");
getSelectionBounds(Xcoor[0],Ycoor[0],wS[0],hS[0]);
// Save coordinate array to variable xc = Xcoor[0];
yc = Ycoor[0];
wt = wS[0];
ht = hS[0];
// Create selection for mask
run("Specify...", "width=wt height=ht x=xc y=yc");
roiManager("Add");
// Create mask to measure from on original image
run("Create Mask");
// Create new image to calculate percentage
imageCalculator("OR create", image, "Mask");
selectWindow("Result of "+image);
// Set threshold to select only green pixels
selectWindow("Threshold Color");
waitForUser("Select Color Threshold and Press OK");
// Measure region of interest
run("Measure");
Pixel = newArray(1);
Pixel[0] = getResult('Area', nResults - 1);
Total = ht*wt;
AvgSpore = 125;
// Make new measures to get percentage and spore count
setResult("Percent", nResults - 1, (Pixel[0]/Total)*100);
setResult("Spores", nResults - 1, (Pixel[0]/AvgSpore));
setResult("Image Name", nResults - 1, image);
updateResults;
// clear ROI manager to move on to next image
roiManager("Delete");
// Clean up
selectWindow("Mask");
run("Close");
selectWindow("Result of "+image);
run("Close");
selectWindow("ROI Manager");
run("Close");
selectWindow(image);
run("Close");
}
```

```
// Save results as an excel file
selectWindow("Results");
saveAs("results");
// Clean up remaining open windows
selectWindow("Threshold Color");
run("Close");
selectWindow("Results");
run("Close");
```