Wheat blast management through identification of novel sources of genetic resistance and understanding of disease dynamics

by

Giovana Cruppe

B.S., Sao Paulo State University, 2010
M.S., Oklahoma State University, 2015

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Plant Pathology
College of Agriculture

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Abstract

Wheat blast (WB), caused by the fungus *Magnaporthe oryzae* pathotype *Triticum* (MoT), emerged as a devastating disease in Brazil in 1985 and now limits wheat production in South America and South Asia. Despite 30-yrs of intensive effort, the $2N^{v}S$ translocation from *Aegilops ventricosa* contains the only useful, although partial and background dependent, source of WB resistance. The greatest yield losses generally occur at early stages of grain development. However, we hypothesize that leaf infection is an important player in the disease epidemiology. Our goals were to (i) identify novel sources of genetic resistance through phenotypic assays in a biosafety-level 3 laboratory in the U.S. and in the field and in growth room experiments in Bolivia and Brazil, and through genome-wide association mapping and (ii) characterize disease dynamics through characterization of MoT population diversity and determining the importance of leaf blast as a source of inoculum. Among the diverse germplasm evaluated, eight non-$2N^{v}S$ genotypes showed moderate levels of WB resistance, four of which were derived from the CIMMYT breeding program and four from the wheat wild relative, *Aegilops tauschii*. Our results showed that newer MoT strains isolated since 2012 have higher levels of aggressiveness compared to the older T-25 isolate from 1988, decreasing the resistance of some $2N^{v}S$-based varieties. The genome-wide association study identified 25 significant SNPs using isolate T-25, in which 21 SNPs were mapped on 2A chromosome. Highly significant linkage disequilibrium was found among these SNPs, suggesting that they may tag the same QTL. The physical position of these SNPs coincides with the $2N^{v}S$ translocation. No significant SNPs were identified with MoT isolates B-71 and 008. Removing the major effect of the $2N^{v}S$ did not reveal additional significant SNPs. QTL pyramiding analyses showed that this strategy might enhance WB resistance in certain backgrounds. The low frequency of genetic resistance coupled with the

increase in aggressiveness of new isolates highlights the threat WB poses to wheat production worldwide. Two field experiments were performed in Bolivia aimed to characterize the disease progress dynamics. We followed disease development in irrigated field plots where 4-week old seedlings were inoculated with Bolivian strain 008 isolated in 2015, as well as in neighboring non-inoculated plots. The results suggested that WB spreads vertically within the plant canopy and horizontally from inoculated to non-inoculated plots. Under high levels of disease intensity, strong correlation between flag leaf and spike blast intensity was found, suggesting that leaf blast can be a reliable predictor of spike severity in a susceptible host population. Finally, whole genome sequencing analyses showed little variation in the core chromosomes between the isolate 008 and the isolates subsequently sampled from blasted spikes. These findings indicate that early-stage leaf blast can play a major role as a source of inoculum for spike infection. Surprisingly, in contrast to core chromosomes, a greater variation was found in the supernumerary mini-chromosomes from these wheat spike isolates, underscoring questions about the role of these dispensable chromosomes in virulence and aggressiveness.

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Approved by:

Major Professor
Barbara S. Valent

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Dedication

For my parents, who always encouraged me to experience the world and its risks, knowing that I would always have the comfort of home to come back to.

"The rewards of the journey far outweigh the risk of leaving the harbor."

Unknown

Chapter 1 - Novel sources of wheat spike blast resistance identified in modern breeding lines and wheat wild relatives

Abstract

Wheat spike blast (W_SB), caused by the fungus *Magnaporthe oryzae* pathotype triticum (MoT), is a devastating disease affecting South America and South Asia. Despite more than 30-yrs of intensive effort, the $2N^vS$ translocation from *Aegilops ventricosa* contains the only useful source of resistance to W_SB effective against MoT isolates. The objective of this study was to identify non-$2N^VS$ sources of resistance to W_SB among elite cultivars, breeding lines, landraces, and wild-relative accessions. Over 780 accessions were evaluated under field and greenhouse conditions in Bolivia, greenhouse conditions in Brazil, and at two biosafety level-3 laboratories in the U.S. The MoT isolates B-71 (2012), 008 (2015), and 16MoT001 (2016) were used for controlled experiments, while isolate 008 was used for field experiments. Resistant and susceptible checks were included in all experiments. Under field conditions, susceptible spreaders were inoculated at the tillering stage to guarantee sufficient inoculum. Disease incidence and severity were evaluated as the average rating for each one meter-row plot. Under controlled conditions, spikes were inoculated after full emergence and individually rated for percentage of diseased spikelets. The diagnostic marker Ventriup-LN2 was used to test for the presence of the $2N^vS$ translocation. Four non-$2N^vS$ spring wheat CIMMYT breeding lines (CM22, CM49, CM52, and CM61) and four wheat wild-relatives, *Ae. tauschii* (TA10142, TA1624, TA1667, and TA10140) were identified as resistant (<5% of severity) or moderately resistant (5 to <25% severity) to W_SB. Experiments conducted at the seedling stage showed little correlation with disease severity at the head stage. MoT isolate 16MoT001 was significantly

more aggressive against $2N^{v}S$-based varieties. The low frequency of $W_{S}B$ resistance and the increase in aggressiveness of newer MoT isolates highlight the threat that the disease poses to wheat production worldwide and the urgent need to identify and characterize new resistance genes that can be used in breeding for durably resistant varieties.

Introduction

Wheat blast, currently a significant threat to wheat (*Triticum aestivum L.*) production, is caused by a specific subpopulation of the fungus *Magnaporthe oryzae* (T.T. Hebert) M.E. Barr (synonym of *Pyricularia oryzae*) (Couch and Kohn 2002; Zhang et al. 2016), specifically the *Triticum* lineage or pathotype MoT (Gladieux et al. 2018; Murakami et al. 2000). For three decades, the disease was restricted to South America (Barea and Toledo 1996; Viedma 2005; Perelló et al. 2015); however, in 2016 the disease spread to Bangladesh, causing significant yield losses and decreasing the wheat area planted in subsequent seasons (Malaker et al. 2016; Mottaleb et al. 2019). Wheat blast epidemics are highly dependent upon environmental conditions such as warm temperatures (25-30°C), moisture (i.e. spike wetness between 25 and 40 h), and high relative humidity (>90%) (Cardoso et al. 2008). Consequently, there is a high year-to-year variability in the magnitude of yield and quality losses due to the disease. In addition, cultivar susceptibility and timing of infection also influence wheat blast severity and potential damage to the crop (Goulart et al. 2008; Urashima et al. 2009). Wheat blast can be partially controlled by foliar fungicides (Goulart et al. 2008; Urashima et al. 2009); however, fungicides add an extra cost to wheat production and the benefits and efficacy are inconsistent. Previous studies have shown that foliar fungicides and other management practices such as fungicide seed treatments and delays in sowing date only provide proper wheat blast control under low to medium-disease pressure, or when resistant cultivars are grown (Mehta et al. 1992; Kohli et al.

2011; Cruz et al. 2015; Coelho et al. 2016). Thus, the use of resistant cultivars is the most sustainable and effective option to control wheat blast.

Genetic resistance to wheat spike blast (W_SB), the most common form of the disease in the field, has relied upon a single source, i.e., the $2N^vS$ translocation (Cruz et al. 2016). This translocation was first introgressed into the wheat variety VPM1 (Jahier et al. 2001; Bariana and McIntosh 2008) from the wild relative *Aegilops ventricosa*. Varieties with the $2N^vS$ translocation has been widely used in wheat breeding programs as a valuable source of resistance to some races of wheat leaf rust (caused by *Puccinia triticina*), stem rust (caused by *Puccinia graminis* f. sp. *tritici*), and stripe rust (caused by *Puccinia striiformis* f. sp. *tritici*) (Helguera et al. 2003). In addition, this translocation contains resistance genes to some pathotypes of the cereal cyst nematode (*Heterodera avenae*) and the root-knot nematode (*Meloidogyne* spp.) (Jahier et al. 2001; Williamson et al. 2013). Conveniently, no wheat grain yield penalty has been associated with the $2N^VS$ translocation (Williamson et al. 2013).

Multiple varieties with $2N^vS$-based resistance are currently used by wheat producers in South American countries and more recently in Bangladesh as the primary source for wheat blast control. Previous research showing variability in disease severity among $2N^vS$-based varieties suggests that the genetic background and/or environment may influence the levels of resistance expression (Cruz et al. 2016). In addition, the strong isolate-by-cultivar interaction (Cruz et al. 2016; Maciel et al. 2013; Urashima et al. 2006) may influence this variability in disease severity. Consequently, relying solely on $2N^VS$-based varieties as the foundation in breeding programs for wheat blast resistance is not recommended (Cruz and Valent 2017). Moreover, the durability of this source of resistance is unclear, especially with the possible emergence of more aggressive MoT races. In addition to the $2N^vS$ translocation, five wheat resistance genes (*Rmg2*, *Rmg3*,

Rmg7, *Rmg8*, and *RmgGR119*) have been identified as effective against some MoT strains (Anh et al. 2015; Tagle et al. 2015; Wang et al. 2018). However, the effectiveness of the majority of these genes (Rmg2, Rmg3, and Rmg7) was significantly reduced by: (i) temperatures higher than 26°C, (ii) newer and more aggressive MoT races, and (iii) infection at the head stage and under field conditions. Anh et al. (2018) revealed that genes *Rmg7*, located on chromosome 2A in tetraploid wheat, and *Rmg8*, located on 2B in hexaploid wheat, do not share the same temperature sensitivity (i.e., *Rmg8* was still effective under temperatures higher than 26°C); however, both of these genes recognize the same *AVR* gene, the *AVR-RMG8*. Wang et al. (2018) demonstrated that there is an additive effect (i.e., enhancement of disease resistance) when combining the genes *Rmg8* and *RmgGR119*, however this still needs to be evaluated under field conditions and against newer MoT isolates.

The effect of chemical and cultural recommendations to control wheat blast is inconsistent (Cruz et al. 2018) and the probability of a profitable operation for wheat producers is uncertain. Within this scenario, host plant resistance arises as one of the most promising strategies to mitigate wheat blast. Considering the significant variability of the $2N^{v}S$-based resistance, the breakdown of some resistance genes by newer and more aggressive isolates, and the inconsistency of cultural practices in controlling the disease, it is imperative to identify and deploy novel sources of resistance to wheat blast. New and unique sources can enhance the current genetic base for resistance, providing valuable new resistance gene(s) for wheat breeding programs. Therefore, our objective was to identify non-$2N^{v}S$ sources of resistance to wheat blast among elite cultivars, breeding lines, landraces, and wheat wild-relative accessions.

Materials and Methods

Plant Materials

A total of 780 wheat accessions including spring and winter elite cultivars, modern breeding lines, landraces from a Nested Association Mapping (NAM) panel, cultivated emmer (Triticum turgidum ssp. dicoccum), and *Aegilops tauschii* (TA) accessions were phenotyped for W_SB resistance either in biosafety level-3 laboratories in the U.S., and/or under field and greenhouse conditions in Bolivia and/or under greenhouse conditions in Brazil. The wheat accessions were genotyped for the presence of the $2N^vS$ chromosome segment. A few selected non-$2N^vS$ resistant and moderately resistant materials were further evaluated at the seedling stage (3-leaf stage) under controlled environments. It is uncertain if the resistance at the head and at the leaf stages are controlled by the same resistance genes.

The spring and winter wheat cultivars encompassed diverse sets of Bolivian, Brazilian, and North American elite cultivars that were selected based on four factors: i) high levels of wheat blast resistance reported in previous experiments using an older and less aggressive blast isolate (i.e. MoT T-25, Cruz et al. 2016); ii) superior yield and quality performance based on regional variety performance tests; iii) Bolivian and Brazilian cultivars that were widely grown in the blast-affected geography; and iv) cultivars that carry resistance genes for other major fungal diseases such as Fusarium head blight, leaf rust, and stripe rust. The breeding lines tested in this study included spring breeding lines from Biotrigo Genetica and CIMMYT breeding programs. The NAM (Yu et al. 2008) landraces were chosen to represent a diverse panel of geographic distribution of wheat across the globe including accessions from 32 different countries. The emmer wheat entries were part of a collection derived from the National Small Grains Collection (NSGC), USDA-ARS, Aberdeen, Idaho. The TA entries were part of a mini

core collection that included entries from 15 different countries and were selected based on preliminary studies using T-25 MoT isolate (Cruz et al. 2016).

Production of inoculum, inoculation procedure, and disease ratings

Highly aggressive monosporic isolates were used in all experiments. Isolate B-71, collected in Okinawa (Bolivia) in 2012 was used for experiments conducted in the U.S.; isolate 008 collected in Quirusillas (Bolivia) in 2015 was used for experiments conducted in Bolivia; and isolate 16MoT001 collected in Passo Fundo – RS (Brazil) in 2016 was used for experiments conducted in Brazil. The three winter wheat cultivars Jagalene ($2N^{v}S$-based), RonL, and Everest, which do not carry the $2N^{v}S$ translocation, were previously selected as the standard set of winter wheat differentials for MoT race identification (Cruz et al. 2012; Cruz et al. 2016) and were used to characterize the aggressiveness of the isolates used in this study (Table 1). Cruz et al. (2012) and Cruz et al. (2016) demonstrated that the same cultivars had substantial differences in disease severity when tested against different isolates, which led to the designation of three different MoT races namely 0, 1, and 2. Within the current set of winter wheat differentials, Everest is highly susceptible to both races 1 and 2, RonL is resistant to race 1 but susceptible to race 2, and Jagalene is resistant to both races 1 and 2. MoT race 0 does not cause disease in any of the three cultivars, thus it is not considered a wheat pathogen (Table 1-1). These cultivars were chosen due to their contrasting reactions with different isolates. All isolates used in this study were identified as race 2, based on reactions on this differential set (i.e. Everest and RonL were grouped as susceptible and Jagalene was grouped as resistant - intermediate) (Table 1-1). Although there was a significant difference in Jagalene' s reaction when tested against the Brazilian isolate 16MoT001 compared to the other two isolates, we are still cautiously classifying this isolate as race 2. MoT isolates used in the U.S. were transported to a biosafety level-3 laboratory under the

requirements stipulated by the United States Department of Agriculture (USDA) Animal and Plant Health Inspection Service.

Inoculum production, inoculation procedure, and disease rating followed protocol previously described by Cruz et al. (2016). MoT cultures were grown on homemade oatmeal agar (Valent et al., 1991) and when sporulating cultures were 8-days old, they were flooded with a mixture of sterile deionized water with 0.42% unflavored gelatin and 0.01% Tween 20, stirred to dislodge conidia from conidiophores, and filtered with four layers of sterile cheesecloth to eliminate the mycelia and any remaining agar medium. The spore concentration was adjusted to 20,000 spores ml^{-1} of suspension. For experiments conducted in controlled environments (i.e., greenhouse and growth chamber), 0.75 ml of the spore suspension was individually applied to each wheat head approximately 2 days after full head emergence (Feekes GS 10.5, Large 1954) using an airbrush (model 69492, Harbor Freight Tools, Camarillo, CA). To provide the optimal conditions for MoT infection, the spikes were covered with black plastic bags (model S-12322BL, ULINE) moistened with water for 48 hours immediately following spore suspension application. Disease severity was evaluated every other day, starting at eight until 14 days after inoculation, where each head was individually rated for the percentage of spikelets affected in a 0 to 100% scale (Cruz et al. 2016). Susceptible and resistant checks were included in all experiments and varied according to country, seed availability, and experiment objective (e.g., spring versus winter). Disease severity was assessed on multiple days to characterize disease progress. However for statistical purposes, we used the data collected when the susceptible check reached 90% or more of disease severity (i.e., usually between 12 and 14 days after inoculation) (Cruz et al. 2016).

Field experiments

Irrigated field experiments were conducted during the 2017 and 2018 wheat growing seasons for a total of five location-years in Bolivia. Field experiments within each location-year were grouped according to wheat classification (e.g. winter vs spring, different breeding programs, etc.), with a total of 12 individual experiments (Table 1-2). The locations selected are considered 'hotspots' for wheat blast development because warm and moist conditions typically occur around wheat heading. In addition, two wheat-growing seasons per year in Bolivia facilitated data collection within a relatively short period. The two growing seasons were i) South American winter (April - August), and ii) South American summer (December - March). In the summer seasons of 2017 and 2018, the experiment was conducted at one location at a farmer's field near Quirusillas (18° 19' 45.92" S, 63° 56' 51.44" W). In the winter season of 2017, the experiment was conducted at two locations: ANAPO's (Association of Producers of Oilseeds and Wheat) Research Station near 26 de Agosto (17° 26' 29.44" S, 62° 36' 27.99" W) and at the Integral Cooperative Agricultural Colonies (CAICO) Research Station near Okinawa (17° 14' 33.83" S, 62° 53' 21.41" W). In the winter season of 2018, the experiments were conducted at CAICO Research Station near Okinawa (17° 14' 33.83" S, 62° 53' 21.41" W).

For all experiments, one meter-row plots with 20 cm row spacing and planting density ranging from 60 (spring wheat entries) to 100 (winter wheat entries) seeds per linear meter were established in a randomized incomplete block design with three to six replications, depending on seed availability. Seeds were treated with the fungicides carboxin 20% plus thiram 20% at a dose of 200 ml/100kg. To control weeds, 0.35 L ha^{-1} fluroxypyr 1-methylheptyl ester1: {[(4-amino-3,5- dichloro-6-fluoro-2-pyridinyl)oxy]acetic acid, 1-methylheptyl ester} and 0.35 L ha^{-1} clodinafop propargil2 {[4-(5-cloro-3-fluropiridin-2-iloxi)fenoxi]propionate de prop-2-inilo}

were applied in all plots three times during the season. Insects were controlled by spraying 0.6 kg ha^{-1} benzoate, split into three applications, and one application of 0.15 kg ha^{-1} thiametozan. Nitrogen was applied as urea split into two applications of 40 kg N ha^{-1} approximately at Feekes GS 2 (tillering initiation) and at Feekes GS 4 (leaf sheath lengthening). Each incomplete block consisted of six 1-m rows, where the outside rows were either a resistant or a susceptible check, and the four inside rows were candidate materials being evaluated. The spring wheat cultivars 'Urubo' or 'Sossego' were used as resistant checks, and 'Atlax' was used as the susceptible check. For the winter wheat experiments, cultivars 'Jagalene' and 'Everest' were used as the resistant and susceptible checks, respectively (Cruz et al. 2012).

Spreader rows were planted surrounding all experimental plots to generate enough inoculum for wheat blast development throughout the season, in order to not rely solely on natural infection. The spreader rows were inoculated with MoT isolate 008 at a concentration of 20,000 spores ml^{-1} at Feekes GS 3 (late tillering stage) using a CO_2 pressurized backpack sprayer. Inoculated rows were covered with plastic tarpaulins for approximately 24 hours. The period that inoculated spreader rows were covered was generally lower under field conditions when compared to controlled environment due to the higher day temperatures to avoid heat damage to the plants. At approximately Feekes GS 11.1 (milky ripe stage of grain development) and Feekes GS 11.2 (soft dough stage of grain development), W_SB incidence was measured as the average percentage of diseased spikes, and disease severity was measured as the average percentage of infected spikelets in each meter row plot (Cruz et al. 2012). Because we measured disease incidence and severity multiple times, stage of grain development was also measured at each evaluation to ensure the optimal timing for wheat blast evaluation (i.e. soft dough stage of grain development).

Experiments at the adult plant stage

Growth chamber experiments were conducted at the Biosecurity Research Institute (BRI) in Manhattan (Kansas, U.S.). Greenhouse experiments (19 total) were conducted at three different locations: the Agricultural Research Service Foreign Disease-Weed Science Research Unit (ARS-FDWSRU) in Fort Detrick (Maryland, U.S.); the ANAPO Research Facility in Santa Cruz de la Sierra (Bolivia); and Biotrigo Genética's Research Facility in Passo Fundo (Brazil) (Table 3). Approximately five spring wheat seeds were sown into 15-cm diameter pots containing a commercial potting medium (Metro-Mix 360, Hummert International, Earth City, MO). For the winter wheat entries, a vernalization period of 8 weeks in a cold room (4°C and 9:15 h light: dark) was required prior to transplanting the seedlings into the pots. At planting, 3.5 g of the fertilizer osmocote (14-14-14) and 1 g of the insecticide imidacloprid {1-[(6-Chloro-3-pyridinyl)methyl]-N-nitro-2-imidazolidinimine} were applied to each pot. Additional insect control occurred as necessary using commercially available insecticides.

For the experiments conducted at the BRI, plants were initially maintained in a greenhouse until the booting stage (Feekes GS 10, Large 1954) and then transferred to the BRI and placed in growth chambers (Conviron, model PGR15, Winnipeg, Canada) at day temperatures of 23 ± 2°C, night temperatures of 18 ± 2°C. Similarly, heading plants at the ARS-FDWSRU were transferred to the Biosafety Level-3 Plant Disease Containment Facility greenhouses, temperatures maintained at 25 ± 4°C. The pots were arranged in an incomplete randomized block design with one to four replications, depending on seed availability and growth chamber space. Wheat spikes were inoculated approximately 2 days after full head emergence and ratings were taken as previously described. After disease rating was completed, the plants were autoclaved and discarded following biosafety level-3 requirements. Experiments

conducted under greenhouse conditions both at the U.S. and South America followed the same inoculum production, inoculation procedure, and disease rating protocols described above.

Experiments at the seedling stage

To complement W_SB evaluations, a few selected non-2N^{v}S wheat accessions classified as resistant and moderately resistant were also evaluated at the seedling stage (3-leaf stage) under greenhouse conditions at ANAPO's Research Facility. Seedlings were grown in soil in six-cell starter trays (model TRTD1404, 9GreenBox). Twelve seeds were sown in each tray, representing a replication. Trays were displayed in a randomized complete block design with three replications. Susceptible and resistant checks were included in all experiments. At the 3-leaf stage, 6 ml of spore suspension at a concentration of 20,000 spores ml^{-1} was sprayed on each tray. Immediately after inoculation, the trays were covered with black plastic bags, moistened with water, for 48 hours. At five and seven days after inoculation, the second leaf was rated for the percentage of leaf affected by wheat blast (Cruz et al. 2012). Wheat blast severity at the seedling stage was compared to disease severity at the heading stage to better characterize non-2N^{v}S resistance and to determine any correlation between resistance and susceptibility at these stages.

Selection of non-2N^{v}S materials

Five different categories were used to group the wheat genotypes according to their level of disease severity: resistant (< 5%), moderately resistant (> 5% and < 25%), intermediate (> 25% and <45%), moderately susceptible (> 45% and < 65%), and susceptible (> 65%). Not all materials were evaluated at all location-years. The genotypes grouped as susceptible in the first year of this study were not evaluated again in the following season. Meanwhile, the non-2N^{v}S

materials showing good levels of resistance (i.e., resistant to intermediate category) were evaluated in more than one experiment.

DNA extraction and molecular marker detection

The wheat genotypes were screened for the presence of the 2AS/2N^{v}S translocation with the objective of identifying promising alternatives to the known 2N^{v}S-based resistance (Cruz et al. 2016). Genomic DNA was extracted from 100 mg of leaf tissue of 10-day-old seedlings using the Qiagen DNeasy 96 Plant Kit (QIAGEN, Hilden, Germany). The quality and quantity of DNA were assessed using a NanoDrop spectrophotometer (NanoDrop Technologies). Polymerase chain reaction (PCR) was performed using the Cleaved Amplified Polymorphic Sequences (CAPS) marker protocol modified from a protocol previously described by Helguera et al. (2003). This protocol detects both the N genome allele and the A genome allele and differentiates homozygous and heterozygous 2AS/2N^{V}S fragments of DNA.; Specifically, PCR reactions consisted of 10 µL of GoTaq Green Mix (Promega Corporation, Madison, WI), 200 ng of genomic DNA, and 10 µM of each 2NVS specific primers, URIC (GGT CGC CCT GGC TTG CAC CT) and ventriup-LN2 (TGC AGC TAC AGC AGT ATG TAC ACA AAA), adjusted to 20 µL total reaction volume. PCR amplification was performed in an Eppendorf thermocycler using the cycling conditions: initial denaturing at 94°C for 45 s; denaturation at 94°C for 45 s, annealing at 64°C for 30 s, and extension at 72°C for 60 s repeated for 38 cycles; and a final extension at 72°C for 7 min. Following amplification with CAPS primers, 5µL of the PCR reaction was digested with DpnII (New England Biolabs, Ipswich, MA) restriction enzyme in a 20 µL reaction, according to manufacturer's protocol. The reaction was incubated at 37°C for one hour. Samples were separated by electrophoresis in 2% agarose gels and the presence of the 285-bp N-allele fragment) and/or the 275-bp A-allele fragment was visualized using the SYBR® Safe

Stain (Invitrogen, Carlsbad, CA) and a LI-COR detection system (LI-COR Biosciences, Lincoln, NE). The presence or absence of the $2N^vS$ segment was also predicted for a subset of these genotypes based on the relative count of wheat and unique $2N^vS$ alien sequencing tags developed by Gao et al. (2018).

Statistical analyses

Data were analyzed using SAS Version 9.2 (SAS Institute, Cary, NC, 2001). For analysis of data from field experiments, disease severity was transformed to logits to linearize the data, although the means and standard errors reported in the results are from the detransformed data. Because disease severity was measured several times at different wheat growth stages, we analyzed it with PROC MIXED using the REPEATED statement for the analysis of repeated measurements. Growth stage was used as a covariate and means of the wheat genotypes of interest were compared to the mean of the susceptible checks measured at soft dough stage of grain development (i.e., the optimal time for wheat blast evaluation). The presence of the $2N^vS$ translocation and genotype within $2N^vS$ were treated as fixed effects. Environment (combination of year and location), experiment within environment, and block within experiment were treated as random effects. For the experiments under controlled environment conditions, we analyzed the disease severity evaluated at 14 days (i.e., susceptible check averaged 90% or more of disease severity) after inoculation using PROC MIXED. In the analysis of field data, the presence of the $2N^vS$ translocation and genotypes within $2N^vS$ were treated as fixed effects. Experiment within environment (e.g., BRI, ANAPO, Biotrigo, or ARS-FDWSRU) and experiment within genotype and $2N^vS$ were treated as random effects.

Results

Field experiments

Mean wheat blast severity across all entries combined was not significantly different among location-years ($p = 0.11$) and among experiments within the same location-year ($p = 0.07$) (Table A1). However, there was a significant difference between incidence and severity of the susceptible versus resistant checks at soft dough stage of grain development among the 12 individual experiments ($p < 0.001$) (Table A2). Disease incidence of the susceptible checks was greater than 50% and disease severity averaged 25% or more in 9 out of 12 experiments. The lowest disease incidence (1.2%) and severity (0.7%) occurred in a field in Quirusillas 2017 (experiment 6), where a moderately resistant check was inadvertently sown instead of a susceptible check. Disease incidence of the resistant checks was lower than 4% in 11 out of 12 experiments and only two experiments averaged disease severity greater than 2%. Due to the known level of resistance and uniform distribution in the field, disease incidence and severity of the susceptible checks provided an estimation of the overall disease intensity within each experiment and were used to standardize the disease variability across the field. Overall, W_SB intensity was low to medium in all location-years of this study. This was due to dry environmental conditions in the region, and the absence of optimal temperatures (25 to 30°C) and high relative humidity (>90%) throughout the wheat growing season. Nevertheless, supplemental irrigation and spreader borders inoculated early in the season made the collected field data a valuable resource.

Of the 526 wheat accessions evaluated under field conditions, 332 were positive and 194 were negative for the presence of the $2N^vS$ translocation. There was a significant difference between the mean disease severity of the $2N^vS$ and the non-$2N^vS$ groups ($p < 0.001$). Within the

2N^{v}S accessions, head blast severity ranged from zero to 87%, with mean severity of 2%. Within the non-2N^{v}S entries, disease severity ranged from zero to 99%, with mean of 16%. Although this difference was statistically significant, the distribution of both groups was skewed towards resistance, probably due to the low disease pressure under field conditions and the unbalanced number of 2N^{v}S and non-2N^{v}S entries (Fig. 1-1a and b). Differences between 2N^{v}S and non-2N^{v}S groups in individual field experiments are shown on Fig. S1. In all experiments, disease incidence and severity of the 2N^{v}S materials were significantly lower than that of non-2N^{v}S genotypes ($p < 0.001$). Wheat blast intensity can vary considerably from year to year even in the 'hot-spot' areas. Given the disease intensity in the field experiments, there was a 90% cumulative probability that 2N^{v}S wheat genotypes scored 5% or less (i.e., resistant category) severity, while the probability was lower (50%) for the non-2N^{v}S genotypes (Fig. 1c).

Greenhouse experiments

The disease severity under controlled environments (greenhouse and growth chamber) was significantly higher than that in field experiments. Under controlled environments, critical environmental variables (i.e. temperature, relative humidity, and leaf wetness) were controlled and spikes were inoculated individually. Similar to field experiments, significant difference was observed between 2N^{v}S and non-2N^{v}S groups across controlled environment experiments. For both groups, disease severity ranged from zero to 100% at 14 days after inoculation. Severity distribution of the 2N^{v}S group was skewed towards resistance, with mean severity of 22%. On the other hand, severity distribution of the non-2N^{v}S group was skewed towards susceptibility, with mean severity of 78% (Fig. 1-2a and b). Based on the data collected from controlled environment experiments, where the disease pressure was higher compared to field experiments, there was a 15% cumulative probability that a 2N^{v}S genotype was in the resistant category. This

probability was null for the non-2N^{v}S genotypes. Considering disease severity at 25%, which is the upper limit for moderately resistant genotypes, there was a 65% cumulative probability that a 2N^{v}S genotype scored 25% or less, and only 5% probability for non-2N^{v}S genotypes (Fig. 1-2c).

Three different isolates were used under controlled environmental conditions in the different experimental sites/countries. Despite the presence of wheat blast, Brazil and Bolivia do not allow the entrance of foreign wheat isolates to avoid the introduction of new and possibly more aggressive races. Resistance of 2N^{v}S and non-2N^{v}S groups and within each group was statistically different for the three isolates. In both groups, the Brazilian isolate, 16MoT001, was the most aggressive (2N^{v}S = 82.1% and non-2N^{v}S = 97.2% mean severity). The non-2N^{v}S group showed similar averaged severity for the two Bolivian isolates (B-71 = 71% and 008= 68%). There was a greater difference within the 2N^{v}S group, with a mean severity of 29.2% for B-71 and of 18.8% for the 008 isolate (Fig. 1-3). Initially, when tested against the set of differentials, the three isolates were classified as race 2. However, these additional experiments have shown a substantially higher level of aggressiveness associated with a Brazilian isolate 16MoT001, especially when tested against moderately resistant 2N^{v}S genotypes. Additional experiments are needed to determine if newer Brazilian isolates are becoming more aggressive and breaking the 2N^{v}S resistance.

Selection of non-2N^{v}S materials

A few non-2N^{v}S genotypes displayed satisfactory levels of wheat blast resistance. Among the spring wheat materials, four non-2N^{v}S CIMMYT lines (i.e., CM 22, CM 49, CM 52, and CM 61) exhibited consistent intermediate to high levels of resistance under both field and controlled environment conditions. The first experiment was conducted at ARS-FDWSRU with 93 CIMMYT lines (Table A3). The best non-2N^{V}S lines were selected and evaluated under field

conditions in Bolivia for a total of four location-years (Fig. 1-4). In the winter wheat season of 2017, five non-2N^{v}S CIMMYT lines plus checks were evaluated in two locations, Okinawa and 26 de Agosto. At both locations, the rank of the materials evaluated was the same. The lines CM 36, CM 52, CM 22 and the resistant 2N^{V}S checks, Urubo and AN120, averaged less than 13% of disease incidence and less than 5% of disease severity. The susceptible check, Atlax, averaged 50% or more of disease incidence, and at least 25% of disease severity. Line CM 43 had the highest incidence (65%) and severity (55%) in both experiments. In 2018, one experiment was conducted in the summer season in Quirusillas and one experiment in the winter season in Okinawa. In addition to the lines tested in 2017, lines CM 27, CM 36, CM 49 and one moderately resistant check (Sossego) were added. In contrast to previous year, genotypes performed differently in the two locations, which can be partially explained by the different seasons (summer vs winter seasons). At both locations, the three resistant checks ranked as the most resistant, but not in the same order. In Quirusillas, the susceptible check averaged 99% of incidence and 87.5% of severity and in Okinawa, averages were 78.3% and 58.8%. Only one of the three additional lines tested in 2018 performed well. The line CM 27 was grouped as resistant in Quirusillas with average severity of 4.4% and as intermediate in Okinawa with severity average of 37.5%. CM 49 was grouped as moderately susceptible, and CM 36 as susceptible in both experiments, while CM 52 was grouped as intermediate. Lines CM 61 and CM 22 were grouped as intermediate and as moderately susceptible in Quirusillas, respectively. The opposite reaction was observed in Okinawa for these two lines.

Under greenhouse conditions, lines CM 22, CM 49, CM 52, and CM 61 averaged less than 41% at 14 days after inoculation (Fig.1-5). The two non-2N^{V}S susceptible checks, Atlax and Bob White, averaged 77.3% and 95.75%, respectively. The moderately-resistant (Sossego) and

the resistant (Urubo) 2N^{v}S checks averaged 8.5% and 1.2%, respectively (Figure 5). To better characterize the non-2N^{v}S resistance in these four CIMMTY lines, seedling resistance was also evaluated in controlled environment (Fig. 1-6). Seedling blast severity of the four lines was lower than both susceptible and resistant checks (Fig. 1-6). There was a weak but significant positive linear relationship ($p < 0.05$) between seedling and head resistance ($r^2 = 0.48$). At the seedling stage, resistant check Urubo, and lines CM 52, CM 49, CM 22, and CM 61 were considered resistant; Atlax, Sossego, and CM 36 were moderately resistant; and CM 43 and Bob White were intermediate. The seedling results are data combined from two independent experiments ($r^2 = 0.78$). At the head stage under controlled experiments, Urubo was placed into the resistant group; Sossego and CM 49 were moderately resistant; CM 52, CM 22, and CM 61 were intermediate; CM 36, CM 43, and both susceptible checks, Atlax and Bob White, were placed in the susceptible group (> 65%). Only the resistant checks, Urubo and Sossego, were placed in the same category at both seedling and head experiments. Surprisingly, both susceptible checks, Atlax and Bob White, did not show high levels of disease severity at the seedling stage. However, Bob White had the greatest disease severity in both experiments.

In addition to the spring wheat lines, one Ae. tauschii line (TA10142) averaged 2.5% severity under controlled experiments (Fig. 1-7). Another three lines, TA1624 (16%), TA 1667 (21.6%), and TA10140 (25.7%) were placed into the moderately resistant category. From the remaining TA lines screened, six lines were classified as intermediate, 9 lines as moderately susceptible, and 49 lines as susceptible. The susceptible check averaged 100% at 14 days after inoculation (Table A4). These lines were not evaluated under field conditions due to the challenge of planting winter wheat in South America. Selected TA lines will serve as parents in

bi-parental and backcrossing populations with a spring background, enabling their further evaluation under field conditions in South America.

Discussion

Screening of over 780 accessions of elite spring and winter wheat cultivars, modern breeding lines, landraces, and wheat wild relatives resulted in the identification of only eight non-2N^{V}S accessions with moderate levels of resistance to W$_{S}$B. This is equivalent to only 1% of the evaluated genotypes offering some level of resistance, highlighting the rare occurrence of inherent protection to this devastating disease. Within the selected materials, four accessions were derived from the CIMMYT breeding program (CM 22, CM 49, CM 52, and CM 61). The resistance in these lines was also characterized at the seedling stage and showed weak correlation with head infection. The largest yield losses are associated with head infection. Our results agree with those of Cruz et al. (2012) and Maciel et al. (2014) that seedling evaluations cannot be used as a surrogate for resistance at the heading stage.

The remaining four promising non-2N^{v}S accessions evaluated were *Ae. tauschii* accessions (TA 10142, TA1624, TA1667, and TA10140), one of the three progenitors of bread wheat. Wild relatives of wheat are a valuable source of genetic diversity and have played an important role in wheat improvement worldwide over the last century (Autrique et al. 1995; Friebe et al. 1996; Hajjar and Hodgkin 2007; Warburton et al. 2006). One of the drawbacks of the introgression of genes from wild relatives is the linkage drag, i.e., the addition of undesirable or deleterious genes that negatively affect grain yield and quality (Zhang et al. 2017). Fortunately, much effort has been made to minimize linkage drag and to increase the use of alien genes in breeding programs (Molnár et al. 2016; Zhang et al. 2017). Nonetheless, introgression

of resistance from these accessions into commercial wheat lines is still laborious and time consuming.

Though a large and highly diverse set of accessions was screened in this study, the frequency of resistance to wheat blast was extremely low, underscoring the threat that wheat blast poses to wheat production worldwide. In addition, we showed preliminary data suggesting an increase in the aggressiveness of a new Brazilian isolate, 16MoT001, which could lead to the breakdown of the only effective source of W_SB resistance currently available, the $2N^VS$ translocation. An increase in aggressiveness of MoT isolates was already observed when comparing isolates collected in the late 1980's to isolates collected after 2010, decreasing the level of resistance of some $2N^vS$-based cultivars under high levels of disease pressure (Anh et al. 2015; Cruz et al. 2012; Cruz et al. 2016; Cruz and Valent 2017). An additional possibility that could help explain the higher levels of disease observed with 16MoT001 in the $2N^vS$ cultivars evaluated would be a strong isolate-by-cultivar interaction, as demonstrated in previous studies (Cruz et al. 2016; Maciel et al. 2013; (Urashima et al. 2006). Because only a limited number of $2N^VS$ accessions were tested against 16MoT001, further experiments are needed to evaluate the reaction of a broader panel of $2N^vS$ materials with isolates recently collected from wheat blast affected regions.

Although not evaluated in this study, five R genes (*Rmg2*, *Rmg3*, *Rmg7*, *Rmg8*, and *RmgGR119*) were previously reported as effective against wheat blast (Anh et al. 2015; Tagle et al. 2015; Wang et al. 2018). However, genes *Rmg2*, *Rmg3*, and *Rmg7* are no longer effective at adult plant stage against newer isolates. As a future goal, we plan to test *Rmg8* and *RmgGR119* under field conditions in Bolivia and against newer and more aggressive MoT isolates under biosafety level-3 laboratories in the United States. If *Rmg8* and *RmgGR119* are indeed effective

sources of W_SB resistance, it is imperative to include these genes in wheat breeding programs and to introgress them into grown 2N^{v}S cultivars. Given the emergence of newer and more aggressive MoT races and the identification of novel sources of resistance, a broader set of wheat differentials must be established. Based on our findings combined with others (Anh et al. 2015; Cruz et al. 2016; Tagle et al. 2015; Wang et al. 2018), we suggest several genotypes including 2N^{v}S and non-2N^{v}S based accessions (including recent findings) with different levels of W_SB resistance that potentially could be included in a new set of wheat differentials (Table A5). It is important that the different blast research programs adopt the same set of wheat differentials for race designation.

An appropriate disease management strategy would include crop rotation with a non-host and rotation of active ingredients in both seed treatment and foliar fungicide applications. Cultural practices such as the removal of secondary hosts or infected residue and the adjustment of planting date could also play an important role in wheat blast control. The strategies cited above must be combined with the use of resistant cultivars, which ideally would carry more than one resistance gene. Critical follow-up to this research is to develop and evaluate several populations from the eight accessions identified in this study to characterize the genetics of resistance in these lines and develop molecular markers linked to resistance genes effective to new and highly aggressive races. These resistant accessions can then be incorporated into wheat breeding programs to introgress resistance into cultivated wheat varieties.

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References

Anh, V. L., Anh, N. T., Tagle, A. G., Vy, T. T. P., Inoue, Y., Takumi, S., Chuma, I., and Tosa, Y. 2015. *Rmg8*, a new gene for resistance to *Triticum* isolates of *Pyricularia oryzae* in hexaploid wheat. Phytopathology 105:1568-1572.

Anh, V. L., Inoue, Y., Asuke, S., Vy, T. T. P., Anh, N. T., Wang, S., Chuma, I., and Tosa, Y. 2018. *Rmg8* and *Rmg7*, wheat genes for resistance to the wheat blast fungus, recognize the same avirulence gene *AVR-Rmg8*. Mol. Plant Pathol. 19:1252-1256.

Autrique, E., Singh, R. P., Tanksley, S. D., and Sorrells, M. E. 1995. Molecular markers for four leaf rust resistance genes introgressed into wheat from wild relatives. Genome 38:75-83.

Barea, G., and Toledo, J. 1996. Identificacion y zonificación de *pyricularia* (*Pyricularia oryzae*) en el cultivo de trigo en el departamento de Santa Cruz. Informe Técnico, Proyecto de Investigación Trigo. 76-86.

Bariana, H. S., and McIntosh, R. A. 2008. Cytogenetic studies in wheat. XV. Location of rust resistance genes in *VPM1* and their genetic linkage with other disease resistance genes in chromosome 2A. Genome 36:476-482.

Cardoso, C. A. de A., Reis, E. M., and Moreira, E. N. 2008. Development of a warning system for wheat blast caused by *Pyricularia grisea*. Summa Phytopathol. 34:216-221.

Castroagudin, V. L., Ceresini, P. C., de Oliveira, S. C., Reges, J. T. A., Maciel, J. L. N., Bonato, A. L. V, Dorigan, A. F., and McDonald, B. A. 2015. Resistance to QoI fungicides is widespread in Brazilian populations of the wheat blast pathogen *Magnaporthe oryzae*. Phytopathology. 105:284-294.

Coelho, M. A. D. O, Torres, G. A. M., Cecon, P. R., and Santana, F. M. 2016. Sowing date reduces the incidence of wheat blast disease. Pesqui. Agropecu. Bras. 51:631-637.

Couch, B. C., and Kohn, L. M. 2002. A multilocus gene genealogy concordant with host preference indicates segregation of a new species, *Magnaporthe oryzae*, from *M. grisea*. Mycologia. 94:683-693.

Cruz, C. D., Bockus, W. W., Stack, J. P., Tang, X., Valent, B., Pedley, K. F., and Peterson, G. L.

2012. Preliminary assessment of resistance among U.S. wheat cultivars to the *Triticum* pathotype of *Magnaporthe oryzae*. Plant Dis. 96:1501-1505.

Cruz, C. D., Kiyuna, J., Bockus, W. W., Todd, T. C., Stack, J. P., and Valent, B. 2015. *Magnaporthe oryzae* conidia on basal wheat leaves as a potential source of wheat blast inoculum. Plant Pathol. 64:1491-1498.

Cruz, C. D., Peterson, G. L., Bockus, W. W., Kankanala, P., Dubcovsky, J., Jordan, K. W., Akhunov, E., Chumley, F., Baldelomar, F. D., and Valent, B. 2016. The 2NS translocation from *Aegilops ventricosa* confers resistance to the *Triticum* pathotype of *Magnaporthe oryzae*. Crop Sci. 56:990-1000.

Cruz, C. D., Santana, F. M., Todd, T. C., Maciel, J. L. N., Kiyuna, J., Baldelomar, D. F., Cruz, A.P., Lau, D., Seixas, C. S., Goulart, A. C., Sussel, A. A., Schipanski, C. A., Chagas, D. F., Coelho, M., Montecelli, T. D. N., Utiamada, C., Custodio, A. P., Rivanadeira, M. G., Bockus, W. W., and Valent, B. 2018. Multi-environment assessment of fungicide performance for managing wheat spike blast (W_SB) in Brazil and Bolivia. Trop. Plant Pathol. 44:183-191.

Cruz, C. D., and Valent, B. 2017. Wheat blast disease: danger on the move. Trop. Plant Pathol. 42:210-222.

Friebe, B., Jiang, J., Raupp, W. J., McIntosh, R. A., and Gill, B. S. 1996. Characterization of wheat-alien translocations conferring resistance to diseases and pests: Current status. Euphytica 91:59-87.

Gao, L., Dorn, K., Rife, T. W., Wang, X., Lemes, C., Clinesmith, M., Silva, P., Fritz, A., Stein, N., Mascher, M. and Poland, J. 2018. Completion of the 'Jagger' wheat genome leads to identification of *Aegilops ventricosa* 2NS translocation and its impact in wheat breeding.

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Gladieux, P., Condon, B., Ravel, S., Soanes, D., Maciel, J. L. N., Nhani, A., Chen, L., Terauchi, R., Lebrun, M. H., Tharreau, D., Mitchell, T., Pedley, K. F., Valent, B., Talbot, N. J., Farman, M., and Fournier, E. 2018. Gene flow between divergent cereal-and grass-specific lineages of the rice blast fungus *Magnaporthe oryzae*. MBio. 9:e01219-17.

Goulart, A. C. P., Sousa, P. G., and Urashima, A. S. 2008. Danos em trigo causados pela infecção de *Pyricularia grisea*. Summa Phytopathol. 33:358-363.

Hajjar, R., and Hodgkin, T. 2007. The use of wild relatives in crop improvement: A survey of developments over the last 20 years. Euphytica 156:1-13.

Helguera, M., Khan, I. A., Kolmer, J., Lijavetzky, D., Zhong-qi, L., and Dubcovsky, J. 2003. PCR assays for the *Lr37-Yr17-Sr38* cluster of rust resistance genes and their use to develop isogenic hard red spring wheat lines. Crop Sci. 43:1839-1874.

Jahier, J. P., Abelard, P., Tanguy, M., Dedryver, F., Rivoal, R., Khatkar, S., Bariana, H. S., Koebner, R. 2001. The *Aegilops ventricosa* segment on chromosome 2AS of the wheat cultivar "VPM1" carries the cereal cyst nematode resistance gene *Cre5*. Plant Breed. 120:125-128.

Kohli, M. M., Mehta, Y. R., Guzman, E., de Viedma, L., and Cubilla, L. E. 2011. Pyricularia blast-a threat to wheat cultivation. Czech J. Genet. Plant Breed. 47:S130-S134.

Large, E. C. 1954. Growth stages in cereals illustration of the Feekes scale. Plant pathol. 3:128-129.

Maciel, J. 2012. Magnaporthe oryzae, the blast pathogen: current status and options for its control. CAB Rev. Perspect. Agric. Vet. Sci. Nutr. Nat. Resour. 264:233-240.

Maciel, J. L. N., Ceresini, P. C., Zala, M., McDonald, B. A., Castroagudin, V. L., and Kema, G.

H. J. 2013. Population structure and pathotype diversity of the wheat blast pathogen *Magnaporthe oryzae* 25 Years after its emergence in Brazil. Phytopathology 104:95-107.

Malaker, P. K., Barma, N. C. D., Tewari, T. P., Collis, W. J., Duveiller, E., Singh, P. K., Joshi, A. K., Singh, R. P., Braun, H. J., Peterson, G. L. and Pedley, K. F., Farman, M. L., and Valent, B. 2016. First report of wheat blast caused by *Magnaporthe oryzae* pathotype *Triticum* in Bangladesh. Plant Dis. 100:2330.

Mehta, Y. R., Riede, C. R., Campos, L. A. C., and Kohli, M. M. 1992. Integrated management of major wheat diseases in Brazil: an example for the Southern Cone region of Latin America. Crop Prot. 11:517-524.

Molnár, I., Vrána, J., Burešová, V., Cápal, P., Farkas, A., Darkó, É., Cseh, A., Kubaláková, M., Molnár-Láng, M., and Doležel, J. 2016. Dissecting the U, M, S and C genomes of wild relatives of bread wheat (*Aegilops* spp.) into chromosomes and exploring their synteny with wheat. Plant J. 88:452-467.

Mottaleb, K. A., Singh, P. K., He, X., Hossain, A., Kruseman, G., and Erenstein, O. 2019. Alternative use of wheat land to implement a potential wheat holiday as wheat blast control: In search of feasible crops in Bangladesh. Land use policy. 82:1-12.

Murakami, J., Tosa, Y., Kataoka, T., Tomita, R., Kawasaki, J., Chuma, I., Sesumi, Y., Kusaba, M., Nakayashiki, H., and Mayama, S. 2000. Analysis of host species specificity of *Magnaporthe grisea* toward wheat using a genetic cross between isolates from wheat and foxtail millet. Phytopathology 90:1060-1067.

Perelló, A., Martinez, I., and Molina, M. 2015. First report of virulence and effects of *Magnaporthe oryzae* isolates causing wheat blast in Argentina. Plant Dis. 99:1177.

Tagle, A. G., Chuma, I., and Tosa, Y. 2015. Rmg7, a new gene for resistance to *Triticum* isolates

of *Pyricularia oryzae* identified in tetraploid wheat. Phytopathology 105:495-499.

Urashima, A. S., Grosso, C. R. F., Stabili, A., Freitas, E. G., Silva, C. P., Netto, D. C. S., Franco, I., and Bottan, J. H. M. 2009. Effect of *Magnaporthe grisea* on seed germination, yield and quality of wheat. Advances in Genetics, Genomics and Control of Rice Blast Disease 267-277.

Urashima, A. S., Lavorent, N. A., Goulart, A. C. P., and Mehta, Y. R. 2006. Resistance spectra of wheat cultivars and virulence diversity of *Magnaporthe grisea* isolates in Brazil. Fitopatol. Bras. 29:511-518.

Valent, B, Farrall, L, and Chumley, F. 1991. Magnaporthe grisea genes for pathogenicity and virulence identified through a series of backcrosses. Genetics 127:87-101.

Viedma, L. Q. 2005. Wheat blast occurrence in Paraguay. Phytopathology 95:S152.

Wang, S., Asuke, S., Vy, T. T. P., Inoue, Y., Chuma, I., Win, J., Kato, K., and Tosa, Y. 2018. A new resistance gene in combination with *Rmg8* confers strong resistance against *Triticum* isolates of *Pyricularia oryzae* in a common wheat landrace. Phytopathology 108:1299-1306.

Warburton, M. L., Crossa, J., Franco, J., Kazi, M., Trethowan, R., Rajaram, S., Pfeiffer, W., Zhang, P., Dreisigacker, S., and Van Ginkel, M. 2006. Bringing wild relatives back into the family: Recovering genetic diversity in CIMMYT improved wheat germplasm. Euphytica 149:289-301.

Williamson, V. M., Thomas, V., Ferris, H., and Dubcovsky, J. 2013. An *Aegilops ventricosa* translocation confers resistance against root-knot nematodes to common wheat. Crop Sci. 53:1412-1418.

Yu, J., Holland, J. B., McMullen, M. D., and Buckler, E. S. 2008. Genetic design and statistical

power of nested association mapping in maize. Genetics 178:539-551.

Zhang, P., Dundas, I. S., Xu, S. S., Friebe, B., McIntosh, R. A., and Raupp, W. J. 2017. Chromosome engineering techniques for targeted introgression of rust resistance from wild wheat relatives. Methods in Mol. Biol. 1659:163-172.

Zhang, N., Luo, J., Rossman, A. Y., Aoki, T., Chuma, I., Crous, P. W., Dean, R., de Vries, R. P., Donofrio, N., Hyde, K. D., Lebrun, M. H., Talbot, N. J., Tharreau, D., Tosa, Y., Valent, B., Wang, Z., and Xu, J. R. 2016. Generic names in *Magnaporthales*. IMA fungus 7:155-159.

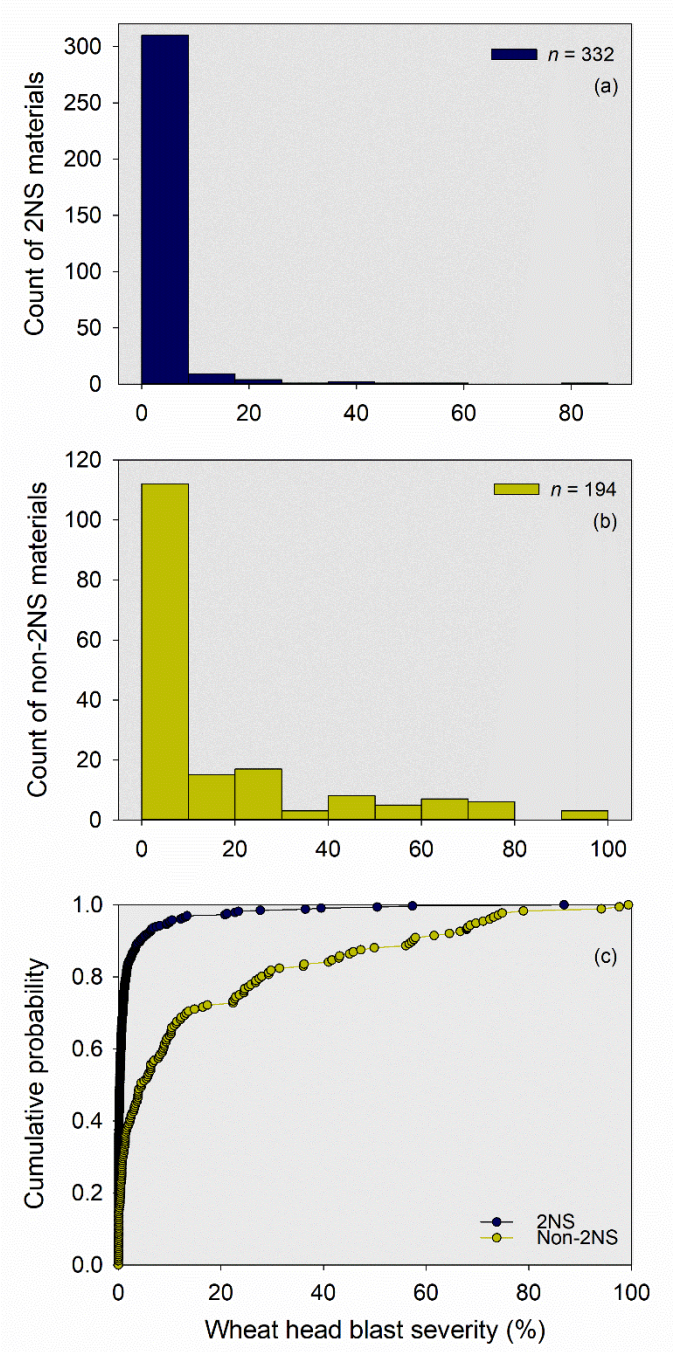


Figure 1-1. Data combined from 12 field experiments. Blast disease severity frequency distribution evaluated at soft dough stage of grain development for (a) 2N^{v}S and (b) non-2N^{v}S genotypes. (c) cumulative probability of head blast severity for 2N^{v}S (blue dots) and non-2N^{v}S genotypes (yellow dots).

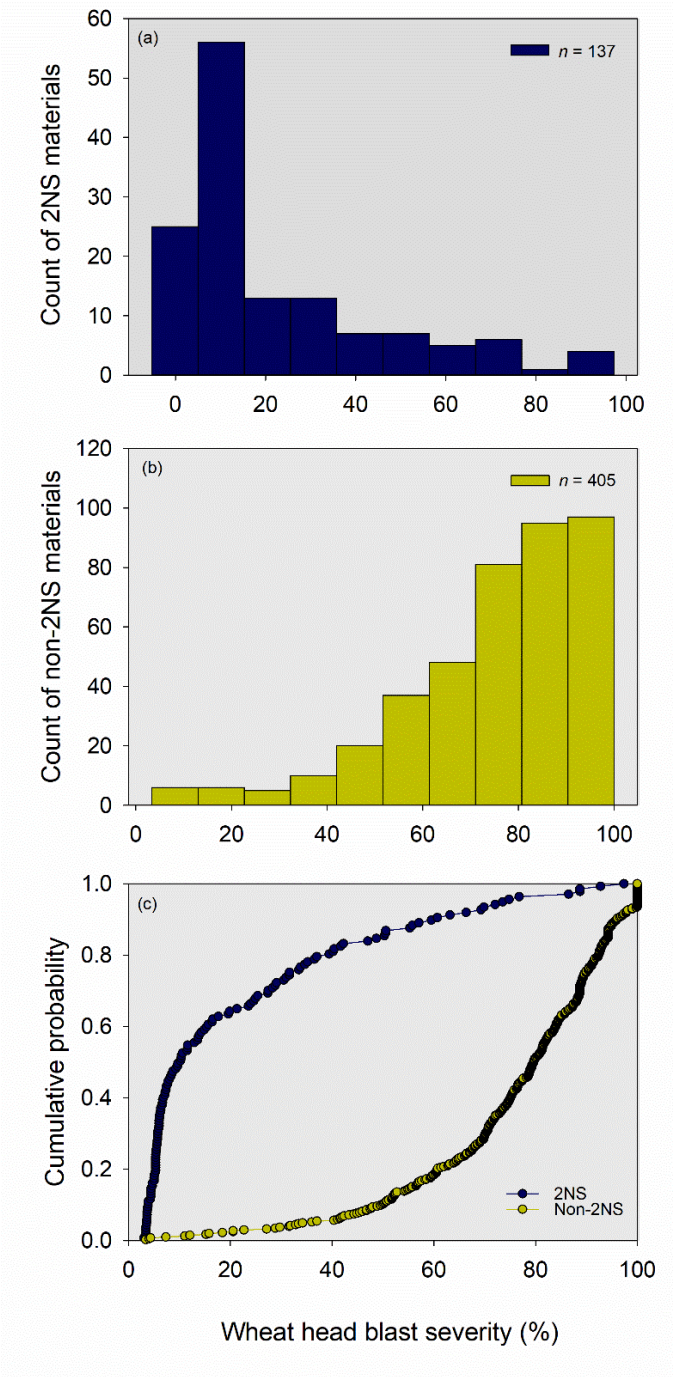


Figure 1-2. Data combined from 17 experiments under controlled environment conditions. Blast disease severity frequency distribution evaluated at 14 days after inoculation for (a) 2N^{v}S and (b) non-2N^{v}S genotypes. (c) cumulative probability of head blast severity for 2N^{v}S (blue dots) and non-2N^{v}S genotypes (yellow dots).

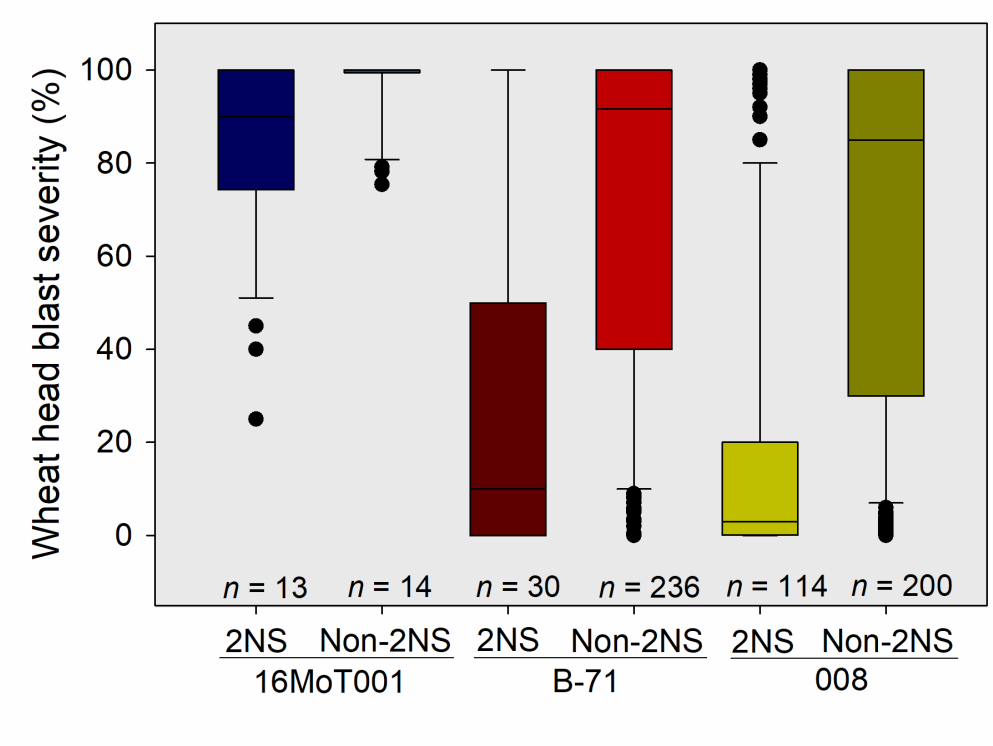


Figure 1-3. Wheat blast disease severity for 2N^{v}S and non-2N^{v}S genotypes tested against three MoT isolates (Brazilian isolate 16MoT001; Bolivian isolate B-71; and Bolivian isolate 008); evaluated in controlled environment experiments.

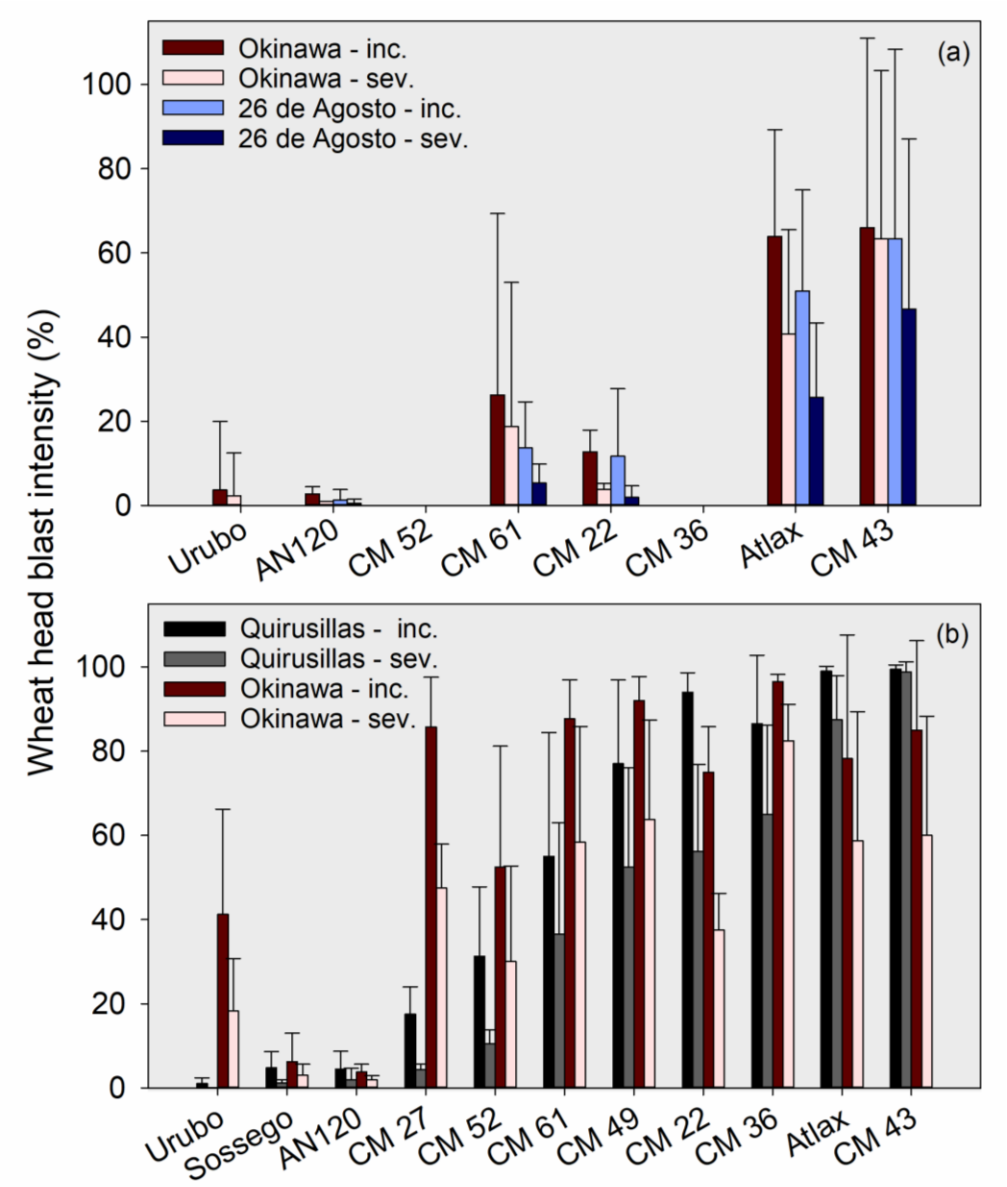


Figure 1-4. Wheat spike blast disease incidence (inc.) and severity (sev.) of CIMMYT breeding lines, plus checks under field conditions at two locations during (a) 2017 and (b) 2018. Atlax was the non-2N^{v}S susceptible check and Urubo, Sossego, and AN120 were the 2N^{v}S resistant checks. Disease intensity was measured at soft dough stage of grain development.

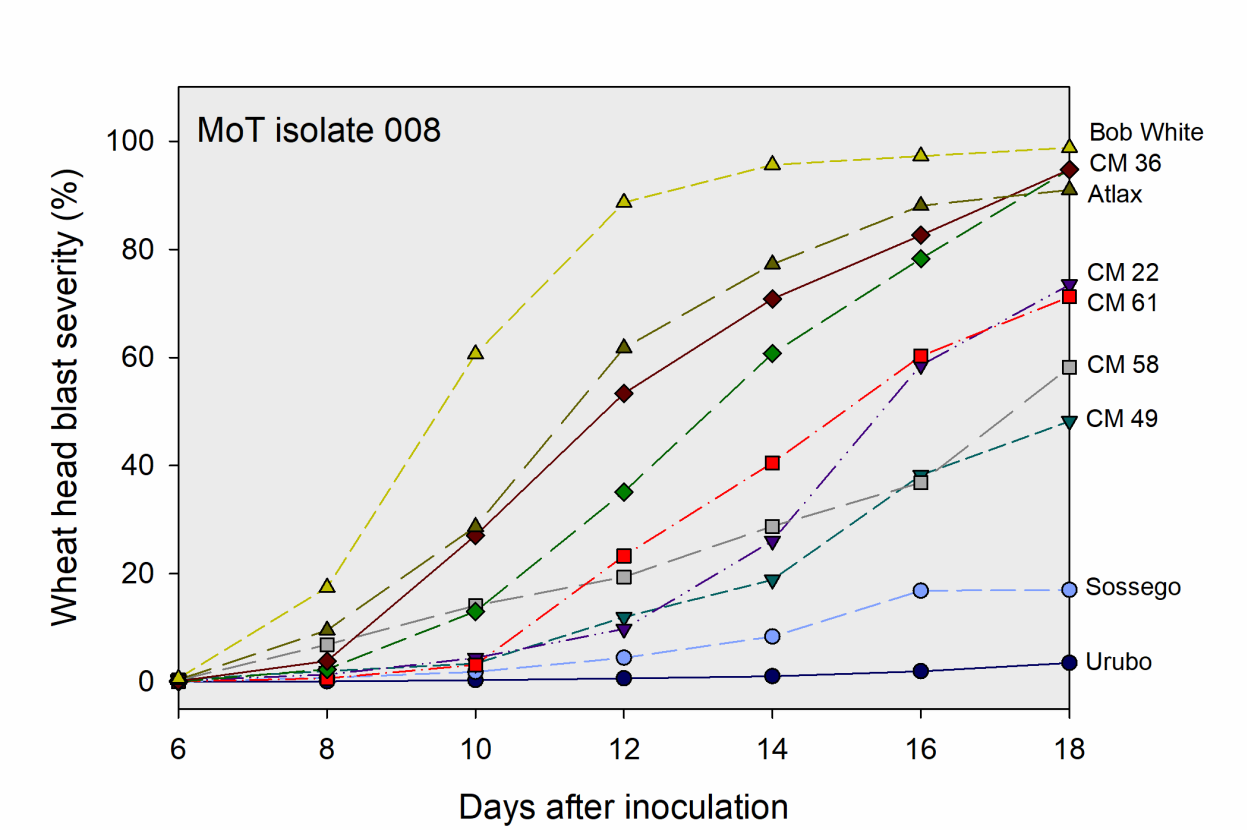


Figure 1-5. Wheat spike blast disease progress curves for non-2N^{v}S CIMMYT lines plus checks under controlled environmental conditions in Bolivia using MoT isolate 008. Urubo and Sossego were the 2N^{v}S resistant checks, and Atlax and Bob White the non-2N^{v}S susceptible checks. Disease assessments were made 14 days after inoculation.

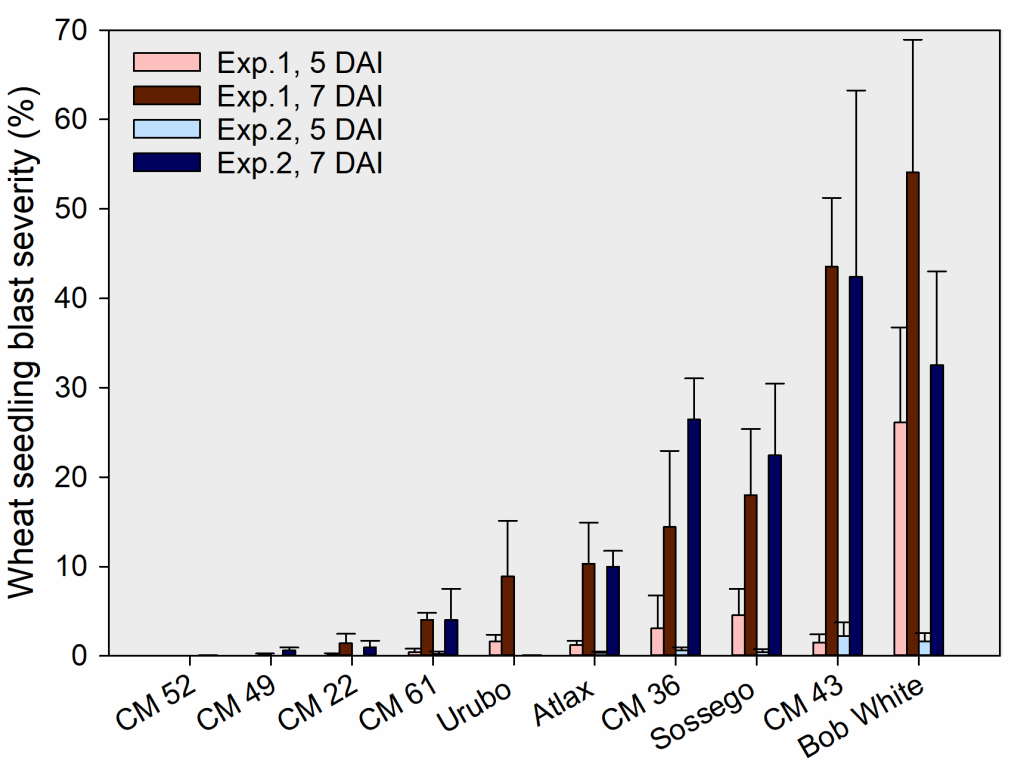


Figure 1-6. Wheat blast disease severity at seedling stage of CIMMYT lines plus checks. Experiments were repeated twice (Exp. 1 and Exp. 2) under controlled environment conditions in Bolivia using MoT isolate 008. Atlax and Bob White were the non-2N^{v}S susceptible checks and Urubo and Sossego the 2N^{v}S resistant checks. Evaluated at five and seven days after inoculation.

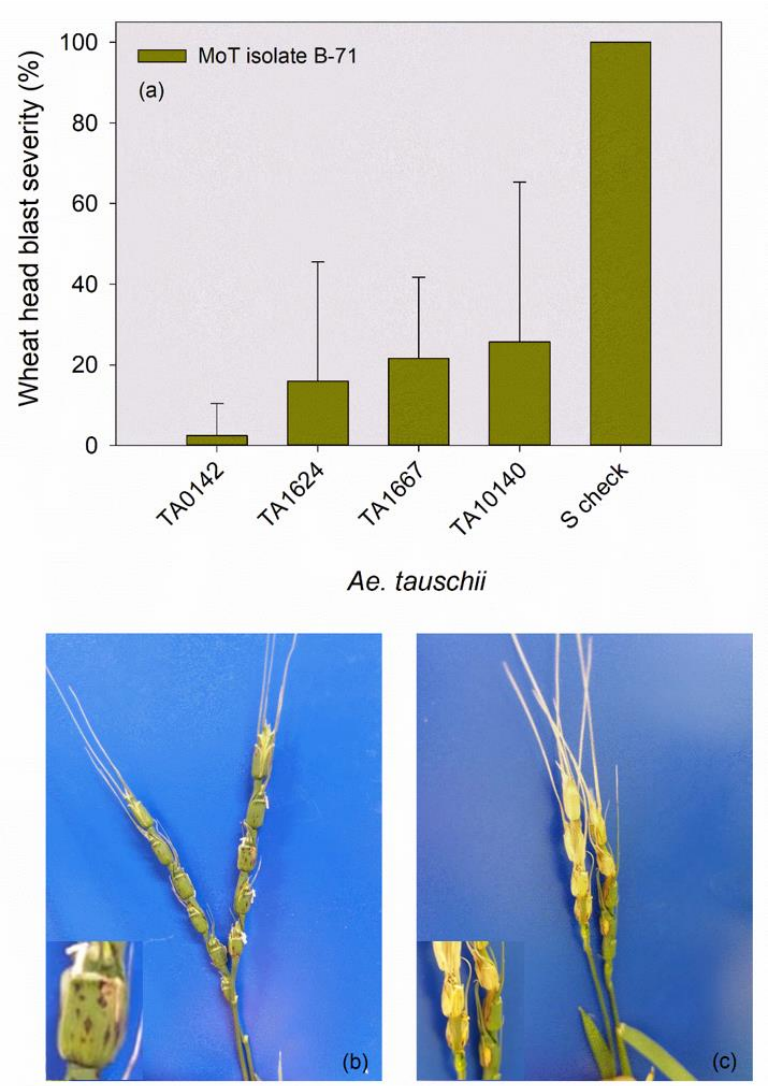


Figure 1-7. (a) Wheat blast disease severity of four *Ae. tauschii* accessions plus the susceptible check Cavalier evaluated 14 days after inoculation under controlled environment conditions using MoT isolate B-71. (b) Dark brown flecks (hypersensitive response) indicates a resistant reaction, and (c) individual large lesions to totally bleached spikes indicates a susceptible reaction.

Table 1-1. MoT race designation and disease phenotype of a set of differential wheat cultivars tested against the three MoT isolates used under controlled environment experiments.

		Cultivar		
MoT race	**MoT isolate[a]**	**Everest (Non-2N^{v}S)**	**Jagalene (2N^{v}S)**	**RonL (Non-2N^{v}S)**
		Race identification		
0	-	R	R	R
1	-	S	R	R
2	-	S	R	S
3	-	S	S	S
		Wheat spike blast severity[b] (%)		
2	B-71	88a[c]	13a	94a
2	008	90a	29a	85a
2	16MoT001	89a	38b	83a

[a] MoT isolates used in this study
[b] Data collected in this study
[c] Means followed by the same letter within the same cultivar are not statistically different at $\alpha = 0.05$.

Table 1-2. Summary of experiments conducted under field conditions in Bolivia.

Experiment number	Genotypes tested	*N*[a]	Year	Location
1	CIMMYT	22	2017	Okinawa
2	Biotrigo	65	2017	Okinawa
3	NAM and EMMER	65	2017	Okinawa
4	CIMMYT	20	2017	26 de Agosto
5	Biotrigo	61	2017	26 de Agosto
6	Biotrigo	192	2017	Quirusillas
7	CIMMYT	24	2018	Okinawa
8	Biotrigo	137	2018	Okinawa
9	CIMMYT	25	2018	Quirusillas
10	Winter wheat	8	2018	Quirusillas
11	Biotrigo	72	2018	Quirusillas
12	Biotrigo	69	2018	Quirusillas

[a] number of wheat breeding lines plus susceptible and resistant checks evaluated. CIMMYT= International Maize and Wheat Improvement Center, Carretera Mexico-Veracruz, Km. 45, El Batan 56237 Texcoco, Mexico; Biotrigo= Estrada do Trigo, 1000 - São José, Passo Fundo - RS, 99052-160, Brazil; NAM= Nested Association Mapping; EMMER= *Triticum turgidum* subsp. *dicoccum*; winter wheat= U.S. breeding lines from several wheat breeding programs.

Table 1-3. Summary of experiments conducted under growth chamber and greenhouse conditions.

Experiment number	Genotypes evaluated	*N*[a]	MoT isolate[b]	Environment[c]	Location
1	Diverse set - spring wheat	9	B-71	BRI	US
2	Diverse set - spring wheat	14	B-71	BRI	US
3	Winter wheat	4	B-71	BRI	US
4	NAM	34	B-71	BRI	US
5	*Ae. tauschii*	16	B-71	BRI	US
6	*Ae. tauschii*	38	B-71	ARS-FDWSRU	US
7	Diverse set - spring wheat	19	008	ANAPO	Bolivia
8	Winter wheat	23	008	ANAPO	Bolivia
9	EMMER	55	008	ANAPO	Bolivia
10	NAM and EMMER	56	008	ANAPO	Bolivia
11	CIMMYT	9	008	ANAPO	Bolivia
12	Biotrigo	27	16MoT001	Biotrigo	Brazil
13	CIMMYT	60	B-71	ARS-FDWSRU	US
14	*Ae. tauschii*	32	B-71	BRI	US
15	*Ae. tauschii*	25	B-71	BRI	US
16	Winter wheat	8	B-71	BRI	US
17	Bi-parental population	188	008	ANAPO	Bolivia

[a] number of lines plus susceptible and resistant checks evaluated. Spring and winter wheat= diverse set of U.S. and Bolivian breeding lines; NAM= Nested Association Mapping; EMMER= Triticum turgidum subsp. dicoccum; Biotrigo= Estrada do Trigo, 1000 - São José, Passo Fundo - RS, 99052-160, Brazil; CIMMYT= International Maize and Wheat Improvement Center, Carretera Mexico-Veracruz, Km. 45, El Batan 56237 Texcoco, Mexico; Bi-parental population=spring whea cross of Sossego and Alvorada (Biotrigo).

[b] B-71= Bolivia, 2012 (race 2); 008= Bolivia, 2015 (race 2); 16MoT001= Brazil, 2016 (race 2).

[c] BRI= Biosecurity Research Institute, Manhattan, KS, U.S.; ARS-FDWSRU=Agricultural Research Service Foreign Disease-Weed Science Research Unit, Ft. Detrick, MD, U.S.; ANAPO= Association of Producers of Oilseeds and Wheat, Santa Cruz de la Sierra, Bolivia.

Chapter 2 - Genome-wide association study of resistance to wheat spike blast identifies potential novel loci conferring resistance

Abstract

Wheat spike blast (W_SB), caused by *Magnaporthe oryzae* pathotype *Triticum* (MoT), is an emerging threat to wheat production. W_SB has relied upon a single major source of resistance, the $2N^vS$ translocation introgressed from the wild relative, *Aegilops ventricosa*. However, this resistance is partial, background dependent, and is being overcome by newer and more aggressive MoT races. With the objective to characterize potential novel loci conferring resistance to W_SB, we conducted a genome-wide association study (GWAS) using a diverse panel of 384 wheat genotypes, including elite wheat varieties, modern breeding lines, and landraces. Genotypes were phenotyped under three controlled environment conditions using MoT isolates T-25 (1988), B-71 (2012), and 008 (2015). Genotyping was carried out using genotyping-by-sequencing and 13,175 SNPs were identified after filtering. Principal components analysis (PCA) identified two clusters based on the presence/absence of the $2N^vS$ translocation and the first three PCAs explained together 13% of the genetic variation. Three individual analyses were performed [full (all genotypes combined), $2N^vS$ genotypes only, and non-$2N^vS$ genotypes only] using a linear mixed model and a threshold of significance of false discovery rate at 5%. Association analysis detected 25 significant SNPs for the full GWAS with MoT isolate T-25. No significant associations were identified with $2N^vS$ and non-$2N^vS$ only GWAS and with isolates B-71 and 008. Twenty-one SNPs were mapped on 2A chromosome with MoT isolate T-25 in the same physical position as the $2N^vS$ translocation. In addition, highly significant linkage disequilibrium was found among these SNPs, suggesting they might tag the

same QTL. Removing the major effect of the 2N^{v}S did not reveal additional significant SNPs. QTL pyramiding analysis showed that the combination of multiple QTL was not statistically different from the individual effect of the 2A QTL. Several potential candidate genes underlying significant SNPs were previously associated with disease resistance. Further efforts to validate these genomic regions will be helpful to breed for broad spectrum and durable wheat blast resistance.

Introduction

Wheat blast, caused by the fungus *Magnaporthe oryzae* pathotype *Triticum* (MoT) is an important disease of wheat (*Triticum aestivum* L.). Since it was first reported in the late 1980s (Igarashi et al. 1986), blast has been one of the most destructive diseases of wheat in South America (Barea and Toledo 1996; Viedma 2005; Perello et al. 2015). In recent years, it also became a significant threat for wheat production in South Asia (Malaker et al. 2016; Mottaleb et al. 2019). The disease is favored by warm (25-30°C), rainy, and high relative humidity (>90%) conditions (Cardoso et al. 2008), and under this scenario, yield losses up to 100% have been reported in individual fields sown with susceptible varieties (Goulart et al. 2012). Chemical and cultural management tactics are either not available or not effective (Cruz and Valent 2017, Cruz et al. 2019). Thus, the use of resistant cultivars is the most effective and sustainable way to control wheat blast.

Despite more than 30 years of intensive efforts investigating wheat genetic resistance to MoT, reliable resistance resources are still scarce (Cruz et al. 2016; Cruz and Valent, 2017; Cruppe et al. 2019). Several wheat genotypes were identified as resistant to specific races of MoT (Cruz et al. 2016; Cruppe et al. 2019); however, the majority of the reported resistance is based on the same source, the 2N^{V}S translocation (Cruz et al. 2016). This chromosome segment

was introgressed into hexaploid wheat from the wild relative *Aegilops ventricosa* and it was previously exploited in wheat breeding programs due to its resistance to the three wheat rusts and to nematodes (Jahier et al. 2001; Helguera et al. 2003; Williamson et al. 2013). A few *R* genes were identified within the $2N^{v}S$ translocation and molecular markers were developed for several important previously mentioned diseases; however, the specific gene(s) associated with wheat blast resistance has not been identified. Thus, the genetic mechanism behind the $2N^{V}S$ wheat blast resistance-trait remains unknown. Based on field and controlled environment experiments, the $2N^{V}S$-based cultivars exhibit characteristics of both qualitative (i.e., complete resistance controlled by a single gene or a few genes with large effects) and quantitative resistance (i.e., incomplete resistance controlled by multiple genes of small effects) (Cruz et al. 2012; Cruz et al. 2016; Cruppe et al. 2019). Qualitative resistance, such as the one conferred by NBS-LRR genes, is often known for its large effects and usually provides a strong selection pressure on the pathogen, leading to the pathogen's rapid evolution and consequently resistance breakdown (Poland et al. 2008). Meanwhile, quantitative resistance tends to be more durable than qualitative resistance (Parlevliet, 2002), because each minor gene has only a small effect on the phenotypic variation, causing a weak selection pressure on the pathogen (Poland et al. 2008).

Resistance to wheat spike blast ($W_{S}B$) most of the times behaves as quantitative due to its continuous variation in disease severity (Maciel et al. 2014; Cruz et al. 2016; Cruppe et al. 2019). In controlled environment experiments encompassing a diverse set of 542 wheat accessions, disease severity from the $2N^{V}S$-based accessions (n = 137) ranged from zero to 100%, with a mean severity of 22% (Cruppe et al. 2019). Within the non-$2N^{V}S$ accessions, the authors reported a similar disease severity range, though with a mean severity of 78%. Notwithstanding, resistant $2N^{V}S$-based cultivars often exhibit a visible defense response usually conferred by *R*

genes and known as the hypersensitive response, which is uncommon for quantitative resistance. This suggests that the mechanism might also be qualitative. This defense mechanism causes the rapid death of plant cells at the site of pathogen entrance, restricting its spread to new cells (Heath, 2000; González et al. 2012). Additional evidence for qualitative nature of W_SB resistance mechanism is the often-observed strong isolate-by-cultivar interaction (Maciel et al. 2014; Cruz et al. 2016). Experiments with near-isogenic lines (NILs, i.e., with vs. without the $2N^VS$ translocation) demonstrated that not all the NILs carrying the $2N^VS$ translocation had a significant reduction in wheat blast when tested against newer and more aggressive MoT isolates, suggesting that the genetic background may influence the effect of the $2N^VS$ resistance (Cruz et al. 2016). It is usually assumed that quantitative resistance is non-race specific, however several race-specific quantitative loci have been identified in other pathosystems such as rice blast (*Magnaporthe oryzae Oryza* B. Couch) (Ballini et al. 2008), barley leaf stripe (*Pyrenophora graminea* Ito and Kuribayashi) (Arru et al. 2003), barley leaf rust (*Puccinia hordei* Otth) (Parlevliet, 1978), and several others. Pyramiding both qualitative and quantitative resistance could be the best strategy to confer adequate levels of wheat blast resistance while increasing the durability of the deployed resistance.

A widely used strategy to identify quantitative trait loci (QTL) associated with resistance against a particular pathogen is through a biparental population, which is also known as linkage analysis (Borner et al. 2002; Quarrie et al. 2005, Horsley et al. 2006; Huang et al. 2006; Wisser et al. 2006; Kumar et al. 2007; Liu et al. 2009; Lu et al. 2009). Although this strategy has been successful for several pathosystems over the years, it is labor intensive and developing recombinant inbred lines or double-haploid populations is very time-consuming (Neumann et al. 2011). In addition, linkage analysis only accesses the alleles segregating between the parents

(i.e., few recombination events), exploring limited genetic diversity and limiting the mapping resolution in most circumstances (Brachi et al. 2011). Consequently, large genomic regions are associated with the trait of interest and they need to be fine-mapped in follow-up experiments (Neumann et al. 2011). The genome-wide association study (GWAS) is a powerful strategy to identify associations between markers and traits based on the linkage disequilibrium (LD) between a marker and the causal polymorphism. The GWAS approach overcomes most limitations of the biparental mapping by using natural populations, which eradicates the need for developing populations, saving time and resources; and by using a diverse germplasm collection (i.e. accounts for historical recombination events), thus offering higher mapping resolution with smaller genomic region intervals (Brachi et al. 2011; Korte and Farlow, 2013; Ogura and Busch, 2015). However, alleles with low frequency are usually not detectable by GWAS; thus, having a balance between resistant and susceptible genotypes is critical to minimize this shortcoming.

Genome-wide association mapping has been successfully applied in wheat to explore genetic improvement for several agronomic traits such as yield and yield components (Lopes et al. 2015; Sukumaran et al. 2015), disease resistance (Gurung et al. 2014; Arruda et al. 2016; Tessmann et al. 2019), and grain quality (Kumar et al. 2018; Liu et al. 2018). To our knowledge, however, only one GWAS has been reported for wheat blast to date (Juliana et al. 2019). In this study, encompassing 271 lines from the CIMMYT International bread wheat screening nursery, two chromosomal regions (2AS and 3BL) were associated with wheat blast resistance under field conditions in Bolivia using a local MoT strain. The authors demonstrated that the 2AS region corresponds to the $2N^{v}S$ translocation, which was previously reported as the only known useful source of wheat blast resistance (Cruz et al. 2016). This study emphasizes the scarce availability of genetic resistance to wheat blast. Given the continuous variability in wheat blast severity

among the 2N^{V}S-based varieties and the limited disease control of the non-2N^{V}S-based varieties, we hypothesize that the wheat blast resistance is an oligogenic trait, i.e., controlled by a few resistance genes, in which the 2N^{v}S translocation has a major effect combined with a few additional resistance genes of smaller effects. We also hypothesize that the 2N^{v}S effect depends on the genetic background, i.e., the additional minor genes. The objective of this study was to identify genomic regions associated with W$_{S}$B resistance among a diverse panel of wheat genotypes. Identifying both minor and *R* genes will be critical to breed for durable resistance. Markers for these genes can be used to rapidly and efficiently introgress novel sources of W$_{S}$B resistance into adapted cultivars.

Materials and methods

Plant materials, experimental design, and phenotypic evaluation of wheat spike blast severity

The 384 spring and winter wheat genotypes used in this study were selected to represent a wide range in response to W$_{S}$B (Cruz et al. 2012; Cruz et al. 2015; Cruppe et al. 2019). In addition to elite cultivars and modern breeding lines, a nested association mapping (NAM) population was also included in this study to represent a diverse panel of geographic distribution of wheat across the globe including accessions from 32 different countries. Wheat spike blast evaluation was conducted at three controlled environment locations: (i) Biosecurity Research Institute (BRI) in Manhattan (Kansas, U.S.); (ii) Agricultural Research Service Foreign Disease-Weed Science Research Unit (ARS-FDWSRU) in Fort Detrick (Maryland, U.S.); (iii) Asociación de Productores de Oleaginosas y Trigo (ANAPO) in Santa Cruz de la Sierra (Bolivia). For all experiments, the pots were arranged in an incomplete randomized block design with two to six replications (depending on seed availability), where each replication consisted of

one pot (producing an average of 3 to 10 wheat spikes per pot). Inoculum production and inoculation procedure are described in detail by Cruz et al. (2016) and Cruppe et al. (2019). Briefly, MoT cultures were grown on homemade oatmeal agar (Valent et al. 1991) and 8-d old cultures were flooded with a mixture of sterile deionized water with 0.42% unflavored gelatin and 0.01% Tween 20, stirred to dislodge conidia from conidiophores, and filtered with four layers of sterile cheesecloth to eliminate the mycelia and any remaining agar medium. The spore concentration was adjusted to 20,000 spores ml^{-1} of suspension and 0.75 ml of the spore suspension was individually applied to each wheat head approximately 2 days after full head emergence (Feekes GS 10.5, Large 1954) using an airbrush (model 69492, Harbor Freight Tools, Camarillo, CA). Immediately after inoculation, the spikes were covered with black plastic bags (model S-12322BL, ULINE) moistened with water and incubated for 48 hours.

Three different MoT isolates were used in this study: (i) T-25, collected in Brazil in 1988, (ii) B-71, collected in Bolivia in 2012, and (iii) 008, collected in Bolivia in 2015. The T-25 isolate is less aggressive and was classified as MoT race 1 (Cruz et al. 2012), while both B-71 and 008 are highly aggressive isolates and were classified as MoT race 2 (Cruppe et al. 2019). The T-25 isolate was tested on 301 genotypes, while B-71 and 008 were tested on 87 and 49 genotypes, respectively. Susceptible and resistant checks were included in all experiments and varied according to location and experiment (i.e., winter vs spring wheat experiment). Wheat spike blast severity was evaluated as the percentage of the spikelets affected (i.e., 1 to 100% scale), and it was measured at least two times at 2-d intervals, with the first rating beginning when the susceptible check reached 90% of disease severity (i.e., usually 10 to 14 days after inoculation) (Cruz et al. 2016).

Statistical analysis of the phenotypic data

Descriptive analyses, analysis of variance, and correlation analysis were performed with both SAS and R software. Phenotypic data for each of the three MoT isolates were analyzed separately considering the difference in aggressiveness between these isolates and the different genotypes tested against each isolate (i.e. not all 384 genotypes were tested against the three isolates). Best linear unbiased estimators (BLUEs) were calculated for each genotype using the *lme4* package in R following the model:

$$Y_{ijk} = \mu + G_i + E_j + R_{k(j)} + \varepsilon_{ijk}$$

where Y_{ijk} is the WSB response for *i-th* genotype in *j-th* environment and *k-th* replication, μ is the overall mean; G_i is the fixed effect of the *i-th* genotype, E_j is the random effect of the *j-th* environment distributed as independent and identically distributed (iid) $E_{(j)} \sim N(0, \sigma_E^2)$, R_k is the random effect of the *k-th* replication nested within the *j-th* environment distributed as iid $R_{k(j)} \sim N(0, \sigma_R^2)$; and ε_{ijk} is the residual error effect distributed as iid $\varepsilon_{(ijk)} \sim N(0, \sigma_\varepsilon^2)$. The broad sense heritability (H^2) was calculated using the following formula:

$$H^2 = \sigma_G^2 \,/\, \left(\sigma_G^2 + \frac{\sigma_\varepsilon^2}{r}\right)$$

where σ_G^2 and σ_ε^2 are the variance of the genotypes and the residuals, respectively, and r is the number of replications calculated as the harmonic mean due to the variable number of replications for each line.

DNA extraction and genotyping-by-sequencing (GBS)

Genomic DNA was isolated and purified from wheat leaves of 10-d old seedlings using BioSprint 96 DNA Plant Kits and a BioSprint 96 robot (QIAGEN, Valencia, CA, United States) following the manufacturer's protocol. The GBS method was performed as previously described

by Poland et al. (2012). Briefly, GBS libraries were prepared in 96-plex, and genomic DNA was digested with *PstI* (CTGCAG) and *MspI* (CCGG) restriction enzymes, ligated with barcode adapters, and amplified by PCR. Pooled GBS libraries were sequenced using Illumina HiSeq2500 equipment. Single nucleotide polymorphisms (SNPs) were called using Tassel 5.0 (Glaubitz et al. 2014). The physical positions of SNP markers were corrected based on the reference genome *161010_Chinese_Spring_v1.0* (IWGSC, 2017). Unanchored SNPs were retained in the genotypic data and assigned to an unknown chromosome (UN). A total of 61,920 SNPs were initially discovered from the 384 wheat accessions. The SNPs were filtered based on the following criteria: (i) markers with minor allele frequency (MAF) greater than 5% were retained, (ii) genotypes having more than 20% missing information were excluded, and (iii) the markers with multi-allelic calls and/or heterozygosity greater than 20% were excluded. As a result, 13,175 SNPs were retained after filtering and used in this study. The presence/absence of the $2N^vS$ translocation was based both on a bioinformatics pipeline using GBS data (Liangliang Gao, *unpublished*) and on the Cleaved Amplified Polymorphic Sequences (CAPS) marker (ventriup-LN2) protocol modified from a protocol previously described by Helguera et al. (2003).

Population structure

One critical step for GWAS analysis is to determine and account for population structure. We used the *A.mat* function from the *rrBLUP* package in R (Endelman, 2011) to impute missing data using the mean method (the mean for each marker). The resulting matrix was used to calculate the eigenvalues and eigenvectors with the *eigen* function to perform principal component analysis (PCA). Given the known effect of the $2N^vS$ translocation, we used it as a criterion to examine any potential population structure based on its presence/absence.

Genome-Wide Association Analysis

Association mapping analysis was performed using a linear mixed model (Yu et al. 2006):

$$y = X\beta + Zg + S_\tau + \varepsilon$$

where y is a n x 1 vector of adjusted means (BLUEs) of W_SB severity, β is a f x 1 vector of fixed effects, g is a n x 1 vector of the genetic background of each genotype as a random effect, τ is a vector of additive SNP effects as a fixed effect, and ε is a vector of residual effects. The respective design matrices: X is a $n\ x\ f$ matrix where f is the number of fixed covariates and n is the number of genotypes; Z is a $n\ x\ n$ matrix relating y to u; S is a $n \times 1$ vector of marker scores. The equation above was applied in the *rrBLUP* package in R (Endelman, 2011), where each marker is independently tested to estimate the effect τ (a scalar), by treating S as a column vector of marker score covariates with values of -1, 0, or 1. First, GWAS was implemented without controlling for population structure effects (Q) and Kinship matrix (K, relatedness between genotypes) (naïve model, data not shown). To account for spurious associations between the SNPs and the trait and the relatedness between individuals, a second GWAS was implemented controlling for both Q (the first three PCs were included) and K effects (Q + K model). Due to the significant effect of the 2N^{v}S translocation in our panel, three GWAS were performed individually [i.e. all genotypes combined (full), only non-2N^{v}S based genotypes, and only 2N^{v}S-based genotypes] for each of the three MoT isolates separately (T-25, B-71, and 008). After splitting the data according to the presence/absence of the 2N^{v}S translocation, the SNPs were filtered again. Significant trait-marker associations were detected using multiple test correction with threshold at 5% false discovery rate (FDR 5%). FDR values were obtained from the GWAS analysis with *rrBLUP*. GWAS results were used to reconstruct Manhattan plots using the *qqman* R package.

Genes underlying significant SNPs and their functional annotations

To find candidate genes or related proteins linked to significant SNP markers, we investigated whether any of the significant SNPs were mapped on genes that have been identified and annotated in the reference genome assembly (International Wheat Genome Sequencing Consortium, IWGSC Ref Seq v1.0). We performed a search in Crop Bioinformatics Adelaide (CroBiAd), using the physical starting point of the marker preceded by the chromosome name and 10kb were added before and after. The 20-kb interval was then explored for predicted genes and annotations that were available from the IWGSC, 2014. Additionally, we performed a BLAST search of the significant associated GBS tags in National Center for Biotechnology Information (NBCI).

Results

Phenotypic data analyses

The frequency distribution and descriptive statistics of W_SB severity showed a wide variation within the diverse panel used in this study (Figure 2-1 and Table 2-1). Among all genotypes, disease severity ranged from zero to 100%, with differences within the three MoT isolates (T-25, B-71 and 008) and the presence/absence of the $2N^vS$ translocation (Table 2-1). The MoT isolate B-71 was significantly more aggressive compared to isolates T-25 and 008 within the $2N^vS$-based genotypes. There was no statistical difference in W_SB severity between the isolates within the non-$2N^vS$ based genotypes. Of the 384 wheat genotypes used in this study, 76 genotypes tested positive and 308 tested negative for the presence of the $2N^vS$ translocation. The presence of this alien chromosome segment significantly decreased W_SB severity when genotypes were tested against MoT isolates T-25 and 008; however, its presence/absence did not

affect W_SB severity with MoT isolate B-71. Broad sense heritability estimates were 0.64 for MoT isolate 008, 0.75 for MoT isolate T-25 and 0.81 for MoT isolate B-71.

Genotypic data and population structure analyses

The number of markers for each chromosome, total length, average and maximum spacing and the percentage of missing loci are described in Table 2-2. From the 13,175 SNP markers in the full GWAS, 5,423 (41.16%) were mapped on A genome, 5,965 (45.27%) on B genome, 1,599 (12.13 %) markers were mapped on D genome, and 188 (1.42%) markers were present on unanchored contigs and labeled as "UN". The SNP markers spanned 14.5 giga base (Gb) of the physical map representing a high coverage of the wheat reference genome, with average spacing of 1.65 mega bases (Mb) and maximum spacing 161.5 Mb. Markers on the D genome had the largest average spacing (2.97 Mb), while A and B genomes had similar averages (approximately 0.93 Mb). Overall, the markers were more concentrated at the end of chromosomes (i.e., sub-telomeric regions) (Figure 2-2). Principal components analysis (PCA) revealed two clusters and the first three PCAs explained together 13% of the total genetic variation with 6% for PC1, 4% for PC2, and 3% for PC3 (Figure 3). The two clusters were highlighted due to the presence/absence of the 2N^{v}S translocation.

Genome-wide association mapping

Three GWAS were performed individually [i.e. all genotypes combined (full), only non-2N^{v}S based genotypes, and only 2N^{v}S-based genotypes] for each of the three MoT isolates separately (T-25, B-71, and 008). Information on significant SNPs, position, *p* values, haplotypes with their respective W_SB severity is shown on Table 2-3. Association analysis detected 25 significant SNPs above the threshold FDR at 5% for the full GWAS. No significant associations were found for the non-2N^{v}S genotypes GWAS and for the 2N^{v}S genotypes GWAS.

With all genotypes combined, i.e. full GWAS, 25 significant SNPs were identified using MoT isolate T-25. Specifically, 21 significant SNPs were mapped on chromosome 2A, one on chromosome 1B, and three on chromosome 1D (0.75 Mb max distance) (Figure 2-4a). For MoT isolate B-71, the three SNPs with the highest *p* values were mapped on chromosome 2A and coincided with the significant SNPs identified with isolate T-25; however, they were not statistically significant (Figure 2-4b). The pairwise linkage disequilibrium (LD) between the 21 SNPs on chromosome 2A is illustrated in Figure 2-5. Highly significant LD was found among these 21 significant SNPs, suggesting that they may tag the same QTL. The physical positions of the SNP markers on the 2A chromosome coincides with the $2N^{v}S$ translocation. The large number of missing data on this locus likely led to different *p* values, which could explain multiple associations being identified (Supplemental Figure B1). Given the large number of SNPs on chromosome 2A, we hypothesized that this QTL could be masking the effect of other QTL of smaller effects in other genomic regions, thus investigating the non-$2N^{v}S$ genotypes independently could reveal additional QTL. However, the removal of the major effect of the $2N^{v}S$ translocation did not reveal additional significant associations with any of the three isolates. The lack of significant SNPs identified with MoT B-71 and MoT 008 in the three association analyses, is most likely due to the small population size tested with these two isolates (*n* = 87 with B-71 and *n* = 49 with 008) (Table 2-2).

Effects of QTL and QTL pyramiding

Pairwise comparison for the full association analyses is shown in Supplemental Table B1. For the full GWAS, W_SB severity ranged from 66% for genotypes not carrying any QTL (*n*= 96) to 22% with the 1B + 2A QTL combination (*n*= 17). The individual effect of the QTL on 2A chromosome (*n*= 32, 25% severity) significantly decreased W_SB severity when compared to the

individual effects of QTL on chromosomes 1B (*n*= 142, 51% severity) and 1D (*n*= 113, 43% severity), and their combination (*n*= 58, 46% severity). W_SB average for the genotypes containing the 1B + 1D + 2A QTL combination was 28% and it was statistically different from the individual effect of 1B (50%), but not different from 1D (43%). The QTL combinations that included the 2A QTL did not differ statistically from its individual effect (2A= 25%, 1B + 2A = 22%, 1D + 2A = 28.6%, and 1B + 1D + 2A = 28) (Figure 2-6). Despite the numerical difference in W_SB severity due to the presence of individual QTL or some specific QTL combinations, there were actually few statistical differences because of the high variability in the response to W_SB severity among the genotypes containing the same QTL or combinations of QTL (Supplemental Table B1).

Genes underlying significant SNPs and their functional annotations

For isolate T-25, six proteins were predicted and these included serine proteinase inhibitor-like allergen, protein detoxification 40-like, arginase gene, xylanase inhibitor, putrescine hydroxycinnamoyl transferase, and uncharacterized acetyltransferase At3g50280-like.

Discussion

Phenotypic and genotypic analyses

Three different MoT isolates with different levels of aggressiveness were used (T-25, B-71, and 008). However, due to the exotic nature of this pathogen, the exchange of MoT isolates is challenging and/or not permitted. Thus, most genotypes were tested with only one isolate. For instance, although wheat blast has been established in Brazil and Bolivia for several years, these countries do not allow the exchange of foreign MoT isolates to avoid the introduction of new and possibly more aggressive MoT races into the country. In the United States, on the other hand, all experiments were performed in BSL-3 laboratories, which have meticulous biosafety protocols

that make the work time-consuming, laborious and space-limited. Due to the aforementioned biosafety constraints, genotypes tested under different environments/institutions were tested with different MoT isolates. Common susceptible and resistant checks were included in all experiments in an attempt to standardize and assess inoculation and disease rating efficacy.

The wheat genotypes used in this study showed a significant level of variation in W_SB resistance with moderate to high heritability. The $2N^vS$ translocation is known as the unique useful source of W_SB resistance (Cruz et al. 2016). However, we expanded these previous findings with data suggesting that other QTL might contribute to increase WSB resistance. Our results, derived from studies with a new independent set of wheat genotypes from previous analysis, confirmed that the $2N^vS$ translocation is the major source of genetic resistance to W_SB. This, in addition to harboring multiple resistance genes in wheat such as *Rkn3* (root-knot nematode), *Cre5* (cereal cyst nematode), *Yr17* (stripe rust), *Lr37* (leaf rust) and *Sr38* (stem rust) (Dyck and Lukow, 1988; Robert et al. 1999; Jahier et al. 2001; Williamson et al. 2013), reinforces its importance within wheat breeding programs. Among our diverse panel of genotypes, the presence of this alien segment decreased W_SB severity by 58% with isolate T-25 (p= <.0001), 35% with isolate 008 (p=0.05), and 11% with isolate B-71 (p=0.36). The lack of statistical significance between $2N^vS$ and non-$2N^vS$ genotypes tested with MoT isolate B-71 reinforced the higher level of aggressiveness of this isolate on $2N^vS$-based cultivars.

The largest gaps in the physical map often occurred in the middle of the chromosomes, i.e., in the centromeric region. Conversely, the SNP markers were more densely distributed at the end of the chromosomes, i.e., in the telomeric regions, which agrees with several other studies in wheat and its relatives (IWGSC, 2014; Avni et al. 2017). Most of the SNP markers were located on A (41.16%) and B (45.27%) genomes, while only 12.13% of the SNP markers were mapped

on the D genome. The low degree of polymorphism on the D genome is consistent with previous studies and is due to: (i) few hybridizations events with *Ae. tauschii* (the DD genome donor) during the evolution of hexaploid wheat; (ii) the highly self-pollinated characteristic (inbreeding coefficients ≥ 90%) of both *Ae. tauschii* and bread wheat leading to limited gene flow between them; and (iii) high levels selection of bread wheat leading to further limited diversity (Akhunov et al. 2010; Jordan et al. 2015; IWGSC 2018; Rosyara et al. 2019). Domestication and successive breeding processes have reduced the genetic diversity in many crops such as wheat, corn, soybean, and rice (Wright et al. 2005; Haudry et al. 2007; Xu et al. 2012; Zhou et al. 2015).

Genome-wide association mapping and QTL effect

The full GWAS identified 25 significant associations, in which 21 were mapped on 2A chromosome with high LD among them, suggesting that they tag the same QTL. These SNPs, which correspond to the $2N^{v}S$ translocation, had the strongest effect on $W_{S}B$ resistance, which reinforces that the $2N^{v}S$ translocation is still the major source of $W_{S}B$ resistance. The lack of blast resistance genes in wheat is in contrast with the ancient rice blast disease, where more than 100 major resistance genes and 350 QTL have been identified (Ballini et al. 2008; Su et al. 2015; Vasudevan et al. 2015; Zheng et al. 2016). In a recent study, Juliana et al. (2019) identified two significant associations for $W_{S}B$ resistance, one on chromosome 2A (which was identified as the $2N^{v}S$ translocation) and one on chromosome 3B (position 757480752 bp). Similar to our experiment, Juliana et al. (2019) evaluated wheat blast using a Bolivian MoT isolate; however, no information about its aggressiveness was reported. In our study the removal of the major effect of the $2N^{v}S$ translocation did not lead to identification of additional significant associations as we had hypothesized.

Resistance genes can sometimes provide resistance to multiple diseases. One well-known example is the *Lr34* gene in wheat, which provides partial resistance to the rust complex (leaf, stripe, and stem) and to powdery mildew in wheat, as well as leaf rust and powdery mildew in barley (Risk et al. 2013). Fusarium head blight is a fungal disease with similar symptoms to wheat blast (bleaching of the spikes) and with similar favorable weather conditions (Wise et al. 2015). If not assessed properly and closely, these two diseases could be easily mistaken (Cruz and Valent 2017, Del Ponte et al. 2017). The wheat 3B chromosome is known to harbor the most effective and stable allele for Fusarium head blight resistance (*Fhb1*) (Waldron et al. 1999; Anderson et al. 2001). Given the similarities between these two diseases, plant pathologists and wheat breeders were optimistic that the same resistance genes could be associated with blast resistance. However, this QTL was not effective in controlling wheat blast (Ha et al. 2016). Previous studies evaluated the effectiveness of several known resistance genes (e.g., *Lr34*, *Lr46*, and *Lr67*) to control wheat blast in the hopes to identify additional pleiotropy; however, these genes were not effective (Bansal, 2014; Bockus, Lagudah and Valent, unpublished results). Additionally, germplasm resistant to the stem rust, UG-99 race, was not resistant to wheat blast. The lack of genetic resistance highlights the threat wheat blast poses to wheat production worldwide.

No significant SNPs identified in this study were common between the MoT isolates, suggesting that the resistance might be isolate specific, which is in agreement with previous studies (Maciel et al. 2013; Cruz et al. 2016; Cruppe et al. 2019). In fact, we only identified significant SNPs using MoT isolate T-25, which could be explained by (i) the resistance identified using the older and less aggressive MoT isolate T-25 was already overcome by the newer and more aggressive MoT isolates, B-71, and/or (ii) the lower number of wheat genotypes

tested with isolate B-71 has limited the discovery of significant associations. Several wheat genotypes previously classified as resistant to isolate T-25 were later grouped as intermediate or susceptible when tested against newer and more aggressive isolates such as B-71 (Cruz et al. 2012; Cruz et al. 2016; Cruppe et al. 2019). Moreover, a MoT isolate sampled in Brazil in 2016 (16MoT01, not used in this study) has shown higher levels of aggressiveness compared to isolate B-71, decreasing the level of resistance of some 2N^{v}S-based genotypes (Cruppe et al. 2019).

Despite being the main source of resistance to W$_{S}$B, the environment and/or the genetic background has a significant influence of the level of resistance of some 2N^{v}S-based cultivars (Cruz et al. 2016; Cruppe et al. 2019). We hypothesized that W$_{S}$B resistance is an oligogenic trait, with the 2N^{v}S translocation as the major effect combined with a few minor genes of smaller effects and that combining multiple QTL would have an additive effect, decreasing W$_{S}$B severity. There was a wide variation in disease severity when comparing genotypes with no QTL to genotypes carrying one or more QTL. However, our results have demonstrated it is about not only the number of QTL in a specific pyramid, but also which specific QTLs are included. For instance, the individual effect of the 2A QTL in the full GWAS with T-25 was greater than the combination of 1B + 1D, but there was a slight increase (not statistically significant) in W$_{S}$B resistance when combining 2A to 1B. These results suggest that stacking one or more QTL of smaller effects to the 2N^{v}S resistance trait might increase W$_{S}$B resistance in certain genetic backgrounds; however, no major differences should be expected. In addition to a significant effect in W$_{S}$B resistance, other factors could be explored. For instance, will the combination of multiple QTL increase the spectrum of MoT isolates that a specific genotype might be resistant? Alternatively, are we protecting the 2N^{v}S resistant trait when combining multiple QTL? Considering there is a continuous increase in the aggressiveness of MoT isolates, it is imperative

to identify and deploy novel genetic sources of resistance and to protect the only known useful source.

Genes underlying significant SNPs and their functional annotations

In the present study, proteins belonging to the multi antimicrobial extrusion family were predicted for SNP marker S1D_11849919 (protein detoxification 40-like). This plasma membrane protein functions as an efflux carrier for plant-derived alkaloids, heavy metal, antibiotics among other toxic compounds as well as in cadmium detoxification (Li et al. 2002). SNP marker S1D_11097633 was observed to lie in the genomic regions harboring subtilisin-chymotrypsin inhibitor CI-1B protein, which has been reported to play a role in serine-type endopeptidase inhibitor activity and response to wounding. *Fusarium culmorum*, one of the causal agents of Fusarium head blight, is known to produce subtilisin-like proteinases, which helps the fungus to colonize grains. Pekkarinen and Jones (2002) and Pekkarinen et al. (2007) demonstrated that barley subtilisin-chymotrypsin CI-1B protein inhibited the production of subtilisin-like proteinases in vitro, suggesting its role in disease resistance. Petti et al. (2010) also showed a significant increase in the production of subtilisin in barley spikes as early as 12 to 48 h post inoculation with the same pathogen.

The SNP S2A_16617246 identified in the full GWAS on 2A chromosome was associated with xylanase inhibitor. This protein has been identified in several crops including wheat, rye, maize, rice, and barley (Elliott et al. 2003; Goesaert et al. 2003, 2004, 2005; Weng et al. 2010). Xylan is one of the most abundant polysaccharides in the cell wall of higher plants and xylanase inhibits endo-β-1, 4-xylanase activity, which degrades this first line of defense against plant pathogens (Sun et al. 2018). Sun et al. (2018) and Weng et al. (2010) reported that xylanase inhibitor gene expression was induced by biotic stress and could be associated with plant defense

via Jasmonic acid mediated signaling pathway in rice and wheat. The Arginase (*ARG*) gene, which has a central role in nitrogen remobilization by conversion of arginine (an N-rich amino acid) to ornithine and urea (She et al. 2017), was predicted in the same 10kb window for the SNP S2A_16617246. Additionally, the *ARG* gene is reported to play an important role in restraining the development of several wheat pathogens including *Puccinia recondita* (leaf rust), *P. graminis* f. sp. *tritici* (stem rust), and *Erysiphe graminis* (powdery mildew) (Weinstein et al. 1987).

Conclusion

Our study identified novel significant SNPs associated with W_SB for MoT isolate T-25. These SNPs provided low to moderate increases in W_SB resistance, indicating they might be minor genes. Further investigation needs to confirm the importance of these genomic regions and the respective genes associated with W_SB. Useful SNP markers linked to W_SB can then be used for marker-assisted introgression of novel resistance into $2N^VS$-based genotypes for a broad spectrum and more durable resistance. Breeding for durable resistance is vital to sustainable agriculture especially with the emergence of new diseases and new virulent pathogen races. It is critical to continue assessing the vulnerability of wheat genotypes and exploring novel sources of genetic resistance, including wheat wild relatives, Ae. *tauschii* and *Ae. ventricosa*. Our results demonstrated the partial effect of the $2N^vS$ translocation, indicating that the resistance mechanism behind the $2N^vS$ trait might be quantitative; however, further experiments are needed. The characterization of the specific genes controlling the disease within this alien segment will elucidate critical aspects of host-pathogen interaction that can be used to breed effectively for W_SB resistance.

References

Akhunov, E. D., Akhunova, A. R., Anderson, O. D., Anderson, J. A., Blake, N., Clegg, M. T., Coleman-Derr, D., Conley, E. J., Crossman, C. C., Deal, K. R. and Dubcovsky, J. 2010. Nucleotide diversity maps reveal variation in diversity among wheat genomes and chromosomes. BMC Genomics 11:702.

Anderson, J. A., Stack, R. W., Liu, S., Waldron, B. L., Fjeld, A. D., Coyne, C., Moreno-Sevilla, B., Fetch, J.M., Song, Q.J., Cregan, P.B. and Frohberg, R. C. 2001. DNA markers for Fusarium head blight resistance QTLs in two wheat populations. Theor. Appl. Genet. 102:1164–1168.

Arru, L., Francia, E., and Pecchioni, N. 2003. Isolate-specific QTLs of resistance to leaf stripe (*Pyrenophora graminea*) in the 'Steptoe' × 'Morex 'spring barley cross. Theor. Appl. Genet. 106:668–675.

Arruda, M. P., Brown, P., Brown-Guedira, G., Krill, A. M., Thurber, C., Merrill, K. R., Foresman, B. J. and Kolb, F. L. 2016. Genome-wide association mapping of Fusarium head blight resistance in wheat using genotyping-by-sequencing. The Plant Gen. 9:1.

Avni, R., Nave, M., Barad, O., Baruch, K., Twardziok, S. O., Gundlach, H., Hale, I., Mascher, M., Spannagl, M., Wiebe, K. and Jordan, K.W. 2017. Wild emmer genome architecture and diversity elucidate wheat evolution and domestication. Science, 357:93–97.

Ballini, E., Morel, J.B., Droc, G., Price, A., Courtois, B., Notteghem, J.L. and Tharreau, D., 2008. A genome-wide meta-analysis of rice blast resistance genes and quantitative trait loci provides new insights into partial and complete resistance. Mol. Plant-Microbe Inter. 21:859–868.

Bansal, A. 2014. Effects of the *Lr34* and *Lr46* Rust-Resistance Genes on Other Diseases of Wheat (doctoral dissertation). University of East Anglia.

Barea, G., and Toledo, J. 1996. Identificacion y zonificación de *pyricularia* (*Pyricularia oryzae*) en el cultivo de trigo en el departamento de Santa Cruz. Informe Técnico, Proyecto de Investigación Trigo. 76–86.

Bariana, H. S., and McIntosh, R. A. 2008. Cytogenetic studies in wheat. XV. Location of rust resistance genes in VPM1 and their genetic linkage with other disease resistance genes in chromosome 2A. Genome 36:476–482.

Borner, A., Schumann, E., Furste, A., Coster, H., Leithold, B., Roder, M. S., and Weber, W. E. 2002. Mapping of quantitative trait loci determining agronomic important characters in hexaploid wheat (*Triticum aestivum* L.). Theor. Appl. Genet. 105:921–936.

Brachi, B., Morris, G. P., and Borevitz, J. O. 2011. Genome-wide association studies in plants: the missing heritability is in the field. Gen. Biol. 12:232.

Cardoso, C. A. de A., Reis, E. M., and Moreira, E. N. 2008. Development of a warning system for wheat blast caused by *Pyricularia grisea*. Summa Phytopathol. 34:216–221.

Cruppe, G., Cruz, C.D., Peterson, G., Pedley, K., Asif, M., Fritz, A., Calderon, L., Lemes da Silva, C., Todd, T., Kuhnem, P. and Singh, P.K., Singh, R.P., Braun, H., Barma, N.C.D., Valent, B. 2019. Novel Sources of Wheat spike blast Resistance in Modern Breeding Lines and Wheat Wild Relatives. Plant Dis. PDIS-05.

Cruz, C. D., and Valent, B. 2017. Wheat blast disease: danger on the move. Trop. Plant Pathol. 42:210–222.

Cruz, C. D., Peterson, G. L., Bockus, W. W., Kankanala, P., Dubcovsky, J., Jordan, K. W., Akhunov, E., Chumley, F., Baldelomar, F. D., and Valent, B. 2016. The 2NS

translocation from *Aegilops ventricosa* confers resistance to the *Triticum* pathotype of *Magnaporthe oryzae*. Crop Sci. 56:990–1000.

Cruz, C. D., Bockus, W. W., Stack, J. P., Tang, X., Valent, B., Pedley, K. F., and Peterson, G. L. 2012. Preliminary assessment of resistance among U.S. wheat cultivars to the *Triticum* pathotype of *Magnaporthe oryzae*. Plant Dis. 96:1501–1505.

Del Ponte, E.M., Valent, B. and Bergstrom, G.C. 2017. A special issue on Fusarium head blight and wheat blast. Trop. Plant Pathol. 42:143–145.

Dyck, P. L., and Lukow, O. M. 1988. The genetic analysis of two interspecific sources of leaf rust resistance and their effect on the quality of common wheat. Can. J. Plant Sci. 68:633–639.

Elliott, G.O., McLauchlan, W.R., Williamson, G., Kroon, P.A. 2003. A wheat xylanase inhibitor protein (XIP-I) accumulates in the grain and has homologues in other cereals. J. Cereal Sci. 37:187–194.

Endelman, J. B. 2011. Ridge regression and other kernels for genomic selection with R Package rrBLUP. Plan Gen. 4:250–255.

Glaubitz, J. C., Casstevens, T. N., Lu, F., Harriman, J., Elshire, R. J., Sun, Q., and Buckler, E. S. 2014. TASSEL-GBS: a high capacity genotyping by sequencing analysis pipeline. PLoS One 9:e90346.

Goesaert, H., Gebruers, K., Courtin, C.M., Delcour, J. A. 2005. Purification and characterization of a XIP-type endoxylanase inhibitor from rice (*Oryza sativa*). J. Enz. Inhib. 20:95–101.

Goesaert, H., Elliott, G., Kroon, P.A., Gebruers, K., Courtin, C.M., Robben, J., Delcour, J.A., Juge, N. 2004. Occurrence of proteinaceous endoxylanase inhibitors in cereals. Biochim. Biophys. Acta 1696:193–202.

Goesaert, H., Gebruers, K., Brijs, K., Courtin, C. M., Delcour, J. A. 2003. XIP-type endoxylanase inhibitors in different cereals. J. Cereal Sci. 38:317–324.

González, A. M., Marcel, T. C., and Niks, R. E. 2012. Evidence for a minor gene–for–minor gene interaction explaining nonhypersensitive polygenic partial disease resistance. Phytopathology. 102: 1086–1093.

Goulart, A. C. P., Sousa, P. G., and Urashima, A. S. 2008. Danos em trigo causados pela infecção de *Pyricularia grisea*. Summa Phytopathol. 33:358–363.

Gurung, S., Mamidi, S., Bonman, J. M., Xiong, M., Brown-Guedira, G., and Adhikari, T. B. 2014. Genome-wide association study reveals novel quantitative trait loci associated with resistance to multiple leaf spot diseases of spring wheat. PLoS One. 9:e108179.

Ha, X., Koopmann, B., and von Tiedemann, A. 2016. Wheat blast and Fusarium head blight display contrasting interaction patterns on ears of wheat genotypes differing in resistance. Phytopathology 106:270–281.

Haudry, A., Cenci, A., Ravel, C., Bataillon, T., Brunel, D., Poncet, C., Hochu, I., Poirier, S., Santoni, S., Glémin, S. and David, J. 2007. Grinding up wheat: a massive loss of nucleotide diversity since domestication. Mol. Biol. Evol. 24:1506–1517.

Heath, M. C. 2000. Hypersensitive response-related death. In Programmed cell death in higher plants. Springer, Dordrecht. 77:90.

Helguera, M., Khan, I. A., Kolmer, J., Lijavetzky, D., Zhong-qi, L., and Dubcovsky, J. 2003. PCR assays for the *Lr37-Yr17-Sr38* cluster of rust resistance genes and their use to develop isogenic hard red spring wheat lines. Crop Sci. 43:1839–1874.

Horsley, R. D., Schmierer, D., Maier, C., Kudrna, D., Urrea, C. A., Steffenson, B. J., Schwarza, P. B., Franckowiaka, J. D., Greena, M. J., Zhangf, B., and Kleinhofs, A. 2006.

Identification of QTLs associated with Fusarium head blight resistance in barley accession CIho 4196. Crop Sci. 46:145–156.

Huang, X. Q., Cloutier, S., Lycar, L., Radovanovic, N., Humphreys, D. G., Noll, J. S., Somers, D. J., Brown, P. D. 2006. Molecular detection of QTLs for agronomic and quality traits in a doubled haploid population derived from two Canadian wheats (*Triticum aestivum* L.). Theor. Appl. Genet. 113:753–766.

Igarashi, S., Utiamada, C. M., Igarashi, L. C., Kazuma, A. H., and Lopes, R. S. 1986. Ocorrência de *Pyricularia* sp. em trigo no estado do Paraná. [Occurrence of *Pyricularia* sp. on wheat in the state of Paraná]. Fitopatologia Brasileira, 11: 351–352.

The International Wheat Genome Sequencing Consortium, Shifting the limits in wheat research and breeding using a fully annotated reference genome. 2018. Science, 361: eaar7191.

Jahier, J. P., Abelard, P., Tanguy, M., Dedryver, F., Rivoal, R., Khatkar, S., Bariana, H. S., Koebner, R. 2001. The *Aegilops ventricosa* segment on chromosome 2AS of the wheat cultivar "VPM1" carries the cereal cyst nematode resistance gene *Cre5*. Plant Breed. 120:125–128.

Jordan, K. W., Wang, S., Lun, Y., Gardiner, L. J., MacLachlan, R., Hucl, P., Sidebottom, C. H. et al. 2015. A haplotype map of allohexaploid wheat reveals distinct patterns of selection on homoeologous genomes. Genome Biol. 16:48.

Juliana, P., Poland, J., Huerta-Espino, J., Shrestha, S., Crossa, J., Crespo-Herrera, L., Toledo, F.H., Govindan, V., Mondal, S., Kumar, U. and Bhavani, S., 2019. Improving grain yield, stress resilience and quality of bread wheat using large-scale genomics. Nat. Genet. 1–10.

Korte, A., and Farlow, A. 2013. The advantages and limitations of trait analysis with GWAS: a review. Plant Meth. 9:29.

Kumar, J., Saripalli, G., Gahlaut, V., Goel, N., Meher, P. K., Mishra, K. K., Mishra, P. C., Sehgal, D., Vikram, P., Sansaloni, C. and Singh, S. 2018. Genetics of Fe, Zn, β-carotene, GPC and yield traits in bread wheat (*Triticum aestivum* L.) using multi-locus and multi-traits GWAS. Euphytica. 214:219.

Kumar, N., Kulwal, P. L., Balyan, H. S., and Gupta, P. K. 2007. QTL mapping for yield and yield contributing traits in two mapping populations of bread wheat. Mol. Breed. 19:63–177.

Large, E. C. 1954. Growth stages in cereals illustration of the Feekes scale. Plant Pathol. 3:128–129.

Li, L., He, Z., Pandey, G. K., Tsuchiya, T., and Luan, S. 2002. Functional cloning and characterization of a plant efflux carrier for multidrug and heavy metal detoxification. Biolog. Chem. 277:5360–5368.

Liu, J., Feng, B., Xu, Z., Fan, X., Jiang, F., Jin, X. Cao, J., Wang, F., Liu, Q., Yang, L. and Wang, T. 2018. A genome-wide association study of wheat yield and quality-related traits in southwest China. Mol. Breed. 38:1.

Liu, S., Hall, M. D., Griffey, C. A., and McKendry, A. L. 2009. Meta-analysis of QTL associated with Fusarium head blight resistance in wheat. Crop Sci. 49:1955–1968.

Lopes, M. S., Dreisigacker, S., Peña, R. J., Sukumaran, S., & Reynolds, M. P. 2015. Genetic characterization of the wheat association mapping initiative (WAMI) panel for dissection of complex traits in spring wheat. Theor. Appl. Genet. 128:453–464.

Lu, Y., Lan, C., Liang, S., Zhou, X., Liu, D., Zhou, G., Lu, Q., Jing, J., Wang, M., Xia, X., and He, Z. 2009. QTL mapping for adult-plant resistance to stripe rust in Italian common wheat cultivars Libellula and Strampelli. Theor. Appl. Genet. 119:1349–1359.

Maciel, J. L. N., Ceresini, P. C., Castroagudin, V. L., Zala, M., Kema, G. H., and McDonald, B. A. 2014. Population structure and pathotype diversity of the wheat blast pathogen *Magnaporthe oryzae* 25 years after its emergence in Brazil. Phytopathology. 104:95–107.

Malaker, P. K., Barma, N. C. D., Tewari, T. P., Collis, W. J., Duveiller, E., Singh, P. K., Joshi, A. K., Singh, R. P., Braun, H. J., Peterson, G. L. and Pedley, K. F., Farman, M. L., and Valent, B. 2016. First report of wheat blast caused by *Magnaporthe oryzae* pathotype *Triticum* in Bangladesh. Plant Dis. 100:2330.

Mottaleb, K. A., Singh, P. K., He, X., Hossain, A., Kruseman, G., and Erenstein, O. 2019. Alternative use of wheat land to implement a potential wheat holiday as wheat blast control: In search of feasible crops in Bangladesh. Land use policy. 82:1–12.

Neumann, K., Kobiljski, B., Denčić, S., Varshney, R. K., & Börner, A. 2011. Genome-wide association mapping: a case study in bread wheat (*Triticum aestivum* L.). Mol. Breed. 27:37–58.

Ogura, T., and Busch, W. 2015. From phenotypes to causal sequences: using genome wide association studies to dissect the sequence basis for variation of plant development. Cur. Op. in Pl. Biol. 23:98–108.

Parlevliet, J. 2002. Durability of resistance against fungal, bacterial and viral pathogens; present situation. Euphytica 124:147–156.

Parlevliet, J. E. 1978. Race-specific aspects of polygenic resistance of barley to leaf rust, *Puccinia hordei*. Netherlands Journal of Plant Pathology. 84:121–126.

Perelló, A., Martinez, I., and Molina, M. 2015. First report of virulence and effects of *Magnaporthe oryzae* isolates causing wheat blast in Argentina. Plant Dis. 99:1177.

Pekkarinen, A. I., Longstaff, C., and Jones, B. L. 2007. Kinetics of the inhibition of Fusarium serine proteinases by barley (*Hordeum vulgare* L.) inhibitors. J. Agric. Food. Chem. 55:2736–2742.

Pekkarinen, A. I. and Jones, B. L. 2002. Trypsin-like proteinase produced by *Fusarium culmorum* grown on grain proteins. J. Agric. Food Chem. 50:3849–3855.

Petti, C., Khan, M., and Doohan, F. 2010. Lipid transfer proteins and protease inhibitors as key factors in the priming of barley responses to Fusarium head blight disease by a biocontrol strain of *Pseudomonas fluorescens*. Func. & Integ. Genom. 10:619–627.

Poland, J. A., Brown, P. J., Sorrells, M. E., and Jannink, J. L. 2012. Development of high-density genetic maps for barley and wheat using a novel two-enzyme genotyping-by-sequencing approach. PloS one 7:e32253.

Poland, J. A., Balint-Kurti, P. J., Wisser, R. J., Pratt, R. C., and Nelson, R. J. 2009. Shades of gray: the world of quantitative disease resistance. Trends Plant Sci. 14:21–29.

Quarrie, S. A., Steed, A., Calestan,i C., Semikhodskii, A., Lebreton, C., Chinoy, C., Steele, N., Pljevljakusic, D., Waterman, E., Weyen, J., Schondelmaier, J., Habash, D. Z., Farmer, P., Saker, L., Clarkson, D. T., Abugalieva, A., Yessimbekova, M., Turuspekov, Y., Abugalieva, S., Tuberosa, R., Sanguineti, M. C., Hollington, P. A., Aragues, R., Royo, A., Dodig, D. 2005. A high-density genetic map of hexaploid wheat (*Triticum aestivum* L.) from the cross Chinese Spring 9 SQ1 and its use to compare QTLs for grain yield across a range of environments. Theor. Appl. Genet. 110:865–880.

Risk, J. M., Selter, L. L., Chauhan, H., Krattinger, S. G., Kumlehn, J., Hensel, G., Viccars, L.A., Richardson, T.M., Buesing, G., Troller, A. and Lagudah, E.S. 2013. The wheat *Lr34* gene

provides resistance against multiple fungal pathogens in barley. Pl. Biotech. J. 11:847–854.

Robert, O., Abelard, C., and Dedryver, F. 1999. Identification of molecular markers for the detection of the yellow rust resistance gene *Yr17* in wheat. Mol. Breed. 5:167-175.

Rosyara, U., Kishii, M., Payne, T., Sansaloni, C. P., Singh, R. P., Braun, H. J., and Dreisigacker, S. 2019. Genetic Contribution of Synthetic Hexaploid Wheat to CIMMYT's Spring Bread Wheat Breeding Germplasm. Sci. Rep. 9:1–11.

She, M., Wang, J., Wang, X., Yin, G., Wang, K., Du, L., and Ye, X. 2017. Comprehensive molecular analysis of arginase-encoding genes in common wheat and its progenitor species. Sci. Rep. 7:6641.

Su, J., Wang, W. J., Han, J. L., Chen, S., Wang, C. Y., Zeng, L. X., Feng, A., Yang, J., Zhou, B. and Zhu, X. 2015. Functional divergence of duplicated genes results in a novel blast resistance gene Pi50 at the Pi2/9 locus. Theor. Appl. Genet. 128:2213–2225.

Sukumaran, S., Dreisigacker, S., Lopes, M., Chavez, P., & Reynolds, M. P. 2015. Genome-wide association study for grain yield and related traits in an elite spring wheat population grown in temperate irrigated environments. Theor. Appl. Genet. 128:353–363.

Sun, R. J., Xu, Y., Hou, C. X., Zhan, Y. H., Liu, M. Q., & Weng, X. Y. 2018. Expression and characteristics of rice xylanase inhibitor OsXIP, a member of a new class of antifungal proteins. Biol. Plant. 62: 569–578.

Tessmann, E. W., Dong, Y., & Van Sanford, D. A. 2019. GWAS for Fusarium head blight traits in a soft red winter wheat mapping panel. Crop Sci. 59:1823–1837.

Valent, B., Farrall, L., and Chumley, F. 1991. *Magnaporthe grisea* genes for pathogenicity and virulence identified through a series of backcrosses. Genetics 127:87–101.

Vasudevan, K., Gruissem, W., and Bhullar, N. K. 2015. Identification of novel alleles of the rice blast resistance gene *Pi54*. Sci. Rep. 5:15678.

Viedma, L. Q. 2005. Wheat blast occurrence in Paraguay. Phytopathology 95:S152.

Waldron, B. L., Moreno-Sevilla, B., Anderson, J. A., Stack, R. W., and Frohberg, R. C. 1999. RFLP mapping of QTL for Fusarium head blight resistance in wheat. Crop Sci. 39:805–811.

Weinstein, L. H., Osmeloski, J. F., Wettlaufer, S. H. and Galston, A. W. 1987. Protection of wheat against leaf and stem rust and powdery mildew diseases by inhibition of polyamine metabolism. Plant Sci. 51:311–316.

Weng, X. Y., Huang, Y. Y., Gao, H., and Sun, J. Y. 2010. Characterization of a xylanase inhibitor TAXI-I from wheat. Biol. Plant. 54:154–158.

Williamson, V. M., Thomas, V., Ferris, H., and Dubcovsky, J. 2013. An *Aegilops ventricosa* translocation confers resistance against root-knot nematodes to common wheat. Crop Sci. 53:1412–1418.

Wise, K., Woloshuk, C., Freije, A. 2015. Fusarium Head Blight (Head Scab). Diseases of Wheat. West Layfayette, Indiana, Purdue Extension.

Wisser, R. J., Balint-Kurti, P. J., & Nelson, R. J. 2006. The genetic architecture of disease resistance in maize: a synthesis of published studies. Phytopathology. 96:120–129.

Wright, S. I., Bi, I. V., Schroeder, S. G., Yamasaki, M., Doebley, J. F., McMullen, M. D., and Gaut, B. S. 2005. The effects of artificial selection of the maize genome. Science 308: 1310–1314.

Yu, J. M., Pressoir, G., Briggs, W. H., Bi, I. V., Yamasaki, M., Doebley, J. F., McMullen, M.D., Gaut, B.S., Nielsen, D.M., Holland, J.B. and Kresovich, S. 2006. A unified mixed-model

method for association mapping that accounts for multiple levels of relatedness. Nat. Genet. 38: 203–208.

Xu, X., Liu, X., Ge, S., Jensen, J. D., Hu, F. Y., Li, X., Dong, Y., Gutenkunst, R. N., Fang, L., Huang, L. and Li, J. 2012. Resequencing 50 accessions of cultivated and wild rice yields markers for identifying agronomically important genes. Nat. Biotech. 30:105–111.

Zhang, H., Mittal, N., Leamy, L. J., Barazani, O., and Song, B. H. 2017. Back into the wild—Apply untapped genetic diversity of wild relatives for crop improvement. Evol. Applic. 10:5–24.

Zheng, W., Wang, Y., Wang, L., Ma, Z., Zhao, J., Wang, P., Zhang, L., Liu, Z. and Lu, X. 2016. Genetic mapping and molecular marker development for Pi65(t), a novel broad-spectrum resistance gene to rice blast using next-generation sequencing. Theor. Appl. Genet. 129:1035–1044.

Zhou, Z. K., Jiang, Y., Wang, Z., Gou, Z. H., Lyu, J., Li, W. Y., Yu, Y., Shu, L., Zhao, Y., Ma, Y., and Fang, C. 2015. Resequencing 302 wild and cultivated accessions identifies genes related to domestication and improvement in soybean. Nat. Biotech. 33:408–414.

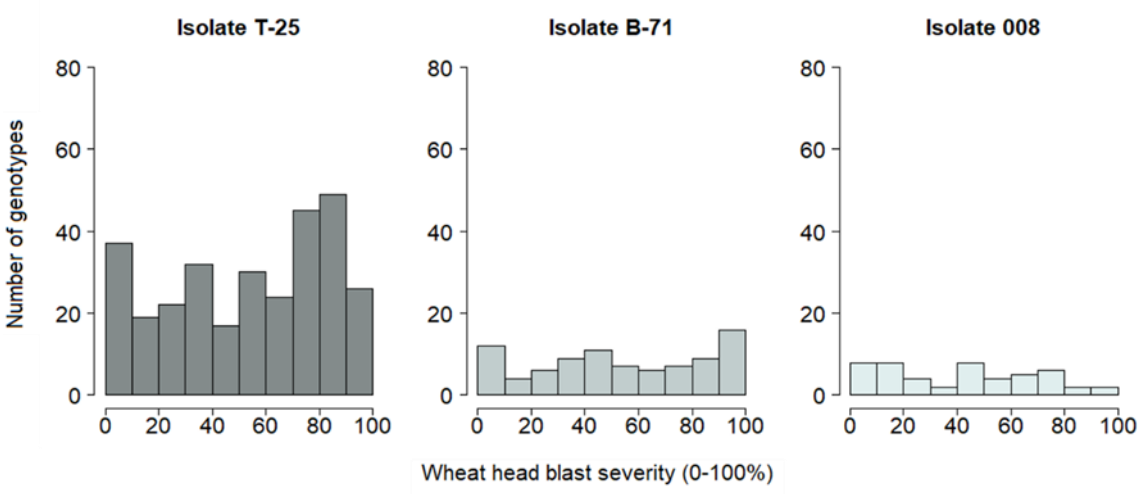


Figure 2-1. Phenotypic distribution of wheat spike blast severity among genotypes used in the genome wide association study for MoT isolates T-25, B-71, and 008.

Figure 2-2. Physical map positions of 13,175 SNP markers. Physical map color-coded by the number of SNPs within 10 Mb windows.

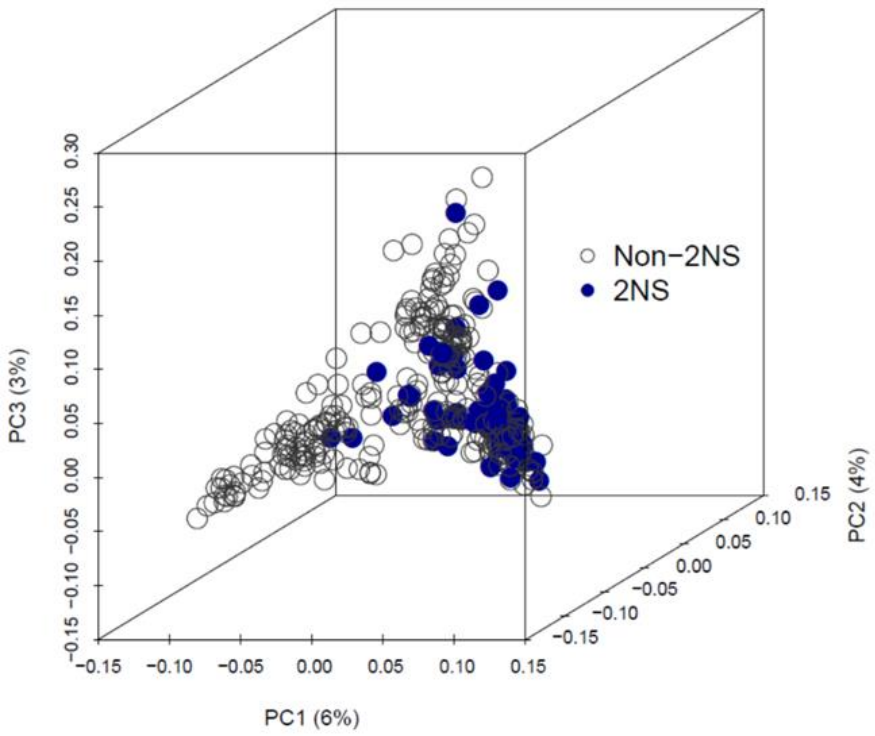


Figure 2-3. Principal component analysis of 13,175 SNP markers.

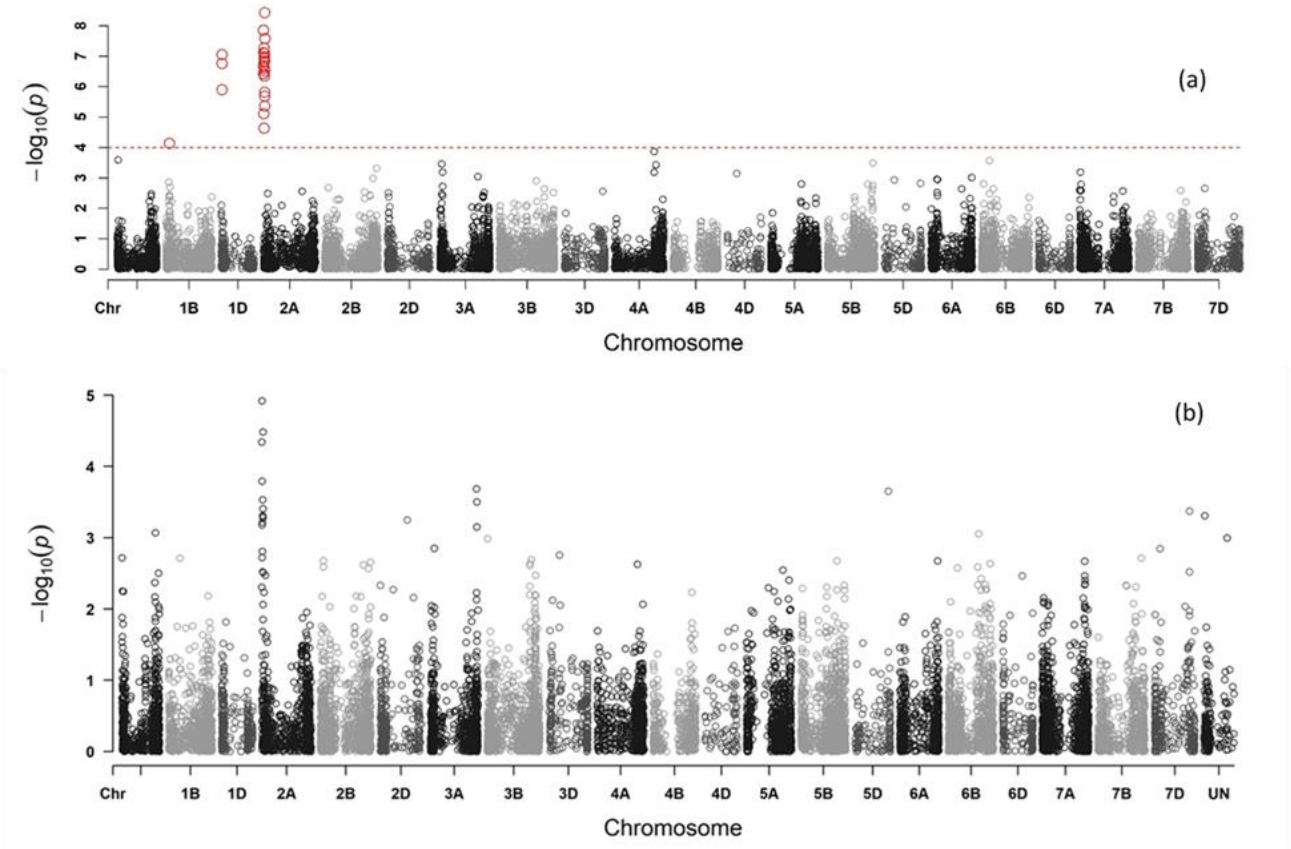


Figure 2-4. Manhattan plots of Wheat spike blast severity of all wheat genotypes combined (full GWAS) using (a) MoT isolate T-25 and (b) MoT isolate B-71 for the seven chromosomes carrying the significant SNP markers detected by MLM models. The red circles above the red dashed line indicate significant SNPs above the threshold -log (P) score of 4.00 (FDR 5%).

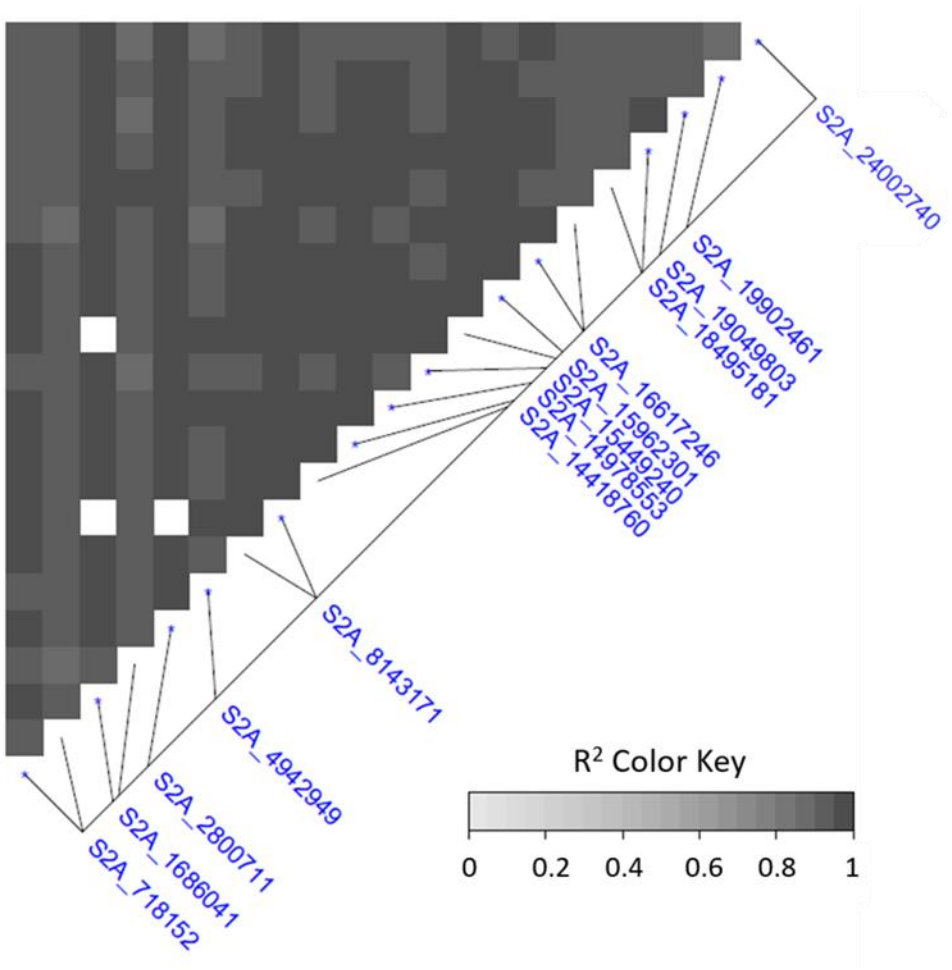


Figure 2-5. Linkage disequilibrium (LD) analysis: heatmap of LD between the significant SNPs detected on chromosome 2A with MoT isolate T-25 by the mixed linear model (at significance level of 5% FDR correction). LD analysis suggests that these 21 SNPs may tag the same QTL.

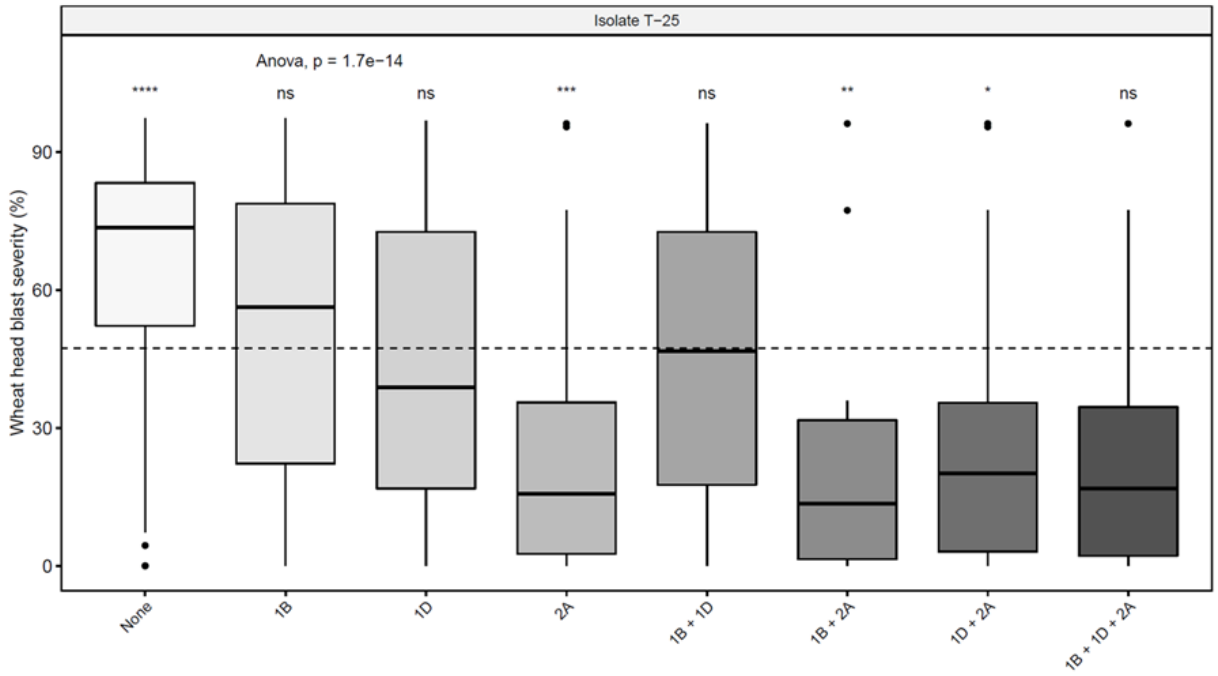


Figure 2-6. Comparison of individual and the combination of multiple QTL effects on W_SB severity with MoT isolate T-25 with the full GWAS provides evidence for quantitative resistance. ns non-significant, * significant at $p<0.05$, ** $p<0.01$, *** $p<0.001$, and **** $p<0.0001$.

Table 2-1. Descriptive statistics and broad sense heritability (H^2) for Wheat spike blast severity.

MoT isolate	$2N^vS$	*n*	Mean severity (%)	SD (%)	Max	Min	H^2
T-25	Absent	266	57.0A	28.4	97.3	0.0	0.75
	Present	35	24.0Ba	27.2	96.3	0.0	
B-71	Absent	45	56.7A	32.6	100.0	0.0	0.81
	Present	42	50.5Ab	31.0	100.0	4.3	
008	Absent	34	47.7A	28.6	98.3	2.0	0.64
	Present	15	31.0Ba	25.8	80.0	1.3	

Note: different capital letters indicate statistical difference at $p<0.05$ using Tukey test due to the presence of the $2N^vS$ translocation within each MoT isolate. Different lower case letters indicate statistical difference at $p<0.05$ among the different MoT isolates within the $2N^vS$ presence.

Table 2-2. Summary of the physical map (bp) of 13,175 SNP markers in the full GWAS panel.

Wheat chr.	N of markers	Length	Average spacing	Maximum spacing	Missing loci
1A	748	592,632,035	793,349	35,928,093	19,422
2A	771	778,581,845	1,011,145	17,902,013	19,023
3A	733	749,892,901	1,024,443	44,407,035	17,190
4A	673	739,903,062	1,101,046	17,256,133	18,415
5A	760	708,537,467	933,514	126,466,403	16,254
6A	620	617,154,432	997,018	36,936,217	16,179
7A	1118	736,145,545	659,038	69,683,191	29,080
1B	788	686,665,173	872,509	26,168,753	23,586
2B	1021	800,243,995	784,552	34,168,501	27,967
3B	1076	829,336,267	771,475	11,358,370	28,492
4B	399	672,512,450	1,689,730	161,542,889	9,525
5B	921	712,632,150	774,600	15,310,640	22,987
6B	937	720,266,590	769,515	17,082,131	26,799
7B	823	750,007,376	912,417	71,947,032	22,833
1D	270	493,900,216	1,836,060	45,026,644	7,118
2D	350	647,552,852	1,855,452	50,736,831	10,253
3D	262	612,581,309	2,347,054	30,973,781	8,717
4D	82	508,134,441	6,273,265	41,402,372	2,644
5D	147	559,814,353	3,834,345	27,220,812	4,466
6D	186	472,599,474	2,554,592	38,697,242	5,100
7D	302	633,203,408	2,103,665	34,387,166	9,070
UN	188	474,668,349	2,538,333	92,299,631	6,203
Overall	13,175	14,496,965,690	1,656,233	161,542,889	351,323

Table 2-3. Information on significant SNPs identified in the full GWAS with MoT isolate T-25.

SNP	Chr.	Position	*p* value	Haplotype	W_SB severity (%)
S1B_50496801	1B	50496801	7.4E-05	C	50.6
				G	55.7
S1D_11097633	1D	11097633	1.7E-07	ACA	56.9
S1D_11646338	1D	11646338	8.9E-08	ACG	68.3
S1D_11849919	1D	11849919	1.3E-06	ATA	77.6
				CCG	54.8
				CTG	43.4
S2A_718152	2A	718152	3.8E-07	CTGTACAGCATCATAACGGCA	24.71
S2A_735436	2A	735436	1.4E-08	TACGGTGCTGCGGCCGGCATG	56.62
S2A_1686041	2A	1686041	2.1E-07		
S2A_1872142	2A	1872142	3.8E-07		
S2A_2800711	2A	2800711	7.7E-08		
S2A_4942949	2A	4942949	7.9E-06		
S2A_8142744	2A	8142744	2.3E-05		
S2A_8143171	2A	8143171	5.5E-08		
S2A_14215359	2A	14215359	1.5E-07		
S2A_14418760	2A	14418760	8.1E-08		
S2A_14978553	2A	14978553	4.6E-07		
S2A_15449240	2A	15449240	1.2E-07		
S2A_15755581	2A	15755581	2.5E-07		
S2A_15962301	2A	15962301	1.0E-07		
S2A_16617246	2A	16617246	3.7E-09		
S2A_16629866	2A	16629866	2.0E-06		
S2A_18468495	2A	18468495	4.4E-06		
S2A_18495181	2A	18495181	1.5E-07		
S2A_19049803	2A	19049803	3.0E-07		
S2A_19902461	2A	19902461	1.5E-06		
S2A_24002740	2A	24002740	2.6E-08		

Chapter 3 - The role of wheat blast lesions as a source of inoculum for spike infection: epidemiological and genomic aspects

Abstract

Despite intensive research efforts and significant advancements, critical aspects of the epidemiology of wheat blast (WB), caused by the *Triticum* pathotype of *Magnaporthe oryzae* (MoT), remains unknown. Our experiments had the dual-purpose to characterize within-field MoT population diversity while better understanding disease dynamics/progress, focusing on the importance of leaf blast as a source of inoculum for spike infection. We measured disease development in irrigated plots where 4-week old wheat seedlings were inoculated with the Bolivian MoT strain 008 (isolated in 2015), as well as in neighboring non-inoculated plots. Our results showed that a low level of seedling blast at the wheat tillering stage can result in severe spike blast infection in a susceptible wheat cultivar under conducive environmental conditions. This results from polycyclic infection of leaves in the upper canopy as well as on spikes. There were strong correlations between flag leaf and spike blast intensity under high disease intensity suggesting that an antecedent leaf blast measure may be a reliable predictor of spike severity under these conditions. An additional outcome of this work was a robust field inoculation protocol that is currently in use for dependable resistance phenotyping plots in the field when natural conditions are not conducive to disease. Genomic analysis of the initial inoculum strain and isolates subsequently recovered from infected spikes in the field supports that *M. oryzae* supernumerary mini-chromosomes provide a highly variable genomic environment to enhance genome variability and the evolutionary potential for this devastating pathogen. Our findings, collectively, will be critical to develop better strategies to control wheat blast.

Introduction

Wheat blast (WB), caused by the *Triticum* (MoT) pathotype of *Magnaporthe oryzae* (Zhang et al. 2016; Valent et al. 2019), is a devastating disease in South America and it was restricted to the subcontinent for over three decades (1985-2016) (Igarashi et al. 1986). Since 2016, the pathogen has spread to wheat producing regions within Bangladesh, South Asia, decreasing the wheat area planted in the subsequent seasons (Islam et al. 2016; Malaker et al. 2016). Despite intensive research efforts and significant advancements, important ecological and epidemiological aspects of the WB pathosystem have yet to be determined, including but not limited to potential sources of inoculum and the dynamics of disease development and spread (Cruz and Valent, 2017). Specifically, the MoT population diversity within a growing season and the importance of leaf infection at early stages of crop growth are components of the WB ecology and epidemiology that must be elucidated. The disease can affect all above ground plant parts at different times in the crop cycle (Gongora-Canul et al. 2019); however, infection at the spike stage is the most common symptom in the field and the greatest yield losses generally occur when infection takes place at early stages of grain development (Igarashi, 1990; Cruz and Valent, 2017; Cruppe et al. 2019). However, infection at early stages of the wheat cycle (i.e. seedling stage) has gained attention of scientists and wheat producers as reports of wheat leaf blast (WLB) symptoms have become more common during epidemic years (). Elucidating these ecological and epidemiological aspects is critical for developing and deploying an effective and sustainable strategy to manage WB.

Given that *M. oryzae* is a seed-borne (Goulart et al. 1995), hemibiotrophic (i.e., initial biotrophic phase followed by a necrotrophic phase) pathogen with a vast host range, a few possible sources of inoculum could be responsible for disease outbreaks. MoT-infected seeds can

be asymptomatic (Kiyuna, M.S. thesis, unpublished), which usually occurs when infection takes place at later stages of grain development or under less conducive weather (Igarashi, 1990). Seedlings from MoT-infected seeds can become diseased after planting (Goulart and Paiva, 1990). Fungicide seed treatment can reduce MoT sporulation and the primary inoculum; however, its effectiveness is limited and it will vary according to active ingredient (Toledo 2015). Similarly, MoT can survive in crop residue and cause disease at early crop stages (Salgado et al. unpublished). However, a recent study conducted in Brazil demonstrated that MoT did not survive more than 5 months in crop residue and thus, probably would not be a strong determinant for WB development and severity in the subsequent season, which is rarely sown within this period (Pizolotto et al. 2019).

Alternatively, other *M. oryzae* host grasses, which are widely cultivated as crops or broadly spread as native weeds and pastures, including "universal hosts" such as tall fescue, Italian ryegrass, and barley (Kato et al. 2000; Tosa et al. 2016), could play an important role in providing primary inoculum (Cruz and Valent, 2017, Gladieux et al. 2017). These alternative hosts grow nearby to wheat fields and may serve as a green-bridge reservoir for pathogen inoculum during wheat fallow periods. Under these circumstances, the MoT population level could be high enough when the wheat crop is sown to cause an epidemic and potential loss of the crop in entire regions (Embrapa, 2019). In addition, the alternative hosts can serve as reservoirs for the pathogen's long-term survival. Moreover, these hosts can facilitate sexual recombination between different but fertile and compatible strains, enabling gene flow between different pathotypes (Maciel et al. 2014). Bangladesh and its neighboring wheat producing regions have implemented "wheat holidays", in which wheat producers should not plant wheat for a certain period of time (i.e. 2 to 3 years) hoping to eradicate the MoT population in those areas (Mottaleb

et al. 2019). However, this strategy might fail in the presence of alternative secondary hosts. WLB intensity and the subsequent development and spread of the disease within season depends upon the level of wheat genetic resistance and the occurrence of conducive weather conditions. These conditions include temperatures ranging from 25 to 30°C, leaf and spike wetness period between 25 and 40h combined with high relative humidity (Cardoso et al. 2008).

Whether MoT has a monocyclic (i.e. one infection cycle per crop cycle) or polycyclic (i.e. more than one infection cycle per crop cycle) life cycle has long been the subject of debate. Reports of WLB symptoms prior to spike symptoms are becoming more common under conducive weather conditions (Diego Baldelomar - ANAPO, personal communication). A recent study under field conditions with artificial MoT inoculation suggested that the pathogen may have spread from plant to plant and may have produced secondary inoculum during the season (Gongora-Canul et al. 2019). However, this study lacked pathogen molecular data comparing the genetic similarity of the MoT population present in different wheat growth stages (e.g., leaf and spike stages), which could provide strong support and validate the polycyclic MoT life style. The report of a near-complete reference genome of a highly aggressive Bolivian MoT B-71 strain has opened the opportunity to further explore its genetics and help to elucidate some of these important epidemiological aspects (Peng et al. 2019).

The genome comparison of old and new MoT strains has unraveled important characteristics of the evolution of this pathogen (Peng et al. 2019). In this study, the authors showed that similar to some MoO strains, the highly aggressive Bolivian MoT strain B-71 and the Paraguayan MoT strain P3 both collected in 2012 have, in addition to the seven core chromosomes, mini-chromosomes (although significant differences were found in the mini-chromosomes of these two MoT strains). Mini-chromosomes, also known as supernumerary or B

chromosomes do not show Mendelian inheritance like the core chromosomes and can be present in different number of copies and sizes. Peng et al. (2019) also demonstrated that in contrast to the new MoT strains, mini-chromosomes were not found in an old MoT strain collected in Brazil in 1988, which is agrees with Orbach et al. (1996) that demonstrated that seven early *M. oryzae* strains did not contain mini-chromosomes. Further, Peng et al. (2019) showed that MoT mini-chromosomes harbor multiple genes associated with host-specificity and virulence, including effector genes (e.g., *PWL2* and *BAS1*) that are present on different copies on the core chromosomes end of MoO strains. No copies of these effector genes were found in the core chromosomes of the MoT strains. In addition to effector genes, mini-chromosomes contain end sequences of core chromosomes that are either duplicated or missing in the core chromosomes. This suggests that mini-chromosomes are acquiring sequences from the end of the core chromosomes, although; the mechanism of this sequence exchange is still unknown. These significant findings will enable the elucidation of basic, yet unknown, epidemiological and ecological aspects of the WB pathosystem with the ultimate goal of delivering an effective and sustainable disease control. The objectives of this study were to characterize the MoT population diversity and dynamics of disease severity within the field during a season and to determine the importance of leaf infection and outside inoculum in the epidemiology and severity of this pathogen. We hypothesize that infection at early wheat stages are the main source of inoculum for outbreaks later in the season, and inoculum from weeds and other grasses plays a secondary role.

Materials and Methods

Field experiments

Irrigated field experiments were conducted during the 2017 wheat-growing season in two Bolivian locations that are considered “hot-spots” for WB development due to warm and rainy conditions during the wheat heading stage. Briefly, the first experiment (Exp. 1) was conducted at ANAPO’s (Association of Producers of Oilseeds and Wheat) Research Station near 26 de Agosto (17° 26' 29.44" S, 62° 36' 27.99" W) and the second experiment (Exp. 2) was conducted at the Integral Cooperative Agricultural Colonies (CAICO) Research Station near Okinawa (17° 14' 33.83" S, 62° 53' 21.41" W). Exp. 1 was sown on April 24 and Exp. 2 on April 25, using certified seeds of a highly WB susceptible cultivar (Atlax). Plot dimensions were 3 x 2 m for Exp.1 and 4 x 3 m and for Exp.2, with planting density of 80 seeds per linear meter and 20 cm of row spacing. Seeds were treated with fungicide carbendazin + thiram at a dose of 150 ml per 100 kg of seed (Toledo, 2015). To control weeds, 0.35 L ha^{-1} fluroxypyr 1-methylheptyl ester1: {[(4-amino-3,5- dichloro-6-fluoro-2-pyridinyl)oxy]acetic acid, 1-methylheptyl ester} and 0.35 L ha^{-1} clodinafop propargil2 {[4-(5-cloro-3-fluropiridin-2-iloxi)fenoxi]propionate de prop-2-inilo} were applied in all plots three times during the season. To control insects, 0.15 kg ha^{-1} benzoate, 0.3 kg ha^{-1} metomil, and 0.3 kg ha^{-1} imidacloprid were applied one time each during the season. Nitrogen was applied as urea split into two applications of 40 kg N ha^{-1} approximately at Feekes GS 2 (tillering initiation) and at Feekes GS 4 (leaf sheath lengthening). No foliar fungicide treatments were applied in these experiments.

Experimental design and treatments

Two treatments (i.e., with and without MoT artificial inoculation) were established in a randomized complete block design with eight replications. Plots were separated by 1 m border

rows planted with corn to minimize any potential interplot interference. MoT isolate 008, which is a highly aggressive isolate originally collected in Quirusillas, Bolivia, in 2015 (Cruppe et al. 2019), was used for inoculations. Based on the reactions of a set of winter wheat differentials including a $2N^vS$-based cultivar Jagalene, and two non-$2N^vS$-based cultivars RonL and Everest, MoT isolate 008 was classified as race 2 (Cruppe et al. 2019; Gongora-Canul et al. 2019). Inoculum production and inoculation procedure followed protocol previously described by Cruppe et al. (2019). Briefly, MoT cultures were grown on homemade oatmeal agar (Valent et al., 1991) and 5 to 7-days old sporulating cultures were flooded with a mixture of sterile deionized water with 0.42% unflavored gelatin and 0.01% Tween 20. Sporulating cultures were gently stirred to dislodge conidia from conidiophores, and resulting conidial suspensions were filtered through four layers of sterile cheesecloth. The spore concentration was adjusted to 24,600 spores ml^{-1} for Exp. 1 and 15,300 spores ml^{-1} for Exp.2. Corresponding plots were inoculated at the tillering stage using a CO_2 pressurized backpack sprayer and immediately covered with plastic tarpaulins for approximately 48 hours.

Wheat leaf and spike blast rating and plant sampling

To characterize the disease development and spread dynamics in the field, WB was evaluated multiple times during vegetative and reproductive stages in inoculated and non-inoculated plots. During the vegetative stages, wheat leaf severity and incidence were evaluated before and after artificial inoculation with MoT for a total of 25 to 50 plants per plot. Disease was assessed prior to MoT inoculation to determine the presence of natural infection of MoT and its potential implications to our study. Disease intensity (i.e. incidence and severity) was measured during the entire season with a total of six disease ratings for Exp. 1 and five ratings for Exp. 2. After spike emergence, 50 to 75 spikes were evaluated in each plot from 35 to 63

days after inoculation [i.e. between Feekes 10.1 (initial heading) and Feekes 11.2 (soft dough stage of grain development)]. Disease incidence consisted of the number of leaves or spikes showing WB symptoms divided by the total number of leaves or spikes evaluated at a given time. Disease severity was determined by the percentage area of leaf or spike affected related to the total area (leaf evaluations) or total number of spikelets (spike evaluations). Nearby wheat fields and other weeds and grasses that could potentially be secondary alternative hosts for M. oryzae were also investigated for WB symptoms.

To characterize the MoT population diversity, three different samplings were conducted during the season. The first sampling was conducted one week after MoT inoculation, the second sampling was conducted at Feekes 10 (booting stage), and the third sampling was conducted between Feekes 11.1 and 11.2 (milk and soft dough stages of grain development). The sampling was conducted in both inoculated and non-inoculated plots. For the first sampling, the first (F-6), second (F-5), and third leaves (F-4) (i.e., lower canopy) of 15 randomly selected plants were collected and placed in individual paper bags (i.e., one bag for each of the 15 plants). For the second sampling, leaves from the upper and mid canopy [i.e. flag leaf and F-1 (upper canopy), F-2 and F-3 (mid-canopy), where F-1 means the first leaf below the flag leaf] from 15 randomly selected plants were collected and placed in individual bags, although the exact number of leaves varied from plant to plant. It was critical that the second sampling occurred prior to wheat heading. For the third sampling, spikes, flag leaf, and leaves from the upper canopy (i.e., same as previously described) were collected from 15 plants and placed separately in paper bags. When possible, leaves in the mid and lower canopy were also collected and separated from the other plant parts. After each sampling, the samples were dried at room temperature for a minimum of five days. Seeds and the isolate used in these experiments, along with the samples collected,

were shipped to the biosafety level 3 (BSL-3) laboratory in Manhattan, Kansas, U.S. following the requirements stipulated by the United States Department of Agriculture (USDA) Animal and Plant Health Inspection Service (APHIS).

Statistical analyses of phenotypic data

Data were analyzed using SAS (version 9.2; SAS Institute, Cary, NC, U.S.A.). One-way ANOVA was performed individually for each location due to the different disease levels in the different experiments. Significance of treatment (inoculated vs. non-inoculated) and plant part (spike, flag leaf, and F-1) and their interaction were analyzed with PROC MIXED COVTEST procedure. Factor significance was evaluated using the SLICE option. Dynamics of disease incidence and severity were analyzed using the REPEATED statement for the analysis of repeated measurements considering a first order auto regressive covariance structure, as nearby measurements were expected to be more correlated than measurements taken further apart (i.e., we used the time as distance between measurements). Replication within treatment and replication within treatment and plant part were treated as random effects. We tested linear and non-linear regression models to predict spike wheat blast (i.e., dependent variable) using wheat blast incidence or severity measured on the flag leaf or F-1 a few days prior (i.e., independent variables). Our goal was to answer the question: *"can the antecedent leaf blast measure predict spike blast incidence?"* The intercept of all models tested were forced to zero to reflect their biological meaning (i.e., there are no negative levels of leaf or spike blast). Some analyses of these data are reported in Gongora-Canul et al. (2019).

Pure culturing and single spore isolation from field samples

Under BLS-3 conditions, spikes showing WB symptoms were selected and threshed (including the upper portion of the stem, here referred as neck) into small pieces and were

incubated on water agar media containing streptomycin sulfate and tetracycline antibiotic to avoid bacterial growth. Plates were incubated under the light for 24 hours for two to four days at 26°C ± 1°C to allow fungal growth. Starting at two days after incubation, plates were observed under dissecting microscope to confirm MoT presence based on morphological observation of MoT conidia and conidiophores. A small percentage of the incubated plates showed MoT growth. The limited growth could have occurred due to the presence of several other fungi such as *Alternaria triticina*, *Bipolaris sorokiniana*, and *Pyrenophora tritici-repentis*. Some bacterial growth was also observed regardless of the antibiotics added to the medium. In addition, MoT sporulation might not have occurred in all samples because they had already sporulated while in the field. After proper identification, MoT conidia were transferred to water agar medium using a sterile loop to eliminate the presence of other pathogens. Immediately, a second transfer of a single spore was performed to an oatmeal agar (OMA) plate and incubated for five to seven days under the same conditions to allow MoT growth. Contaminated plates were discarded and pure MoT cultures were maintained to perform DNA extraction.

DNA extraction from isolated MoT strains and wheat seed

To prepare MoT cultures for DNA extraction, colonized agar pieces cut from an approximately 2 x 3 cm square of five-to-seven day old mycelium were placed inside a 250 ml Erlenmeyer containing 150 ml of 3-3-3 media (3 g of glucose, 3 g of yeast extract, and 3 g of casamino acids) and incubated in the dark for 72 hours at 25°C and rpm of 150. After 72 hours, the mycelial suspensions were filtered into a new Erlenmeyer flask through two layers of miracloth filter to eliminate the 3-3-3 medium. Mycelia collected on the miracloth filters were dried using autoclaved paper towels to eliminate any remaining liquid and to preserve the dried mycelia. The dried mycelia were then placed in autoclaved foil envelopes and stored in -80°C

until DNA extraction. Genomic DNA was extracted from approximately 200 mg of MoT mycelia using the ZR Fungal/Bacterial DNA MiniPrep (Zymo Research, Irvine, CA). DNA was labeled and maintained at -80°C until proof of inactivation of MoT was completed and the samples could be removed from BSL-3 containment (see below).

With the objective to verify that the certified seeds used in these experiments were MoT free, DNA was extracted from wheat seeds and MoT presence/absence was determined by Polymerase Chain Reaction (PCR) using the MoT3 diagnostic assay (Pieck et al. 2017). Approximately 30 g of seeds were sampled and ground out of a total of 1 kg of seeds available. Five grams of ground seeds were added to 10 ml of sterile deionized water, vortexed for approximately 30 seconds and filtered using four layers of cheesecloth (Kiyuna, M.S. thesis 2019). DNA was extracted using 200 µl of this solution using the ZR Fungal/Bacterial DNA MiniPrep (Zymo Research, Irvine, CA). DNA was extracted from three independent 30 g samples and kept at -80°C until proof of MoT inactivation was completed.

Validation of MoT inactivation in DNA samples

To transfer MoT and seed DNA out of our BLS-3 laboratory, 2 µl of each DNA sample was streaked onto OMA media and potato dextrose agar media (PDA) to check for complete pathogen inviability. In addition to DNA samples, two MoT isolates and a negative control (i.e. ddH2O) were added and replicated twice for each medium. All the plates were incubated under the lights for seven days at 26°C ± 1°C. After seven days and confirmation of inactivation of MoT in the DNA, samples were transported to our BSL-1 laboratory in Throckmorton Hall. Preliminary quality and quantity of DNA were assessed using a NanoDrop spectrophotometer (NanoDrop Technologies).

Presence/absence of MoT on wheat seeds

End-point PCR was performed to determine the presence or the absence of MoT in the seeds used in the field experiments. Specifically, PCR reactions consisted of 5 µl of wheat seed DNA sample, 1 µM of each of the specific primers, MoT3-F and MoT3-R (Pieck et al. 2017), 12.5 µl of GoTaq® G2 Green master mix (Promega, Madison, US), adjusted to 25 µl total reaction volume. PCR amplification was performed in a Gradient Thermocycler (BIO RAD, U.S.) using the cycling conditions: initial denaturation at 94°C for 90 s; followed by 30 cycles of 94°C for 30 s, 62°C for 30 s, and 72°C for 60 s; and a final extension at 72°C for 120 s (Pieck et al. 2017). Samples were separated by electrophoresis in 2% agarose gel at 90 V for 60 min and stained with ethidium bromide ultraviolet illumination. MoT positive and negative controls were added. Three biological replicates were performed for each wheat seed DNA.

MoT whole genome sequencing and analysis

The quality of DNA samples was measured with the Agilent 2200 TapeStation using the Genomic DNA Analysis Tapes. The sample quantification was performed with the Qubit 2.0 Fluorometer (Thermo Fisher Scientific). Nucleic acid purity (A260/280 and A260/230) and quantity was assessed using the NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific). We sequenced a total of 48 MoT DNA samples, in which 44 samples were MoT isolated from wheat spikes (22 from each experiment and both sampled from inoculated and non-inoculated plots) and four samples were from the original MoT isolate used for field inoculations (2 samples from each experiment). The highly aggressive Bolivian MoT B-71 was used as the reference genome (Peng et al. 2019). Genomic DNA libraries were generated using the Illumina's Nextera DNA Flex kit and indexed using the Nextera DNA CD Indexes (8 bp long barcodes) according to the manufacturer’s protocol. Libraries were sequenced using NextSeq

500 Mid Output Kit v2.5 (2 x 150 bp run) at the K-State Integrated Genomics Facility (IGF). For error estimate, 1% of PhiX control (Illumina pre-made library) was spiked-in in the run. Raw sequencing reads were processed to remove adapters and low-quality bases with Trimmomatic (version 0.38) (Bolger et al. 2014) with the default parameters. Cleaned reads with at least 60 bp were aligned to the B-71 reference genome with BWA (0.7.12-r1039) (Li and Durbin, 2014), followed by alignment filtering with an in-house script that required at least 50 bp matches, less than 5% mismatches, and less than 5% of reads that were not aligned (Peng et al. 2019). SNPs relative to the reference genome were discovered for each strain with the module of "Haplotypecaller" in GATK 4.0 (Walker et al. 2014). The R package *Ape* (5.3) was used to construct phylogenetic relations (Paradis et al. 2004). Phylogenetics trees were optimized with the online tool (itol.embl.de). Copy number analysis was performed using the approach described in Peng et al. (2019). Briefly, normalized read depths per region of each strain was compared to the average normalized read depth of all strains. The difference of normalized read depths indicated the dissimilarity in copy number or the polymorphism level of the region between a strain and the average.

Results

Seedling blast in inoculated field plots

We used conidial suspensions of the Bolivian strain 008 (24,600 or 15,300 spores ml^{-1}) to produce seedling blast at the wheat tillering stage (2 to 3 leaves) in field plots at two locations. Both air temperatures and relative humidity impacted subsequent disease development. Day (6:40 am to 6:39 pm) and night (6:40 pm to 6:39 am) air temperatures, and relative humidity for each location at key wheat stages are presented in Table 3-1. Irrigation was provided at both locations as needed. Overall, weather conditions were similar at both locations during the season.

On the day of field inoculation and during the following days, average day and night temperatures were 18.9 and 15.9°C, respectively, with RH of 82.4 and 94.3%, respectively, in 26 de Agosto (Exp. 1). In Okinawa (Exp. 2), average temperature was 20.2°C during the day with RH of 81.7% and 16.2°C with RH of 92.7% during the night. Maximum day temperatures reached 33°C in Exp. 1 and 27.1°C in Exp. 2. During the stages of grain development (i.e. heading to soft dough), day temperatures ranged from 21.7°C (watery – milky stage) to 29.4°C (soft dough stage), with maximum temperature of 37.6°C (soft dough stage) in Exp.1. In Exp.2, temperatures were slightly lower with averages from 20°C (soft dough stage) to 27.3°C (anthesis) with maximum temperature of 37.9°C (watery –milky). These environmental conditions were conducive to continued wheat blast disease development throughout the season.

Disease rating in the upper canopy (flag and F-1 leaves) and spikes

Disease ratings performed in the lower and mid canopy (F-6 to F-2) are not presented due to the large number of leaf senescence during the evaluations; thus, these results only focused on the upper canopy and on the spikes.

Wheat blast progress was fit by a quadratic polynomial model (incidence: r^2= 0.82 for Exp. 1 and 0.86 for Exp. 2, and severity: 0.86 for Exp. 1 and 0.85 for Exp. 2). Disease incidence and disease severity for both experiments are shown in Figures 3-1 and 3-2. For Exp. 1, disease incidence for F-1 leaves in the MoT inoculated plots began at 4.5% at the first rating (27 DAI), and reached a peak of 57.1% at 41 DAI (Fig. 3-1c). Meanwhile, in the non-inoculated plots, disease incidence ranged from 2.5% at 31 DAI to 8.7% at 47 DAI. Due to high leaf senescence (86% senescence in inoculated and 66% in the non-inoculated plots) during the last rating, the data were not considered in the analysis. While there was a consistent increase in disease severity in the F-1 leaves in the inoculated plots ranging from zero to 9.4% (Fig. 3-1f), disease severity

remained close to zero (maximum of 0.3%) in the non-inoculated plots during all evaluations. Disease incidence at the flag leaf stage followed a similar pattern as observed in the F-1 leaves. Incidence increased continuously from 9.8% (31 DAI) to 54% (58 DAI) in the inoculated plots, while the non-inoculated plots followed the same trend but with lower incidence levels (Fig. 3-1b). Flag leaf senescence in the last rating was 31.5% and 14% in the inoculated and non-inoculated plots, respectively. Disease severity at the flag leaf ranged from zero to 14.6% in the inoculated plots and stayed close to zero in the non-inoculated plots (Fig. 3-1e). During the first two disease ratings (27 and 31 DAI), spikes were not fully emerged, hence no disease rating was performed. During heading, 4.9% of the spikes were showing blast symptoms in the inoculated plots, while no symptoms were found in the non-inoculated plots. At anthesis (47 DAI), disease incidence was 18.8% and 3.4% in the inoculated and non-inoculated plots, respectively. Disease continued to develop during the stages of grain elongation and at milky - soft dough stages and averaged 91.2% in the inoculated plots and 58.8% in the non-inoculated plots (Fig. 3-1a). Disease severity began around 2% in the inoculated plots at heading and reached 76.4% at soft dough stage. For the non-inoculated plots, severity averaged 15.3% and 32.4% at milk and soft dough stages, respectively (Fig. 3-1c). Overall, disease severity was significantly higher for spike evaluations in the inoculated plots only at 47, 58, and 63 DAI. Despite the numerical differences, there were no statistical differences between the treatments for flag leaf and F-1 leaves.

Overall WB development in Exp. 2 followed a similar trend as described in Exp. 1. Disease incidence of F-1 leaves in the inoculated plots began at 7% in the first rating at 25 DAI, and reached its peak of 25.5% at 48 DAI. Likewise, maximum incidence in the non-inoculated plots occurred at 28 DAI (7.8%) (Fig. 3-2c). In the last disease evaluation, F-1 leaf senescence was high in both treatments. In the inoculated plots, senescence was 74.2%, while in the non-

inoculated plots F-1 senescence was 58.5%. F-1 severity increased from 0.24% to 19% in the MoT inoculated plots during the season; however, severity in the non-inoculated plots remained low, with maximum 1.5% severity in the last rating (Fig. 3-2f). Likewise, there was a consistent increase in WB incidence in the flag leaf. Maximum blast incidence average was 36% in the inoculated plots and 11% in the non-inoculated plots at 48 DAI (Fig. 3-2b). Flag leaf severity was slightly higher in Exp. 2 compared to Exp. 1, but trends were similar (Fig. 3-2e). Wheat spikes started to show blast symptoms at 41 DAI in both treatments [inoculated plots (12%) and non-inoculated plots (0.8%)]. Between watery and soft dough stages of grain development, blast incidence increased from 42% to 94% in the inoculated plots and from 25.5% to 72.3% in the non-inoculated plots (Fig. 3-2a). Final spike severity averaged 78% in the inoculated plots and 46.7% in the non-inoculated plots (Fig. 3-2d). Disease severity was significantly higher in the inoculated plots at 48 and 56 DAI for F-1 leaves, flag leaf, and spikes. No statistical differences were found at 25, 35, and 41 DAI for any of the different plant parts assessed (Fig. 3-2).

Wheat blast severity increase within inoculated and non-inoculated plots

We developed predictions (i.e., time-to-event) of WB incidence and severity increase within each plant part for both MoT inoculated and non-inoculated plots (Figures 3-1 and 3-2). Specifically, we aimed to answer the following question: *"How many days after inoculation (i.e., pathogen exposure) would it take for a particular plant part to reach a specific WB incidence/severity in the inoculated and non-inoculated plots?"* In both experiments, the difference in days to reach a specific severity between inoculated and non-inoculated plots is significantly higher for the upper canopy (flag and F-1 leaves) compared to the spikes. For instance, in Exp. 2 (Figure 3-2), it would take 58 and 63 days to reach 100% spike severity in the inoculated and non-inoculated plots, respectively. However, while it would take 70 days for the

flag leaf to reach 50% severity in the inoculated plots, the number of days would have to double (144 days) to reach the same severity in the non-inoculated treatment. This difference increases in the F-1 leaves (72 vs. 176 days). The prediction for spike severity increase was similar for Exp. 1, but predictions were limited for flag leaf and F-1 leaves in the non-inoculated plots due to the low disease pressure. Predictions for disease incidence followed similar pattern.

Correlation between leaf and spike MoT incidence and severity

The disease intensity in the upper canopy (F-1 and flag leaf) was lower when compared to the spikes in both treatments and experiments. However, there were multiple strong and significant correlations between disease ratings in different plant parts during the season (Fig. 3-3 and 3-4). We used the disease incidence or severity of either F-1 leaves or flag leaf at one specific rating time to correlate with disease incidence or severity in the following rating time of either flag leaf or spike (spike vs. flag leaf, spike vs. F-1, and flag leaf vs. F-1). For blast incidence in Exp. 1, there was a linear and significant correlation between spike vs. flag leaf in the inoculated plots (r^2= 0.96) (Fig. 3-3a), and spike vs. F-1 in both treatments (r^2= 0.94 and 0.90 inoculated and non-inoculated, respectively) (Fig. 3-3b). The correlation between flag leaf and F-1 was sigmoidal (r^2= 0.99, p<0.001) in the inoculated plots and exponential (r^2= 0.86, p<0.001) in the non-inoculated plots. For blast severity in Exp. 1, there was significant sigmoidal correlation between disease severity in the spike vs. flag leaf (r^2= 0.99, p<0.001) and spike vs. F-1 (r^2= 0.99, p=0.001) in the inoculated plots (Fig. 3-3d and 3-3e). Disease severity increase at both flag leaf and F-1 leaves in the first two ratings had small effect in the spike disease severity mostly because the spikes were not fully emerged (bottom plateau). The sigmoidal model suggested that the inflection point at which the increase in spike blast rate was maximized was at flag leaf or F-1 severity of 4.13-5.06%, and that spike blast severity reached an asymptotic

maximum of ~76% (i.e., the second plateau, which coincided with high percentage of flag and F-1 leaves senesced). The relationship between flag leaf and F-1 leaves was linear (r^2= 0.95, p<0.001) (Fig. 3-3f). On the other hand, no significant correlations were found in the non-inoculated plots most likely due to the low disease severity in the flag and F-1 leaves.

For Exp. 2, spike blast incidence showed a linear and significant correlation with flag leaf incidence in both treatments (Fig. 3-4a). On the other hand, while the correlations between spike vs. F-1 and flag leaf vs. F-1 were linear in the non-inoculated plots, both correlations were exponential in the inoculated plots (Fig. 3-4b and 3-4c). In Exp. 2, disease severity correlations were linear and significant for all comparisons (r^2 varied from 0.94 for flag leaf vs. F-1 in the non-inoculated plots to 0.99 for spike vs. flag leaf in both treatments) (Fig. 3-4d, 3-4e, and 3-4f). We note in passing that the linear relationship between blast severity in the flag leaf vs. spike, and in the F-1 leave vs. spike, might be masked by one fewer measurement in between days 56 and 63. Had we performed this additional measurement, a sigmoidal relationship similar to that in Exp. 1 might have been the best fit, as otherwise the remaining measurements followed a similar pattern. The potential for a previous leaf blast incidence/severity measure to predict a subsequent spike blast incidence/severity warrants future research.

Whole genome sequencing of inoculated and sampled blast isolates

We sequenced and assembled genomes for 48 MoT samples, including four samples of strain 008 (from Quirusillas, Bolivia, in 2015) that were used for the initial field inoculations, and 44 samples isolated from infected wheat spikes late in the season. Wheat seeds used to plant these field experiments tested positive for the presence of MoT using PCR analysis. However, we did not sequence this contaminating fungal DNA because the DNA sample was extracted from the wheat seeds and not from the isolated fungus recovered from the seeds. The mix

between wheat seed DNA and fungus DNA would not provide the quantity and quality of MoT DNA required for whole genome sequencing. In addition, it is unlikely that the MoT present in the seeds and the isolate used for field inoculation (MoT isolate 008) were the same because the seeds were treated with fungicide prior to planting and no wheat blast symptoms were identified prior to artificial inoculation. The final 008 genome assembly (Table 3-2) was comprised of ~44.25 Mb in seven core chromosomes and five unanchored scaffolds corresponding to a dispensable supernumerary mini-chromosome in the MoT reference genome of strain B-71 (Peng et al. 2019). All 48 isolates exhibited high sequence similarity in the core chromosomes, indicating they are likely to be clonal (Figure 3-5). In addition, the 48 MoT isolates from this study were compared to eight previously sequenced *M. oryzae* strains (Py221, B2, P29, P3, P28, B-71, B51, and Py5020) (Peng et al. 2019). Surprisingly, strain 008, which was not previously sequenced, was closely related to Py5020, which was isolated in Brazil in 2005, and not closely related to other strains sequenced from Bolivia (Figure 3-5). Together these results indicate that the same MoT strain we used for seedling inoculations at the tillering stage of corresponding treatment caused spike blast later in the season in both inoculated and non-inoculated plots, consistent with polycyclic nature MoT as previously described in Gongora-Canul et al. (2019).

Based on our MoT reference genome for strain B-71 (Peng et al. 2019), we predict that the 008 genome scafolds 1-5 correspond to at least one mini-chromosome in this strain, and the 008 mini-chromosome(s) contains at least two effector genes, *PWL2* and *BAS1*, found in the B71 mini-chromosome. Therefore, our sequencing of 48 strains representing blast infection through one crop cycle has provided an intriguing hypothesis for future follow-up. That is, based on phylogenetic analyses of the five unanchored scaffolds separately from analysis of the core chromosome sequences, there appeared to be around 10-fold more genetic variation in the

putative mini-chromosome sequences among the 48 different strains than in the core chromosome sequences (Figure 3-6). To further detect genomic copy number variation (CNV) in specific sequences among the isolates, a read depth approach was applied focusing on the identification of genomic regions with conserved copy number (CNequal), lower copy number (CNminus) or higher copy number (CNplus) (data not shown). Overall, the majority of the sequence exhibited CNequal in the core chromosomes with a small percentage of CNplus, while in scaffolds 1-5 (hypothesized as mini-chromosomes), there was higher variation in copy number with a high percentage of CNplus for some isolates. These results require verification through electrophoretic separation [Via Contour-clamped homogeneous electric field (CHEF) gels] of the chromosomes in individual strains, recovery of separated mini-chromosome DNA from the gel and sequencing of individual mini-chromosomes as previously reported (Peng et al. 2019). When verified, our results will support the larger hypothesis that mini-chromosomes represent a highly variable genome region with major impact on the ability to *M. oryzae* to become more aggressive and evade resistance in current hosts, as well as to jump to new hosts.

Discussion

Wheat blast development is highly dependent upon conducive environmental conditions, thus there is a high year-to-year variability in disease incidence and severity. Prior to the establishment of these field experiments, we aimed to sample *Magnaporthe oryzae* isolates from nearby wheat fields as well as from weeds and other grasses that could serve as alternative hosts and potential alternative sources of inoculum. However, the non-conducive weather conditions did not allow blast development during the wheat winter season in Bolivia in 2017 (ANAPO, 2017); thus, no additional *Magnaporthe oryzae* isolates were collected outside of our irrigated field plots. Therefore, this study does not address whether outside inoculum plays an important

role for the epidemiology of this disease. Instead, we present epidemiological and genomic data documenting a role for autoinfection (i.e., infection resulting from inoculum produced on the same wheat host unit) in the development of wheat spike blast. Isolation of the same fungal strain causing disease at the tillering and heading stages of wheat supports evidence for the polycyclic nature of the MoT fungus. Our findings agree and complement those reported by Gongora-Canul et al. (2019), who demonstrated that spike blast epidemics in a highly susceptible host population do not follow a monocyclic pattern.

Despite the warm temperatures and high precipitation at sowing, dry conditions during wheat heading (June and July) inhibited wheat blast development under natural conditions. Nonetheless, the daily supplemental irrigation together with MoT artificial inoculation in a highly susceptible variety allowed the disease to develop and spread in both inoculated and non-inoculated plots in our experiments. Temperatures between 25 and 30°C combined with moist and high relative humidity conditions during the wheat heading stage often favor MoT infection (Cardoso et al. 2008). In both experiments, average day temperatures at heading and anthesis were close to 25°C, but reached above 30°C on several days. Conducive temperatures continued for the remaining season in both experiments. In commercial wheat fields, blast symptoms during vegetative stages are not often reported (Maciel et al. 2013, Cruz and Valent, 2017; Gongora-Canul et al. 2019), which can be explained by the non-conducive weather, low pathogen population (inoculum is still building up), or a low correlation between seedling and spike infection. In our experiments, average temperatures below 20°C prior to heading could have limited MoT infection during vegetative stages and impacted disease development. Nevertheless, these experiments prove the efficacy of our field inoculation procedure for blast resistance phenotyping even under nonconducive weather conditions.

Although much extensive progress has been made to explain epidemiological factors for the rice blast pathosystem, critical aspects about the epidemiology of wheat blast are still unknown. A few studies demonstrated that infection at the seedling stage can occur in some specific genotype by isolate combinations, but with a low correlation with spike infection (Maciel et al. 2013; Martinez et al. 2018; Cruppe et al. 2019). However, results from these studies are derived either from seedling and spike severity being evaluated in different experiments or under controlled environment conditions. In our study, we performed a single MoT inoculation at tillering stage and we provided the optimal conditions for blast development up to the spike stage. The results reported in this paper strengthen the understanding of the role of leaf infection in the spread and severity of wheat blast in a susceptible host population (Gongora-Canul et al., 2019). Cruz et al. (2015) reported that the lower canopy of moderately resistant varieties produced significantly less inoculum (measured as number of conidia per g of dry basal leaves) compared to susceptible varieties. Still the amount of conidia produced by the moderately resistant varieties was high enough to cause an epidemic later in the season. As follows, we report multiple approaches to determine the relationship between leaf and spike infection.

First, we followed wheat blast spread from tillering (inoculation time) to maturity in both inoculated and non-inoculated plots. Blast symptoms began in the lower and mid canopy (data not shown) and in time spread to new emerging leaves (upper canopy) and to the spikes. Symptoms were found either first in the inoculated plots or at higher levels compared to non-inoculated plots. It was difficult to determine if more lesions were produced in the upper or in the lower canopy due to the high percentage of leaf senescence in the lower canopy when disease ratings were performed. Ghatak et al. (2013) reported that young expanding rice leaves were

more susceptible to blast compared to older leaves, which contrasts with reports for wheat blast (Cruz et al. 2015). In our study, flag leaves and F-1 leaves had similar levels of disease incidence and severity within treatment and within experiment.

Furthermore, we determined the correlation between leaf and spike incidence and severity. Our findings indicate that leaf severity in the upper canopy (flag leaf and F-1 leaves) can be a reliable estimator of spike severity when leaf blast severity is moderate to high and the environment is conducive. However, correlations were weak and not significant under low disease severity conditions (non-inoculated plots), despite the same conducive environment; suggesting that current disease pressure should be considered. Other critical factors such as current and forecast weather conditions as well as the susceptibility of the variety will also determine the prediction's accuracy. Several studies have demonstrated a low correlation between seedling and spike resistance, thus predictions should be cautiously used (Maciel et al. 2013, Cruz et al. 2015, Cruppe et al. 2019). Estimating future wheat blast spike incidence and severity based on a previous leaf incidence and severity measure could help wheat farmers to make better decisions about optimal timing to apply foliar fungicide, i.e., applications earlier in the season could reduce the MoT population and consequently the severity in the spike stage, the most critical stage for MoT infection. Since the upper leaves contribute greatly to yield, it is important to understand how wheat blast spreads up within wheat canopy. It is still unclear whether the partial control of wheat blast at the spike stage by foliar fungicides is due to the insufficient rate or inadequate coverage, inherently low fungicide effectiveness, improper timing, or a combination of these factors (Damicone and Smith, 2009; Cruz and Valent, 2017).

Finally, we documented through whole genomic sequencing that the strain we inoculated on wheat seedlings at the tillering stage was the same strain that was isolated from blasted wheat

spikes later in the season. This was true in independent experiments in two separate locations during the Bolivian winter season in 2017. The fungus isolated from wheat leaves (sampling two) was not sequenced because wheat blast did not occur naturally during the 2017 wheat-growing season, thus no variation was expected. The seven core chromosomes from the genome assemblies of the 44 spike blast isolates were nearly identical, within the limits of sequencing accuracy, to the strain used for field inoculations. Interestingly, although there was little sequence variation in the core chromosomes, dispensable mini-chromosome sequences in the initial strain and recovered isolates showed enhanced variation. It has recently been shown that MoT mini-chromosomes are enriched in blast effector genes and undergo frequent sequence exchanges with the ends of the core chromosomes, which are also enriched in known and putative effector genes (Peng et al. 2019). Mini-chromosomes are enriched for transposable elements, which comprise 52.8% of the B71 mini-chromosome sequence, compared to 9.7% of the core chromosomes, which is likely to contribute to the apparent genetic instability of the mini-chromosomes. All current data suggest that these dispensable extra chromosomes in the blast fungus provide an environment for rapid evolution of genes, including effector genes that potentially impact pathogen aggressiveness and host specificity. Our results show that field studies such as ours can play a critical role in tracking mini-chromosome evolution, and in defining the evolutionary potential of the wheat blast pathogen, as well as other host-adapted strains of *M. oryzae*.

References

Bain, D., Patel, B., Patel, M. 1972. Blast of ryegrass in Mississippi. Plant Disease Reporter. 56:210.

Bockus, W. W., Cruz, C. D., Stack, J. P., and Valent, B. 2015. Effect of seed-treatment

fungicides on sporulation of *Magnaporthe oryzae* from wheat seed. Plant Dis. Manag. Rep 9:ST004.

Bolger, A.M., Lohse, M. and Usadel, B. 2014. Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics. 30:2114–2120.

Cardoso, C. D. A., Reis, E. M., and Moreira, E. N. 2008. Development of a warning system for wheat blast caused by *Pyricularia grisea*. Summa Phytopathol. 34:216–221.

Carver, R., Rush, M., and Lindberg, D. 1972. An epiphytotic of ryegrass blast in Louisiana. Plant Disease Reporter. 56:157–159.

Chuma, I., Isobe, C., Hotta, Y., Ibaragi, K., Futamata, N., Kusaba, M., Yoshida, K., Terauchi, R., Fujita, Y., Nakayashiki, H., Valent, B., and Tosa, Y. 2011. Multiple translocation of the AVR-Pita effector gene among chromosomes of the rice blast fungus *Magnaporthe oryzae* and related species. PLoS Pathogens. 7:e1002147.

Cook, D.E., Mesarich, C.H. and Thomma, B.P.H.J. 2015. Understanding plant immunity as a surveillance system to detect invasion. Annu. Rev. Phytopathol. 53: 541–563.

Couch, B. C., Fudal, I., Lebrun, M-H., Tharreau, D., Valent, B., van Kim, P., Notteghem, J-L., Kohn, L. M. 2005. Origins of host-specific populations of the blast pathogen *Magnaporthe oryzae* in crop domestication with subsequent expansion of pandemic clones on rice and weeds of rice. Genetics. 170:613–630.

Cruz, C. D., Peterson, G. L., Bockus, W. W., Kankanala, P., Dubcovsky, J., Jordan, K. W., Akhunov, E., Chumley, F., Baldelomar, F. D., and Valent, B. 2016. The 2NS translocation from *Aegilops ventricosa* confers resistance to the *Triticum* pathotype of Magnaporthe oryzae. Crop Sci. 56:990–1000.

Cruz, C. D., and Valent, B. 2017. Wheat blast disease: danger on the move. Trop. Pl. Path.

42:210–222.

Cruz, C.D., Santana, F.M., Todd, T.C., Maciel, J.L., Kiyuna, J., Baldelomar, D.F., Cruz, A.P., Lau, D., Seixas, C.S., Goulart, A.C. and Sussel, A.A., 2019. Multi-environment assessment of fungicide performance for managing wheat head blast (WHB) in Brazil and Bolivia. Trop. Plant Pathol.183-191.

Dong, Y., Li, Y., Zhao, M., Jing, M., Liu, X., Liu, M., Guo, X., Zhang, X., Chen, Y., Liu, Y. and Liu, Y. 2015. Global genome and transcriptome analyses of *Magnaporthe oryzae* epidemic isolate 98-06 uncover novel effectors and pathogenicity-related genes, revealing gene gain and lose dynamics in genome evolution. PLoS Path. 11:e1004801.

Ghatak, A., Willocquet, L., Savary, S., and Kumar, J. 2013. Variability in aggressiveness of rice blast (*Magnaporthe oryzae*) isolates originating from rice leaves and necks: a case of pathogen specialization? PLoS One. 8:e66180.

Gongora-Canul, C., Salgado, J., Singh, D., Cruz, A., Cotrozzi, L., Couture, J.J., Rivadeneira, M.G., Cruppe, G., Valent, B., Todd, T. and Poland, J., and Cruz, C. C. 2019. Temporal Dynamics of wheat blast epidemics and agreement between remotely sensed data measurements and visual estimations of wheat spike blast (W_SB) under field conditions. Phytopathology. (ja).

Goulart, A. C. P., and Paiva, F. A. 1990. Transmission of *Pyricularia oryzae* by wheat Triticum aestivum seeds. Fitopatol. Bras. 15:359–362.

Goulart, A. C. P., Paiva, F. A., and Andrade, P. J. M. 1995. Relação entre a incidência da brusone em espigas de trigo e a presença de *Pyricularia grisea* nas sementes colhidas. Fitopatol. Bras. 20:184-189.

Heath, M. C. 2000. Hypersensitive response-related death. In Programmed cell death in higher

plants. 77–90. Springer, Dordrecht.

Igarashi, S., Utiamada, C. M., Igarashi, L. C., Kazuma, A. H., and Lopes, R. S. 1986. Ocorrência de *Pyricularia* sp. em trigo no estado do Paraná. [Occurrence of Pyricularia sp. on wheat in the state of Paraná]. Fitopatologia Brasileira, 11: 351–352.

Igarashi, S. 1990. Update on wheat blast (*Pyricularia oryzae*) in Brazil. In: Saunders D, ed. Proceedings of the International Conference – Wheat for the Nontraditional Warm Areas. Mexico DF, Mexico: CIMMYT, 480–3.

Islam, M. T., Croll, D., Gladieux, P., Soanes, D. M., Persoons, A., Bhattacharjee, P., Hossain, M. S., Gupta, D. R., Rahman, M. M., Mahboob, M. G., Cook, N., Salam, M. U., Surovy, M. Z., Sancho, V. B., Maciel, J. L. N., Nhani, A,, Castroagudin, V. L., Reges, J. T. D., Ceresini, P. C., Ravel, S., Kellner, R., Fournier, E., Tharreau, D., Lebrun, M. H., McDonald, B. A., Stitt, T,, Swan, D., Talbot, N. J., Saunders, D. G. O., Win, J, Kamoun, S. 2016. Emergence of wheat blast in Bangladesh was caused by a South American lineage of *Magnaporthe oryzae*. BMC Biol. 14:84.

Kang, S., Sweigard, J. A., and Valent, B. 1995. The PWL host specificity gene family in the blast fungus *Magnaporthe grisea*. Mol. Pl. Microb. Inter. 8: 939–948.

Kato, H., Yamamoto, M., Yamaguchi-Ozaki, T., Kadouchi, H., Iwamoto, Y., Nakayashiki, H., Tosa, Y., Mayama, S., and Mori, N. 2000. Pathogenicity, mating ability and DNA restriction fragment length polymorphisms of *Pyricularia* populations isolated from Gramineae, Bambusideae and Zingiberaceae plants. J. Gen. Plant. Pathol. 66:30–47.

Kiyuna J. 2019. Detection of wheat blast within and on wheat seeds and preliminary risk evaluation tool for the detection of MoT infected seeds. M.S. Kansas State University, Manhattan, Kansas.

Lenné, J. M., Takan, J. P., Mgonja, M. A., Manyasa, E. O., Kaloki, P., Wanyera, N., Okwadi, J., Muthumeenakshi, S., Brown, A. E., Tamale, M., Sreenivasaprasad, S. 2007. Finger millet blast disease management - A key entry point for fighting malnutrition and poverty in East Africa. Outlook on Ag. 36:101–108.

Li, H. and Durbin, R., 2010. Fast and accurate long-read alignment with Burrows–Wheeler transform. Bioinformatics. 26:589–595.

Maciel, J. L. N., Ceresini, P. C., Castroagudin, V. L., Zala, M., Kema, G. H., and McDonald, B. A. 2014. Population structure and pathotype diversity of the wheat blast pathogen Magnaporthe oryzae 25 years after its emergence in Brazil. Phytopathology. 104:95–107.

Malaker, P. K., Barma, N. C. D., Tiwari, T. P., Collis, W. J., Duveiller, E., Singh, P. K., Joshi, A. K., Singh, R. P., Braun, H. J. , Peterson, G. L., Pedley, K. F., Farman, M. L. , Valent, B. 2016. First report of wheat blast caused by *Magnaporthe oryzae* pathotype *triticum* in Bangladesh. Plant Dis. 100:2330–2330.

Marangoni, M. S., Nunes, M. P., Fonseca, N. Jr, and Mehta, Y. R. 2013. *Pyricularia* blast on white oats: a new threat to wheat cultivation. Trop. Plant. Pathol. 38:198–202

Mottaleb, K. A., Singh, P. K., He, X., Hossain, A., Kruseman, G., Erenstein, O. 2019. Alternative use of wheat land to implement a potential wheat holiday as wheat blast control: In search of feasible crops in Bangladesh. Land Use Policy 82:1–12.

Orbach, M.J., Chumley, F.G. and Valent, B., 1996. Electrophoretic karyotypes of *Magnaporthe grisea* pathogens of diverse grasses. MPMI. 9:261–271.

Paradis, E., Claude, J. and Strimmer, K. 2004. APE: analyses of phylogenetics and evolution in R language. Bioinformatics. 20:289–290.

Peng, Z., Oliveira-Garcia, E., Lin, G., Hu, Y., Dalby, M., Migeon, P., Tang, H., Farman, M.,

Cook, D., White, F. F., Valent, B., and Liu, S. 2019. Effector gene reshuffling involves dispensable minichromosomes in the wheat blast fungus. PLOS Genetics 15:e1008272.

Pieck, M.L., Ruck, A., Farman, M.L., Peterson, G.L., Stack, J.P., Valent, B. and Pedley, K.F. 2017. Genomics-based marker discovery and diagnostic assay development for wheat blast. Plant Dis. 101:103-109.

Pizolotto, C. A., Maciel, J. L. N., Fernandes, J. M. C., & Boller, W. 2019. Saprotrophic survival of *Magnaporthe oryzae* in infested wheat residues. Europ. J. of Pl. Pathol. 153:327–339.

Rovenich, H., Boshoven, J. C. and Thomma, B.P.H.J. 2014. Filamentous pathogen effector functions: of pathogens, hosts and microbiomes. Curr. Opin. Plant Biol. 20:96–103.

Sweigard, J. A., Carroll, A. M., Kang, S., Farrall, L., Chumley, F.G. et al. 1995. Identification, cloning, and characterization of *Pwl2*, a gene for host species-specificity in the rice blast fungus. The Plant Cell. 7:1221–1233.

Toledo, J. 2015. Tratamiento de Semillas. In. Manual de recomendaciones técnicas – Cultivo de trigo. Santa Cruz, Bolivia. Asociación de Productores de Oleaginosas y Trigo (ANAPO), 140 p.

Tosa, Y., Hirata, K., Tamba, H., Nakagawa, S., Chuma, I., Isobe, C., Osue, J., Urashima, A. S. , Don, L. D., Kusaba, M., Nakayashiki, H., Tanaka, A., Tani, T., Mori, N., and Mayama, S. 2004. Genetic constitution and pathogenicity of Lolium isolates of *Magnaporthe oryzae* in comparison with host species-specific pathotypes of the blast fungus. Phytopathology. 94:454–462.

Tosa, Y., Inoue, Y., Trinh, T. P. V., and Chuma, I. 2016. Genetic and molecular analyses of the incompatibility between Lolium isolates of *Pyricularia oryzae* and wheat. Physiol. Mol. Plant. Pathol. 95:84–86

Urashima, A., Grosso, C., Stabili, A. 2009. Effect of Magnaporthe grisea on seed germination, yield and quality of wheat. In: Wang G, Valent B, eds. Advances in Genetic, Genomics and Control of Rice Blast Disease. New York, NY, USA: Springer Science and Business Media, 267–77.

Valent, B., Farrall, L., and Chumley, F. G. 1991. *Magnaporthe grisea* genes for pathogenicity and virulence identified through a series of backcrosses. Genetics. 127:87–101.

Viji, G., Wu, B., Kang, S., Uddin, W., Huff, D. R. 2001. *Pyricularia grisea* causing gray leaf spot of perennial ryegrass turf: population structure and host specificity. Plant Dis. 85:817–826.

Walker, B.J., Abeel, T., Shea, T., Priest, M., Abouelliel, A., Sakthikumar, S., Cuomo, C.A., Zeng, Q., Wortman, J., Young, S.K. and Earl, A.M. 2014. Pilon: an integrated tool for comprehensive microbial variant detection and genome assembly improvement. PloS one. 9:e112963.

Wang, G-L. and Valent, B. (ed). 2009. Advances in genetics, genomics and control of rice blast disease. Springer Science and Business Media, New York, New York.

Wang, B. H., Ebbole, D. J., Wang, Z. H. 2017. The arms race between *Magnaporthe oryzae* and rice: Diversity and interaction of Avr and R genes. J. of Integ. Ag. 16:2746–2760.

Zhang, N., Luo, J., Rossman, A. Y., Aoki, T., Chuma, I., Crous, P. W., Dean, R., de Vries, R. P., Donofrio, N., Hyde, K. D., Lebrun, M-H., Talbot, N. J., Tharreau, D., Tosa, Y., Valent, B., Wang, Z., and Xu, J-R. 2016. Generic names in *Magnaporthales*. IMA Fungus. 7:155–159.

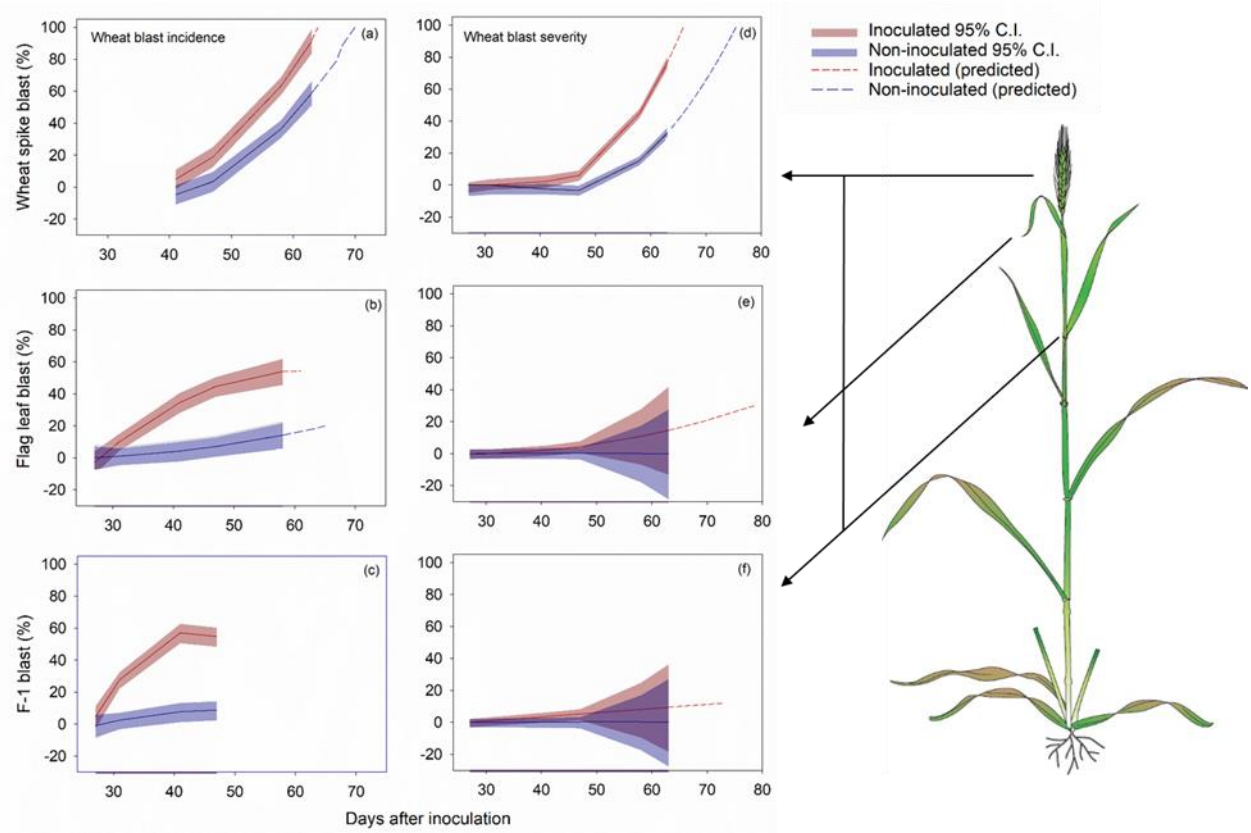


Figure 3-1. Observed and predicted (dash lines) blast incidence (left) and severity (right) development on (a) and (d) wheat spikes, (b) and (e) flag leaf, and (c) and (f) F-1 leaves for Experiment 1. Red lines indicated MoT (isolate 008) inoculated plots and blue lines indicate non-inoculated plots. Wheat image credit: Wheat Growth and Development, Lollato 2018.

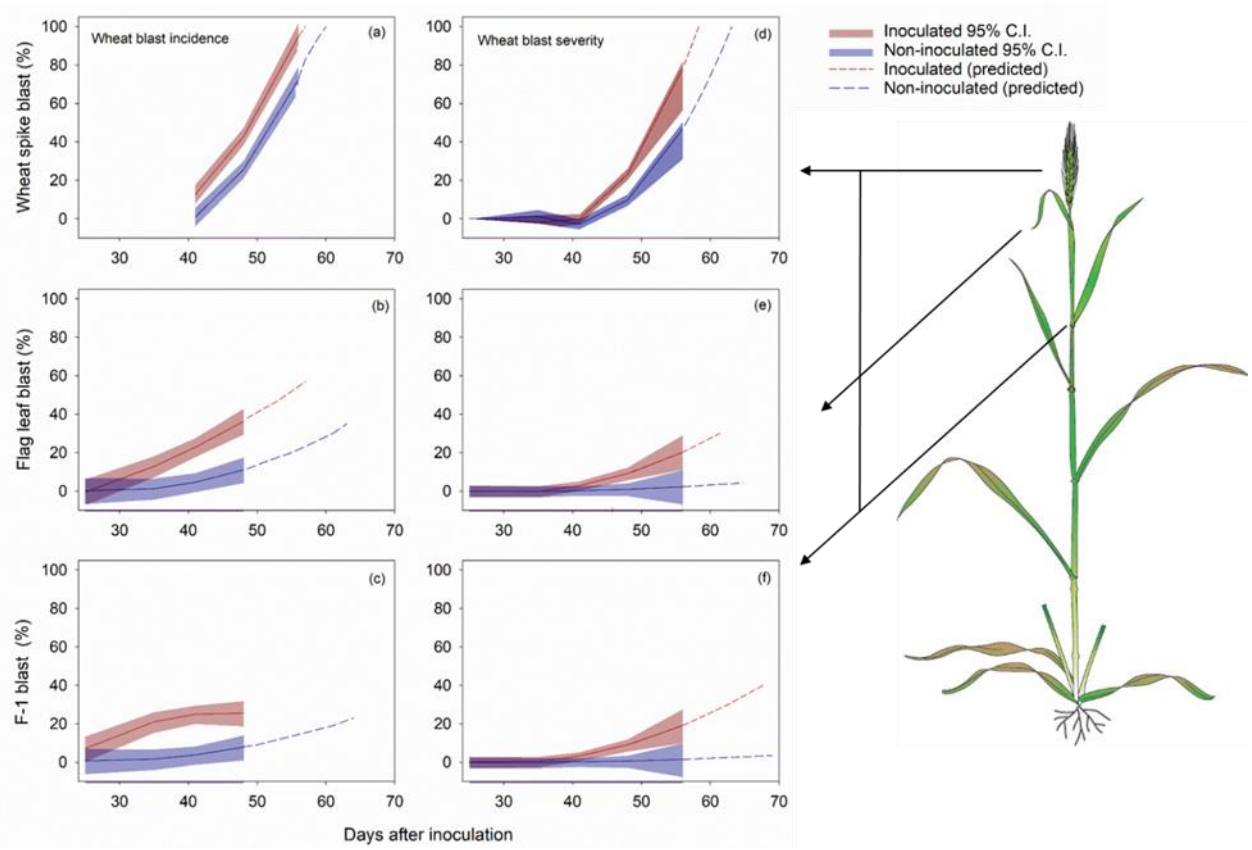


Figure 3-2. Observed and predicted (dash lines) blast incidence (left) and severity (right) development on (a) and (d) wheat spikes, (b) and (e) flag leaf, and (c) and (f) F-1 leaves for Experiment 2. Red lines indicated MoT inoculated (isolated 008) plots and blue lines indicate non-inoculated plots. Wheat image credit: Wheat Growth and Development, Lollato 2018.

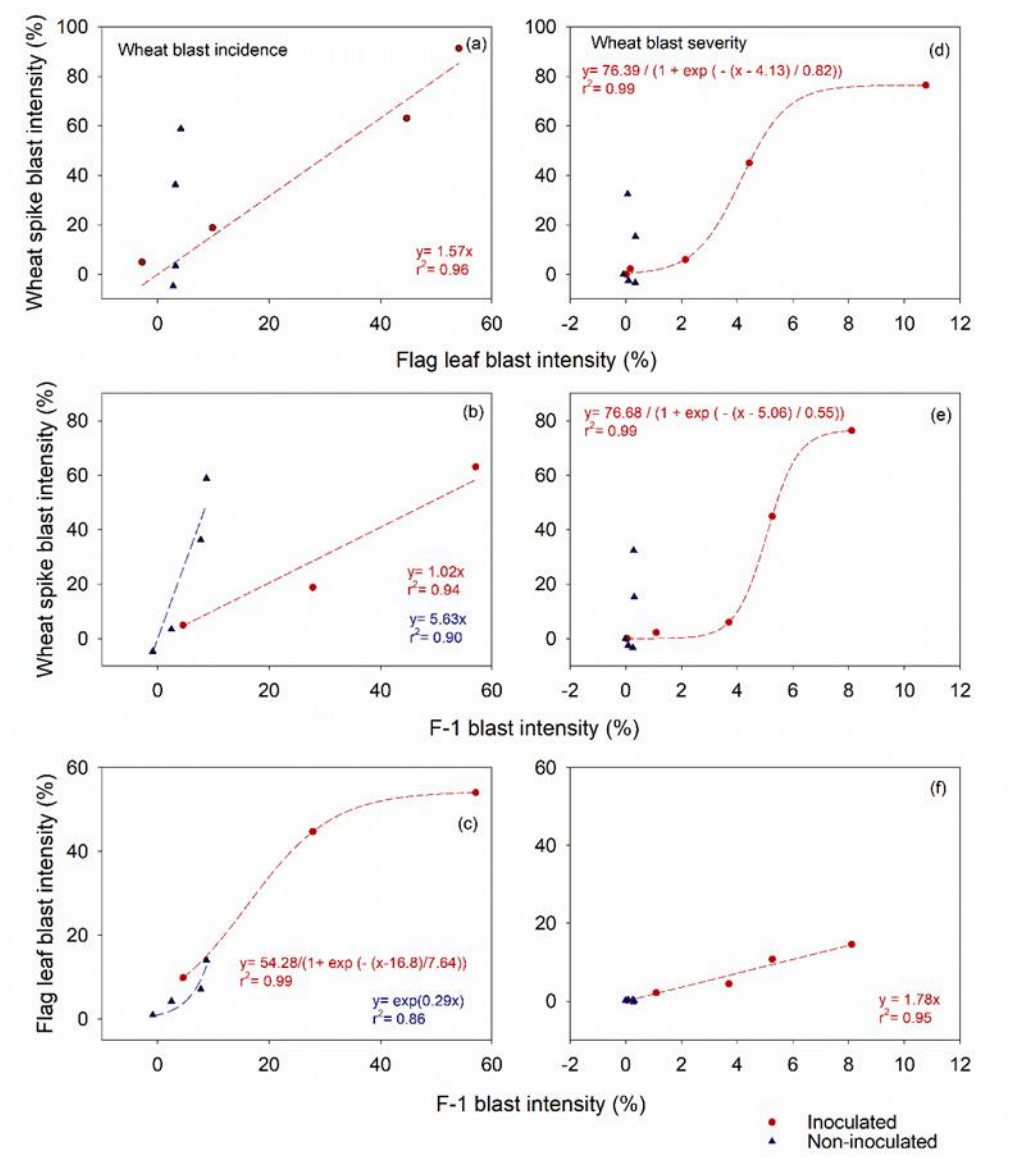


Figure 3-3. Relationship between wheat spike incidence (left) and severity (right) and either flag leaf or F-1 (a, b, d, and e) and flag leaf and F-1 (c and f) for Experiment 1. Red lines indicate MoT inoculated plots and blue lines indicate non-inoculated plots. Disease rating at one specific timing was used to predict spike or flag leaf incidence/severity at the following disease rating timing (e.g., flag leaf severity at 58 DAI was used to predict spike severity at 63 DAI).

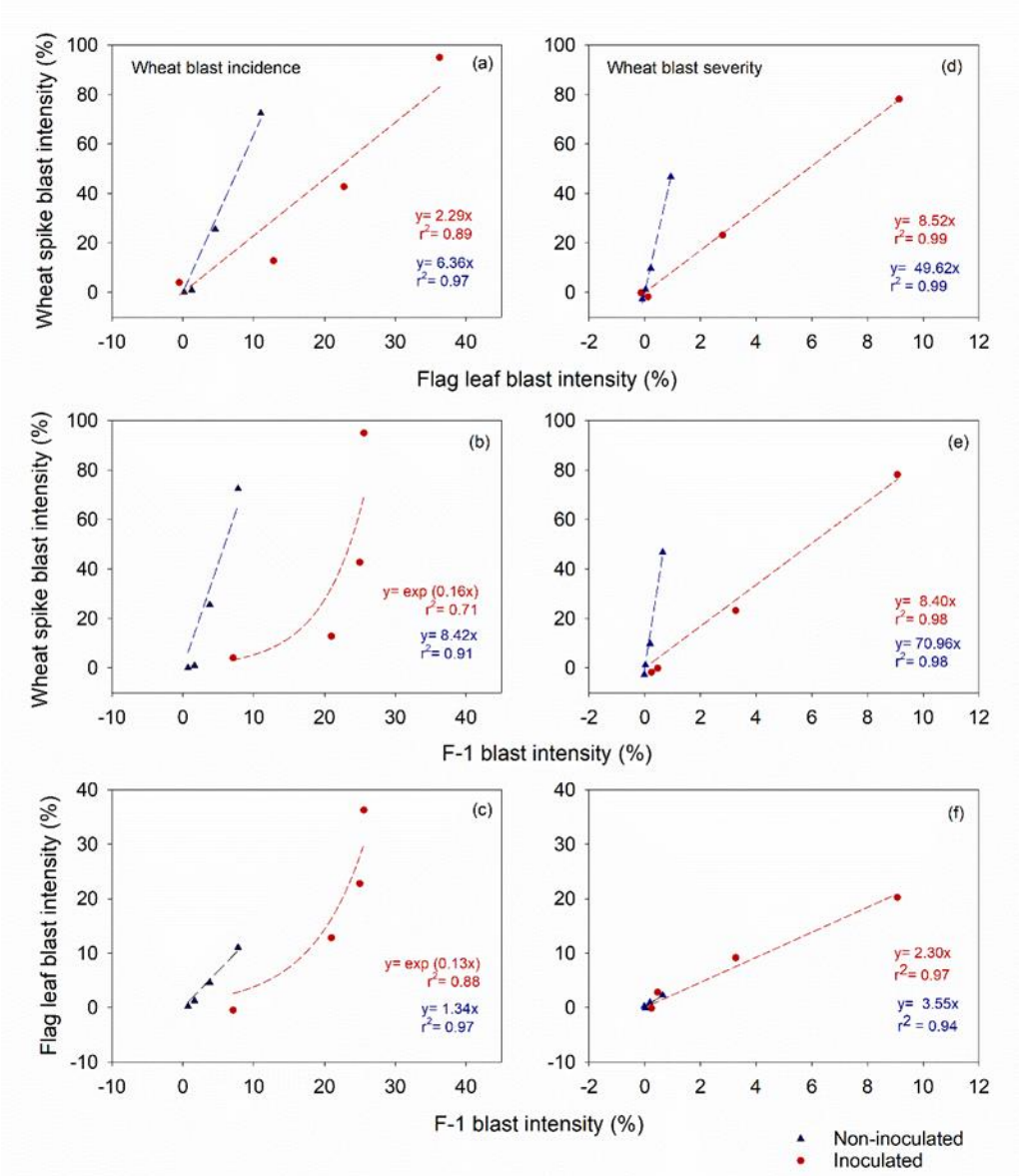


Figure 3-4. Relationship between wheat spike incidence (left) and severity (right) and either flag leaf or F-1 (a, b, d, and e) and flag leaf and F-1 (c and f) for Experiment 2. Red lines indicate MoT inoculated plots and blue lines indicate non-inoculated plots. Disease rating at one specific timing was used to predict spike or flag leaf incidence/severity at the following disease rating timing (e.g., flag leaf severity at 58 DAI was used to predict spike severity at 63 DAI).

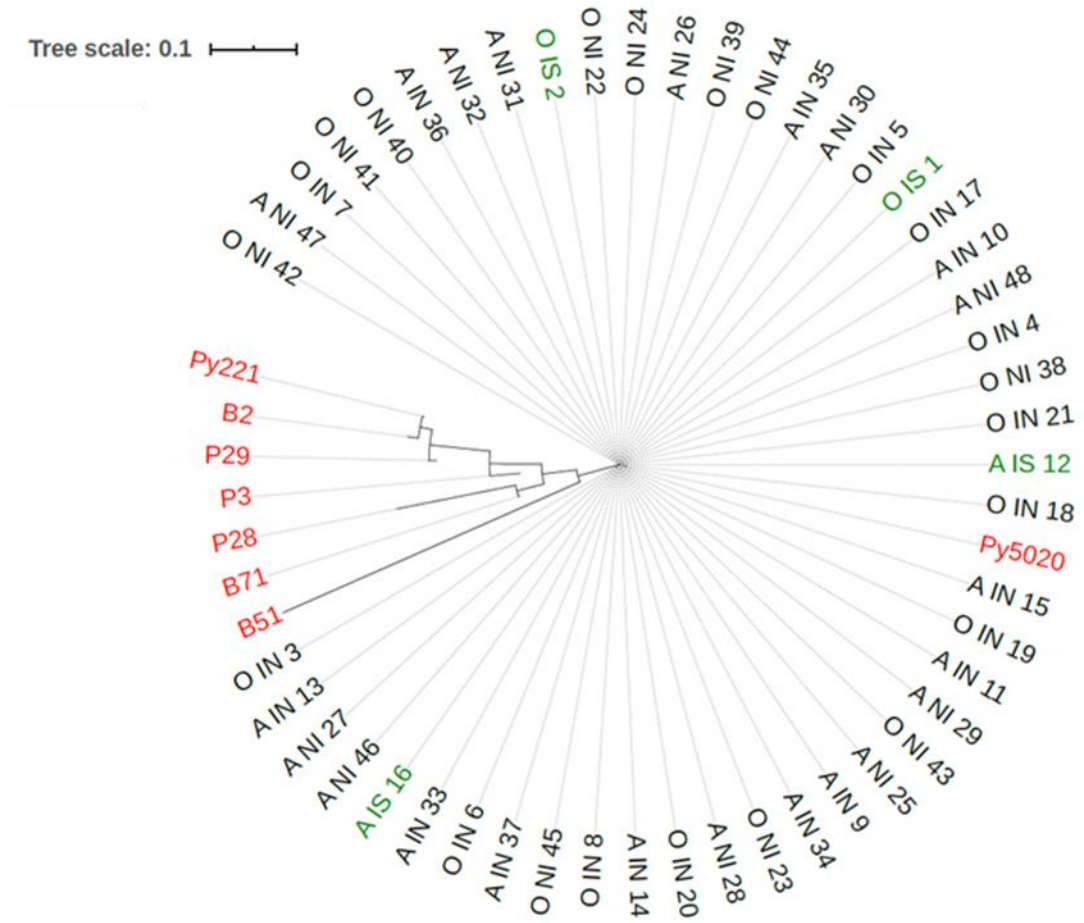


Figure 3-5. Comparison between the 48 MoT isolates sampled from this study and eight additional *Magnaporthe oryzae* isolates sampled from different countries. Strain names from this experiment include: A: experiment 1; O: experiment 2; IS: isolates used for field inoculations; IN: isolates sampled from inoculated plots; NI: isolates sampled from non-inoculated plots. Strains highlighted in red are the eight additional isolates from Peng et al. (2019) used for comparison. These are *Triticum* pathotype strains Py22.1 (Brazil, 2007); B2 (Bolivia, 2011); P29 (Paraguay, 2014); P3 (Paraguay, 2012) and B71 (Bolivia, 2012); *Lolium* pathotype strain P28 (Paraguay, 2014); and *Eleusine* pathotype strain B51 (Bolivia, 2012). The tree scale ratio indicates 10% of polymorphic sites showing differences.

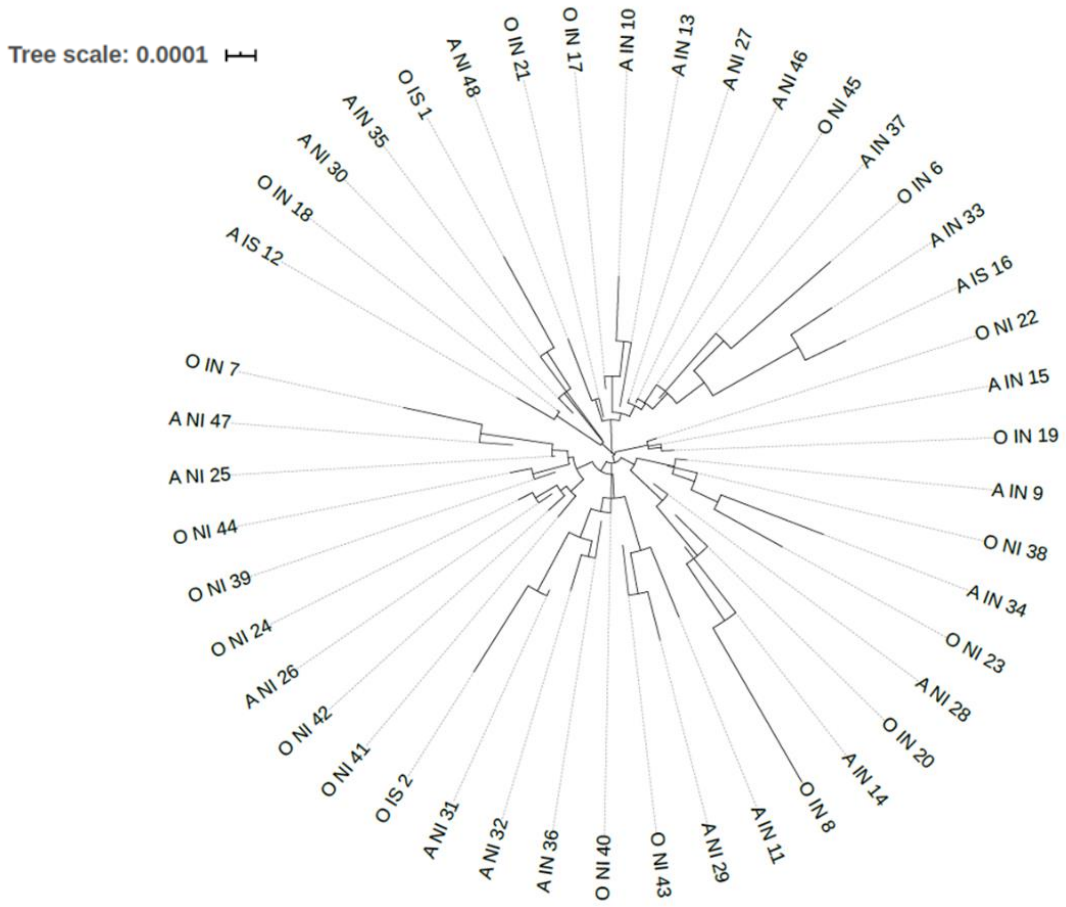


Figure 3-6. Comparison of scaffolds 1 to 5 (hypothesized as one or more mini-chromosomes) for the 44 MoT samples from wheat spikes plus four MoT samples used for field inoculations. Strains names include: A: experiment 1; O: experiment 2; IS: isolates used for field inoculations; IN: isolates sampled from inoculated plots; NI: isolates sampled from non-inoculated plots. The tree scale ratio indicates differences at 0.01% of polymorphic sites in the mini-chromosomes. In contrast, differences in core chromosomes were visualized using a tree scale corresponding to 0.001% of polymorphic sites.

Table 3-1. Weather conditions [average, maximum, and minimum temperatures and average relative humidity (RH)] at key wheat developmental stages for field experiments 1 (26 de Agosto) and 2 (Okinawa) in Bolivia, 2017. Data were collected in the center of the field experiment.

Location	Wheat stage	Temperature Day (°C)			RH (%)	Temperature night (°C)			RH (%)
		Average	Maximum	Minimum	Average	Average	Maximum	Minimum	Average
26 de Agosto (Exp. 1)	Tillering (inoculation)	18.9	33	12.4	82.4	15.9	24.2	12	94.3
	Heading	26.7	34.7	11.1	73.2	18.5	23.1	11.5	96.5
	Anthesis	25.7	32.6	13.5	66.2	18.1	24.6	13.2	84.6
	Watery - Milky	21.7	34.4	0.8	50	11.9	24.7	1	98.8
	Milky - Soft dough	29.4	37.6	13.9	56.9	17.2	21.3	12.5	94.7
Okinawa (Exp. 2)	Tillering (inoculation)	20.2	27.1	12.8	81.7	16.2	19.9	12.8	92.7
	Heading	24.1	32.3	12.5	81	17.6	22.4	11.8	97.1
	Anthesis	27.3	35.4	18.8	81.1	19.6	23.2	16.3	98.1
	Watery - Milky	26.8	37.9	13	70.6	17	23	13	92.3
	Milky - Soft dough	20	34	1.9	51.6	8.5	17.9	1.1	81.9

Table 3-2. Lengths of assembled sequences for strain 008.

Chromosome	Length (bp)
chr1	6,415,455
chr2	7,886,897
chr3	8,204,532
chr4	5,387,683
chr5	4,427,654
chr6	6,080,059
chr7	4,013,678
scaf1	933,767
scaf2	711,306
scaf3	99,578
scaf4	43,290
scaf5	52,600

Note: strain was sequenced using NextSeq 500 Mid Output Kit v2.5 (2 x 150 bp run). The highly aggressive MoT strain B-71 was used as the reference genome.

Chapter 4 - Prospects and Conclusions

Novel sources of genetic resistance to wheat blast

Prior to the studies outlined in this dissertation, the $2N^vS$ translocation from *Aegilops ventricosa* was the only useful known source of wheat blast resistance identified (Cruz et al. 2016b). However, this alien chromosome segment provides partial and background-dependent resistance. Additionally, our findings have shown that newly isolated strains are decreasing the resistance of some specific $2N^vS$-based genotypes and thus the imperative need to identify and deploy novel sources of wheat blast resistance.

Results from Chapters 1 and 2 highlighted the scarce availability of genetic resistance to wheat blast. Data combined from both chapters included phenotypic assessment of more than 1000 wheat genotypes, encompassing U.S. and international elite cultivars and breeding lines, landraces, and wild wheat relatives tested against several MoT isolates with different levels of aggressiveness. Experiments were carried out both at seedling and spike stages in biosafety-level 3 laboratories in the U.S., and in growth room, greenhouse, and field conditions in Bolivia and Brazil. To guarantee quality and consistency of the data collected, we performed extensive training across the different institutions and countries involved, and implemented standardized protocols (e.g., common susceptible and resistance checks, inoculation procedure, disease evaluation, etc.). Despite the large number of genotypes tested in our study, we only identified a limited number of promising genotypes, underlining the threat that wheat blast poses to wheat production in regions where it is already established and, perhaps in the future, worldwide.

In Chapter 1, we identified eight non-$2N^vS$ genotypes with moderate levels of resistance to wheat blast, four of which were derived from the CIMMYT breeding program (CM 22, CM 49, CM 52, and CM 61) and four of which were derived from the wheat wild relative *Aegilops*

tauschii (TA 10142, TA1624, TA1667, and TA10140). A critical follow up to these studies is to develop and evaluate populations from these eight genotypes to characterize the unknown resistances. Along these lines, future research will focus on crossing the resistant CIMMYT genotypes to non-2N^{v}S susceptible genotypes with different genetic backgrounds to map novel sources of resistance that differ from the 2N^{v}S translocation, as well as to validate resistance QTL and better delineate the chromosomal regions containing the resistance QTL. Complementarily, future research will develop additional populations by crossing the CIMMYT lines to moderately resistant 2N^{v}S-based cultivars to evaluate any additive effects (i.e., increase in wheat blast resistance) when combining 2N^{v}S with additional resistance genes. Given the limited resources and the large number of possible populations, priority in parent selection will be based on levels of wheat blast resistance and genetic diversity (i.e., it is not known if these four lines carry the same resistance genes). Bi-parental and backcross populations with the TA10142 *Aegilops tauschii* line (crossed with 2N^{v}S and non-2N^{v}S genotypes) were already developed by the K-State Wheat Breeding program and will also be evaluated. Spring wheat populations (CIMMYT) will be evaluated under field conditions in Bolivia, while winter wheat populations (*Aegilops tauschii*) will be evaluated under controlled environment only. Among the diverse germplasm evaluated in chapter 1 was a Nested Association Mapping (NAM) panel consisting of landraces representing a geographically diverse collection from 32 different countries (Jordan et al. 2018). Currently, additional experiments seeking genetic resistance to wheat blast are being conducted under controlled environment conditions using these landraces, and populations have also been developed using these landraces as parents. If promising NAM genotypes are identified with enhanced resistance to wheat blast, the respective populations will be evaluated. In addition, a diverse panel of wheat genotypes has been evaluated for multiple

agronomic traits (yield, quality, and diseases). In collaboration with Dr. Mohammad Asif from Heartland Plant Innovation institute, we will perform a genome-wide association mapping analyses to wheat blast (both at seedling and spike stage) using this panel.

The low frequency of resistance to wheat blast found in common wheat led us to search for sources of genetic resistance in wild wheat relatives. Fifty-one lines are currently being tested under controlled environment conditions, from which 24 are *Aegilops ventricosa* ($2N^vS$ translocation donor) and 27 are the wild grass *Aegilops uniaristata* Vis. As wild relatives of wheat have proven to be a powerful source of useful genes for multiple agronomic traits, including the major source of wheat blast resistance, we hope to find other promising sources of resistance to head blast.

In addition, we will also evaluate recently reported resistance genes and genome-edited knockout wheat lines. Dr. Yukio Tosa's group from Kobe University (Japan) recently reported two resistance genes associated with wheat blast resistance (*Rmg8* and *RmgGR119*) (Anh et al. 2015; Wang et al. 2018). However, these genes were only tested at either seedling stage or using old and less aggressive MoT isolates. In collaboration with Dr. Tosa, we are currently evaluating wheat genotypes IL191 (*Rmg8)* and GR119 (*Rmg8* + *RmgGR119*) at the spike stage using aggressive and recently collected MoT isolates. Finally, in collaboration with Dr. Brian Staskawizc from University of California, we will determine if a CRISPR-Cas9 genome editing strategy of knocking out putative wheat susceptibility genes (i.e., wheat orthologs of the rice *Pi21* and *Bsrk-1* genes) will confer resistance to wheat blast at seedling and spike stages using aggressive MoT isolates.

From all the experiments above, we hope to (i) identify novel genomic regions associated with wheat blast both at seedling and spike stages, (ii) determine any additive effect when

pyramiding 2N^{v}S translocation to additional resistance genes, (iii) develop molecular markers tightly linked to resistance genes/QTL to facilitate gene introgression into adapted cultivars, and (iv) identify new resistant genotypes that could be used for future population development. The main goal is to deliver an effective and broad-spectrum wheat blast resistance to wheat breeding programs and ultimately to wheat producers.

MoT aggressiveness evolution and host specificity

Wheat blast was first reported in Brazil in 1985, and since then, there has been a significant increase in the aggressiveness of MoT isolates. A winter wheat set of differentials [Jagalene (2N^{v}S-based) plus RonL and Everest (non 2N^{v}S-based)] have been used to characterize the aggressiveness of MoT isolates (Cruz et al. 2012; 2016). However, due to the recent findings in terms of both pathogen diversity and reported blast resistance genes (under evaluation), we proposed the establishment of a new set of differentials using both spring and winter wheat genotypes described in detail in chapter 1 (Cruppe et al. 2019). Phenotypic and genomic characterization of MoT pathogen diversity are critical factors in the selection of isolates for resistance screening assays and breeding efforts, as well as for studies on pathogen evolution. We plan to use a diverse collection of MoT isolates sampled from 1985 to present from all blast-affected regions, including South Asia, to conduct assays at both seedling and spike stages using the new proposed set of differentials. To complement the disease severity assessment, we will evaluate pathogen aggressiveness traits such as latent period, and lesion number and size; all of which are critical information still lacking for MoT epidemiology.

The fungus *Magnaporthe oryzae* is divided into host-specialized lineages that together cause disease in more than 50 grass species (Zhang et al. 2016; Gladieux et al. 2018). Highly aggressive lineages that cause recurring serious disease epidemics on major cereal and turf crops

are referred to as pathotypes, i.e., the *Triticum* pathotype and the *Lolium* pathotype (Bain et al. 1972; Carver et al. 1972; Viji et al. 2001; Tosa et al. 2004; Wang et al. 2009; Marangoni et al. 2013; Cruz and Valent, 2017; Gladieux et al. 2018; Valent et al. 2019). The host specificity of each pathotype is determined, in large part, by the presence of *AVR* genes, meaning that the loss of an *AVR* gene can allow a pathotype to cause disease in a host that was not susceptible before (Chuma et al. 2011; Wang et al. 2017; Peng et al. 2019). The closely related *Lolium* (MoL) pathotype is already established in the U.S. and is a major concern for forage production (Bain et al. 1972; Carver et al. 1972). Primary hosts for the *Lolium* pathotype include perennial and annual ryegrasses and tall fescue. However, our experiments (not included in this dissertation) combined with studies conducted at The Ohio State University by Dr. Pierce Paul's group and at the USDA-ARS by Gary Peterson's group demonstrated that MoL isolates can cause disease on wheat, but at lower levels when compared to MoT isolates. The presence of the MoL pathotype in the U.S. coupled with its high genetic similarity with the MoT pathotype and its capability of causing disease in wheat, represent a high risk of wheat blast establishment in the U.S. through a host jump from MoL isolates (the event that most probably led to the emergence of wheat blast in Brazil, Inoue et a. 2017).

Critical upcoming experiments will include determining the aggressiveness of MoL isolates on wheat, using a large panel of isolates and a set of wheat differentials (discussed in chapter 1) plus additional U.S. wheat cultivars, primarily focused on those grown in high-risk areas with disease conducive environments such as Louisiana. Another critical experiment would involve determining the MoT host range, including the primary MoL hosts (e.g., perennial and annual ryegrass and tall fescue) and other crops such as oats, barley, and cereal rye. The characterization of MoT host range is critical in the case of an exotic introduction of MoT from

South America or South Asia, because it indicates possible alternative hosts other than wheat that could facilitate pathogen establishment and spread in the U.S. Finally, in collaboration with Dr. Boyd Padgett from Louisiana State University, we will screen wheat, forage pastures, and weeds grown nearby wheat fields for blast symptoms when conditions are conducive for blast development. We will collect *M. oryzae* isolates from these crops and use them in the experiments for pathogen characterization and host specificity described above. According to previously reported epidemiological studies, the state of Louisiana has the most conducive climate conditions for a potential wheat blast introduction and establishment in the U.S. (Cruz et al. 2016a). Therefore, characterizing the cropping systems and their vulnerabilities in the state is critical.

Sources of inoculum

A few possible sources of inoculum could contribute to wheat blast outbreaks, some of which originate within the field and others outside the field. Within the field, potential sources of inoculum include MoT infected seeds and infected crop residue, which could lead to leaf blast infection at early growth stages (Goulart and Paiva, 1990; Goulart et al. 1995). Alternative hosts such as other grasses and weeds grown near wheat fields could provide outside inoculum. The importance of each source of inoculum is still the subject of debate.

In Chapter 3, we aimed to determine the role of both leaf infection and outside inoculum coming from other grasses and weeds. For leaf infection, we inoculated our plots at the tillering stage (Feekes 2) and we provided the optimal conditions for disease development through irrigation throughout the remaining season. For the outside inoculum, we planned to isolate the fungus from potential alternative hosts and, through whole genome sequencing, compare these isolates to the MoT population present in our plots. Unfortunately, due to the non-conducive

weather conditions, no natural wheat blast infection occurred during the period when these experiments were conducted, thus no outside isolates were sampled. However, due to daily supplemental irrigation combined to artificial inoculation, wheat blast developed well in our plots. Phenotypic and genomic analyses demonstrated that the strain we inoculated at the seedling stage caused extensive head blast in both inoculated and neighboring non-inoculated plots. This shows that leaf infection can be a major source of inoculum for disease outbreaks in a susceptible host population when weather conditions are conducive. Interestingly, while limited genetic diversity was observed in the core chromosomes within the isolates sampled from infected spikes in our plots, greater genetic diversity was found in the supernumerary mini-chromosomes in these strains. This result is consistent with recent characterization of highly variable mini-chromosomes in MoT and other *M. oryzae* strains, and our working hypothesis that mini-chromosomes contribute to enhanced genetic diversity in *M. oryzae* (Peng et al, 2019). The role of mini-chromosomes in wheat blast virulence and evolution needs further investigation in field studies as well as in the laboratory.

As previously mentioned, MoT infected seeds could also play an important role in disease outbreaks (Kiyuna, M.S. thesis 2019, Kansas State University). It is hypothesized that the 2016 wheat blast outbreak in Bangladesh occurred due infected grain imported for food from South America. Kiyuna et al. (M.S. thesis, 2019, Kansas State University) investigated the detection threshold in MoT infected seeds using quantitative and conventional PCR. The second goal of their study was to develop a preliminary risk assessment to determine the probability of disseminating MoT infected seeds. Combining the results from chapter 3 with findings from the Kiyuna thesis, we plan to determine the MoT transmission rate from infected seeds to seedlings. Preliminary experiments will be performed under controlled environment conditions to define

the treatments including, but not limited to (i) MoT concentration on the seeds; (ii) protocol for seed inoculation; (iii) MoT infected seeds incubation period, among others. After the establishment of the optimal treatments, we will perform experiments under field conditions using genotypes with different levels of resistance. MoT infected seeds can be asymptomatic, which aggravates the risk of wheat spread into new regions.

Conclusions

Wheat blast has proven hard to control where the disease is established. The lack of genetic resistance, the limited efficacy of chemical and cultural control, the pathogen's mixed reproduction system (high evolutionary potential), and pathogen diversity are just a few examples of why wheat blast is a major concern in regions where the disease is present. Furthermore, the vast host range, the genetic similarity between some pathotypes, and the air and seed-borne characteristics of *Magnaporthe oryzae* underscore the high risk of wheat blast introduction and establishment into new regions, including the U.S. An integrated disease management strategy must be adopted in blast-affected regions. This system should include crop rotation with non-hosts, rotation of active ingredients used in fungicide seed treatment and foliar fungicide applications, removal of infected crop residue, and adjustment of planting date. The strategies mentioned above must be combined with cultivars with moderate levels of resistance. Non-affected areas must avoid exotic introduction of the pathogen by implementing an efficient protocol for MoT detection, especially on asymptomatic seeds and under low levels of disease incidence and severity. However, non-affected areas such as the U.S. are still at risk of an introduction through a host jump from pathotypes already established in the country (*Lolium* pathotype). Thus, a preparedness and recovery plan must be in place, including surveillance in high-risk areas (e.g., Louisiana and Mississippi) during blast conducive years, training of

extension specialists for proper disease identification, and rapid detection and diagnosis protocols.

References

Anh, V. L., Anh, N. T., Tagle, A. G., Vy, T. T. P., Inoue, Y., Takumi, S., Chuma, I., and Tosa, Y. 2015. *Rmg8* , a new gene for resistance to *Triticum* isolates of *Pyricularia oryzae* in hexaploid wheat. Phytopathology. 105:1568–1572.

Bain, D., Patel, B., Patel, M. 1972. Blast of ryegrass in Mississippi. Plant Disease Reporter. 56:210.

Carver, R., Rush, M., and Lindberg, D. 1972. An epiphytotic of ryegrass blast in Louisiana. Plant Dis. Reporter. 56:157–159.

Cruppe, G., Cruz, C.D., Peterson, G., Pedley, K., Asif, M., Fritz, A., Calderon, L., Lemes da Silva, C., Todd, T., Kuhnem, P. and Singh, P.K., Singh, R.P., Braun, H., Barma, N.C.D., Valent, B. 2019. Novel Sources of Wheat spike blast Resistance in Modern Breeding Lines and Wheat Wild Relatives. Plant Dis. PDIS-05.

Cruz, C. D., Bockus, W. W., Stack, J. P., Tang, X., Valent, B., Pedley, K. F., and Peterson, G. L. 2012. Preliminary assessment of resistance among U.S. wheat cultivars to the *Triticum* pathotype of *Magnaporthe oryzae*. Plant Dis. 96:1501–1505.

Cruz, C. D., Magarey, R. D., Christie, D. N., Fowler, G. A., Fernandes, J. M., Bockus, W. W., Valent, B., Stack, J. P. 2016a. Climate suitability for *Magnaporthe oryzae Triticum* pathotype in the United States. Plant Dis. 100:1979–1987.

Cruz, C. D., Peterson, G. L., Bockus, W. W., Kankanala, P., Dubcovsky, J., Jordan, K. W., Akhunov, E., Chumley, F., Baldelomar, F. D., and Valent, B. 2016b. The 2NS

translocation from *Aegilops ventricosa* confers resistance to the *Triticum* pathotype of *Magnaporthe oryzae*. Crop Sci. 56:990–1000.

Chuma, I., Isobe, C., Hotta, Y., Ibaragi, K., Futamata, N., Kusaba, M., Yoshida, K., Terauchi, R., Fujita, Y., Nakayashiki, H., Valent, B., and Tosa, Y. 2011. Multiple translocation of the *AVR-Pita* effector gene among chromosomes of the rice blast fungus *Magnaporthe oryzae* and related species. PLoS Pathogens. 7:e1002147.

Gladieux, P., Condon, B., Ravel, S., Soanes, D., Maciel, J.L.N., Nhani, A., Chen, L., Terauchi, R., Lebrun, M.H., Tharreau, D. and Mitchell, T., Pedley, K.F., Valent, B., Talbot, N.J., Farman, M., and Fournier, E. 2018. Gene flow between divergent cereal-and grass-specific lineages of the rice blast fungus *Magnaporthe oryzae*. MBio. 9:01219–17.

Goulart, A. C. P., and Paiva, F. A. 1990. Transmission of *Pyricularia oryzae* by wheat *Triticum aestivum* seeds. Fitopatol. Bras. 15:359–362.

Goulart, A. C. P., Paiva, F. A., and Andrade, P. J. M. 1995. Relação entre a incidência da brusone em espigas de trigo e a presença de *Pyricularia grisea* nas sementes colhidas. Fitopatol. Bras. 20:184–189.

Inoue, Y., Vy, T.T., Yoshida, K., Asano, H., Mitsuoka, C., Asuke, S., Anh, V.L., Cumagun, C.J., Chuma, I., Terauchi, R. and Kato, K., 2017. Evolution of the wheat blast fungus through functional losses in a host specificity determinant. Science. 357:80–83.

Jordan, K. W., Wang, S., He, F., Chao, S., Lun, Y., Paux, E., Sourdille, P., Sherman, J., Akhunova, A., Blake, N. K., Pumphrey, M. O., Glover, K., Dubcovsky, J., Talbert, L., and Akhunov, E. D. 2018. The genetic architecture of genome-wide recombination rate variation in allopolyploid wheat revealed by nested association mapping. The Plant Journal 95:1039–1054.

Kiyuna J. 2019. Detection of wheat blast within and on wheat seeds and preliminary risk evaluation tool for the detection of MoT infected seeds. M.S. Kansas State University, Manhattan, Kansas.

Marangoni, M. S., Nunes, M. P., Fonseca, N. Jr, and Mehta, Y. R. 2013. *Pyricularia blast* on white oats: a new threat to wheat cultivation. Trop. Plant. Pathol. 38:198–202.

Peng, Z., Oliveira-Garcia, E., Lin, G., Hu, Y., Dalby, M., Migeon, P., Tang, H., Farman, M., Cook, D., White, F. F., Valent, B., and Liu, S. 2019. Effector gene reshuffling involves dispensable minichromosomes in the wheat blast fungus. PLOS Genetics 15:e1008272.

Tosa, Y., Hirata, K., Tamba, H., Nakagawa, S., Chuma, I., Isobe, C., Osue, J., Urashima, A. S. , Don, L. D., Kusaba, M., Nakayashiki, H., Tanaka, A., Tani, T., Mori, N., and Mayama, S. 2004. Genetic constitution and pathogenicity of Lolium isolates of *Magnaporthe oryzae* in comparison with host species-specific pathotypes of the blast fungus. Phytopathology. 94:454–462.

Urashima, A. S., Alves, A. F., Silva, F. N., Oliveira, D., and Gazaffi, R. 2017. Host range, mating type and population structure of *Magnaporthe* sp. of a single barley field in São Paulo state, Brazil. J. of Phytopathology. 165:414–424.

Valent, B., Farman, M., Tosa, Y., Begerow, D., Fournier, E., Gladieux, P., Islam, M.T., Kamoun, S., Kemler, M., Kohn, L.M. and Lebrun, M.H., Stajich, J.E., Talbot, N.J., Terauchi, R., Tharreau, D., and Zhang, N. 2019. # *Pyricularia graminis-tritici*# is not the correct species name for the wheat blast fungus: Response to Ceresini et# al#(MPP 20: 2). Mol. Plant Pathol. 20:173–179.

Viji, G., Wu, B., Kang, S., Uddin, W., Huff, D. R. 2001. *Pyricularia grisea* causing gray leaf spot of perennial ryegrass turf: population structure and host specificity. Plant Dis. 85:817–826.

Wang, S., Asuke, S., Vy, T. T. P., Inoue, Y., Chuma, I., Win, J., Kato, K., and Tosa, Y. 2018. A new resistance gene in combination with *Rmg8* confers strong resistance against *Triticum* isolates of *Pyricularia oryzae* in a common wheat landrace. Phytopathology. 108:1299–1306.

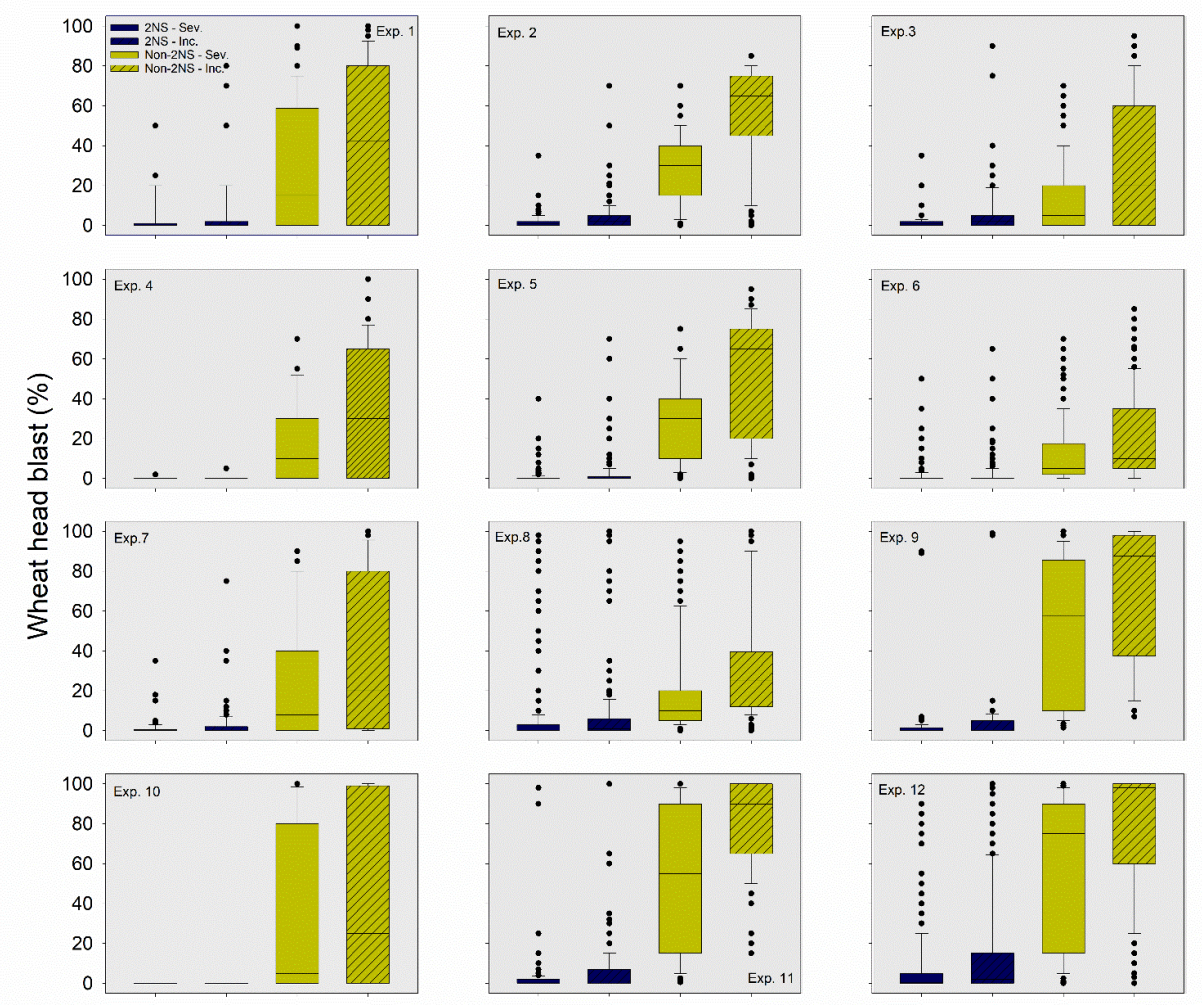
Wang, G-L. and Valent, B. (ed). 2009. Advances in genetics, genomics and control of rice blast disease. Springer Science and Business Media, New York, New York.

Wang, B. H., Ebbole, D. J., Wang, Z. H. 2017. The arms race between *Magnaporthe oryzae* and rice: Diversity and interaction of Avr and R genes. J. of Integ. Ag. 16:2746–2760.

Zhang, N., Luo, J., Rossman, A. Y., Aoki, T., Chuma, I., Crous, P. W., Dean, R., de Vries, R. P., Donofrio, N., Hyde, K. D., Lebrun, M-H., Talbot, N. J., Tharreau, D., Tosa, Y., Valent, B., Wang, Z., and Xu, J-R. 2016. Generic names in *Magnaporthales*. IMA Fungus. 7:155–159.

Appendix A - Supplementary Figures and Tables from Chapter 1

Appendix A. Tables and Figures



Supplementary Figure A1. Wheat blast disease severity of 2N^{v}S (blue bars) and non-2N^{v}S (yellow bars) groups for the 12 field experiments evaluated at soft dough stage of grain development. Medians are indicated by the horizontal lines.

Supplementary Table A1. Analysis of variance of field experiments on wheat spike blast severity.

Source of variation	Wheat spike blast severity
Environment[a]	NS
Experiment (environment)	NS
Incomplete block (experiment x environment)	***
Experiment x line ($2N^VS$)	***
Growth stage x experiment x line ($2N^VS$)	***
Residual	***

[a] combination of year and location

Supplementary Table A2. Mean disease severity (sev.) and incidence (inc.) of susceptible and resistant checks sown under field conditions.

Experiment	Susceptible checks					Resistant checks				
	n^y	Sev. (%)	Std Dev	Inc. (%)	Std Dev	*n*	Sev. (%)	Std Dev	Inc. (%)	Std Dev
1	26	40.7	24.8	63.9	25.3	24	2.3	10.2	3.7	16.3
2	91	33.3	14.7	65.2	17.2	88	0.5	0.8	1	1.6
3	73	40.5	18.7	79.3	11.5	73	4.3	6	26.7	25.7
4	25	25.7	17.7	51	24	24	0.2	1	1.5	6.5
5	91	37.9	16.3	70.2	18.7	89	0.7	4.3	1.4	7.7
6	145	-	-	-	-	146	0.1	0.3	0.2	0.7
7	49	17.3	4.9	9.7	10.7	49	0.4	0.8	0.9	1.6
8	109	7.6a	4.4	17.3	8.9	109	0.7	1.4	1.6	3.8
9	49	55.3	35.7	68.9	33.6	48	0.9	1	2.5	2.7
10	13	46.1	43.9	57.4	45.2	12	0	0	0	0
11	109	41.9	31.7	75.7	20.7	108	0.6	1.1	1.4	2.8
12	109	51.6	32.2	75.8	25.4	108	0.8	4.8	1.8	7.9

[Y] number of checks in each experiment

Supplementary Table A3. Wheat spike blast disease severity of 93 CIMMYT breeding lines, plus the susceptible checks Cavalier, Everest, and Glenn. Experiments were conducted at ARS-FDWSRU, US. Pedigree information for each line included.

Material	*n*	Severity (%)	St dev	Cross
CM59	7	0.00	0.00	MILAN/MUNIA
CM61	4	0.00	0.00	SUPER 152
CM86	10	0.00	0.00	SUP152/3/INQALAB 91*2/TUKURU//WHEAR
CM91	11	0.00	0.00	ATTILA*2/PBW65*2//KACHU/3/TRCH/HUIRIVIS #1
CM94	9	0.00	0.00	BORL14//KFA/2*KACHU
CM100	10	0.00	0.00	KSW/SAUAL//SAUAL/3/BORL14
CM8	9	0.78	2.33	MUTUS//KIRITATI/2*TRCH/3/WHEAR/KRONSTAD F2004
CM87	10	1.00	3.16	SUP152/3/TRCH/SRTU//KACHU
CM93	6	6.17	15.11	BORL14//KFA/2*KACHU
CM98	16	6.25	25.00	KSW/SAUAL//SAUAL/3/BORL14
CM22	10	6.50	14.15	QUAIU #1
CM58	7	6.71	13.86	VOROBEY
CM14	8	8.25	23.33	KIRITATI//HUW234+LR34/PRINIA/3/FRANCOLIN #1/4/BAJ #1
CM54	3	8.33	14.43	BORLAUG100 F2014
CM90	8	9.00	17.37	KFA/2*KACHU/3/ATTILA*2/PBW65//MURGA
CM97	8	11.00	17.41	KSW/SAUAL//SAUAL/3/BORL14
CM48	9	11.11	12.04	BAW-1260
CM13	9	11.22	22.40	KENYA SUNBIRD/2*KACHU
CM60	5	12.80	19.47	FRANCOLIN #1
CM63	4	13.75	16.01	TOB/ERA//TOB/CNO67/3/PLO/4/VEE#5/5/KAUZ/6/FRET2/7/VORB/8/MILAN/KAUZ//DHARWAR
CM15	9	16.00	20.92	DRY/3/BAV92
CM95	10	17.30	24.38	BORL14//KFA/2*KACHU
CM36	8	17.45	17.02	BARI GOM 30
CM76	10	18.50	26.78	SWSR22T.B./2*BLOUK #1//WBLL1*2/KURUKU
CM43	6	18.83	20.30	BAW-1208
CM65	6	20.33	20.56	BECARD/KACHU

Supplementary Table A3. Continued

CM96	10	20.80	41.82	KACHU/KIRITATI//BORL14
CM89	10	21.30	22.45	KSW/SAUAL//SAUAL/3/BORL14
CM25	6	25.00	38.86	FRANCOLIN #1
CM52	4	25.00	50.00	TEPOCA T 89
CM56	3	25.33	35.57	SUP152/BAJ #1
CM17	6	26.50	32.03	FRET2*2/BRAMBLING//KIRITATI/2*TRCH/3/FRET2/TUKURU//FRET2
CM82	8	29.75	44.41	BAJ #1/3/TRCH/SRTU//KACHU
CM49	8	32.00	45.27	SOKOLL/ROLF07
CM74	10	32.20	39.55	SUP152/FRNCLN
CM2	10	34.90	45.66	KIRITATI//HUW234+LR34/PRINIA/3/BAJ #1
CM45	3	38.00	54.15	BAW-1219
CM27	14	39.00	44.07	DANPHE/PAURAQUE #1//MUNAL #1
CM84	10	40.30	50.01	BAJ #1/5/ATTILA/3*BCN//BAV92/3/TILHI/4/SHA7/VEE#5//ARIV92
CM88	8	40.63	40.31	ATTILA*2/PBW65//KACHU/3/UP2338*2/KKTS*2//YANAC
CM21	12	51.67	40.14	ND643/2*WBLL1//VILLA JUAREZ F2009
CM57	7	52.00	46.01	ATTILA
CM81	9	55.44	49.57	FRANCOLIN #1/BAJ #1
CM77	10	55.80	48.79	FRANCOLIN #1*2//ND643/2*WBLL1
CM9	9	56.11	48.85	BAJ #1*2/TINKIO #1
CM3	4	56.25	51.54	FRANCOLIN #1*2//ND643/2*WBLL1
CM6	10	56.70	47.42	PAURAQ/4/WHEAR/KUKUNA/3/C80.1/3*BATAVIA//2*WBLL1/5/PAURAQUE #1
CM41	12	57.17	45.00	BAW-1202
CM75	10	57.60	48.62	FRNCLN/3/ND643//2*PRL/2*PASTOR/4/FRANCOLIN #1
CM68	10	57.90	45.61	NAC/TH.AC//3*PVN/3/MIRLO/BUC/4/2*PASTOR/5/KACHU/6/KACHU
CM20	13	59.15	40.08	BAVIS/3/ATTILA/BAV92//PASTOR/5/CROC_1/AE.SQUARROSA (205)//BORL95/3/PRL/SARA//TSI/VEE#5/4/FRET2
CM24	10	59.80	37.80	TAM200/PASTOR//TOBA97/3/FRNCLN/4/WHEAR//2*PRL/2*PASTOR
CM85	10	61.00	44.75	BAJ #1/3/TRCH/SRTU//KACHU

Supplementary Table A4. Wheat spike blast severity (%) of 64 *Ae. tauschii accessions*, plus the susceptible check Cavalier evaluated 14 days after inoculation under controlled conditions.

Genotype	N of spikes	Wheat spike blast severity (%)	Std Dev
TA1584	1	33	0
TA2575	12	33.3	24.1
TA1644	11	36.4	50.5
TA1642	12	41.7	51.5
TA2378	27	42.1	43.8
TA10077	7	43.7	32
TA1707	29	50.6	36.8
TA2398	8	52	51.6
TA10124	12	54.4	32.3
TA2536	34	56.8	39.6
TA10179	20	58.7	35.1
TA1596	20	59.7	39.2
TA1613	8	62.6	37.2
TA1651	29	64.6	29.1
TA10174	7	64.7	40.3
TA1668	20	66.1	35.5
TA10172	7	68.1	43.1
TA2474	41	69.7	32.8
TA10212	28	70.1	31.1
TA2530	16	70.7	36.9
TA10155	5	71.2	27.9
TA1673	21	74	38.5
TA1578	5	76	40.2
TA2468	27	76.7	23.6
TA1631	6	79	24.2
TA1659	5	80	44.7
TA10185	3	80.7	33.5
TA10108	7	82.7	25.7
TA10106	12	82.8	33.3
TA2538	5	84	35.8
TA10330	24	84.8	30.3
TA2387	7	86	24.7
TA10162	12	86.5	25.9
TA2374	17	87.5	24.3
TA2420	8	87.5	35.4
TA2545	27	88.2	16.1
TA2395	38	89.1	31.4
TA1655	7	89.3	28.3
TA10141	13	89.8	18.8
TA1665	10	90	12.7

Supplementary Table A4. Continued

TA1680	4	90.5	19
TA1717	6	91.7	20.4
TA1605	27	92.2	13
TA2448	17	92.6	7.3
TA10144	15	98.2	3.7
TA2413	7	99.3	1.9
TA10210	10	99.3	1.6
TA2435	16	99.5	0.9
TA2376	10	99.5	1.6
TA2514	10	99.5	1.6
TA10320	11	99.5	1.5
TA1669	10	99.8	0.6
TA2586	10	99.8	0.6
Cavalier (S check)	7	100	0
TA10099	10	100	0
TA1666	7	100	0
TA2381	7	100	0
TA2388	11	100	0
TA2401	1	100	0
TA2422	2	100	0
TA2431	29	100	0
TA2433	11	100	0
TA2485	1	100	0
TA2488	10	100	0
TA2508	9	100	0

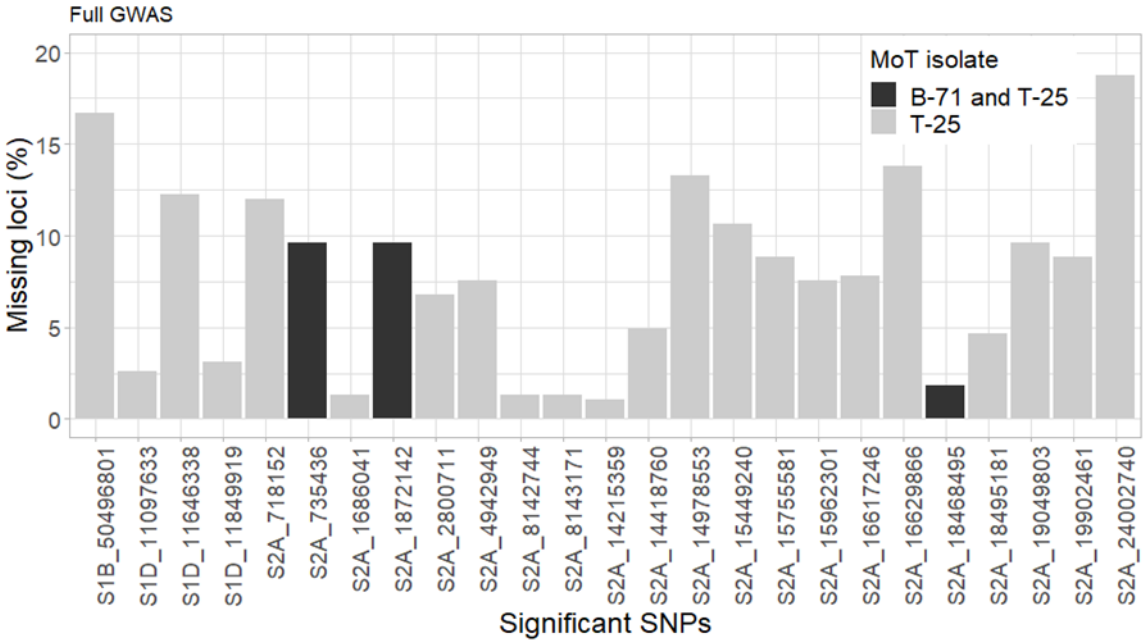
Supplementary Table A5. Potential candidates accessions that could be included in the new set of wheat differentials for MoT race designation.

Accession	Alternatives[x]	Resistant group[y]	Resistance source	Data source
Bob White	Atlax, Everest	S	None	Cruppe et al. (this report); Cruz et al. 2016
Urubo	AN-120	R - MR	$2N^{v}S$	Cruppe et al. (this report); Cruz et al. 2016
Sossego	Jagalene, Sintonia	MR - I	$2N^{v}S$	Cruppe et al. (this report); Cruz et al. 2016
St24	-	Unknown	*Rmg7*	Tagle et al. 2015
S-615	-	Unknown	*Rmg8*	Anh et al. 2015
GR119	-	Unknown	*RmgGR119* + *Rmg8*	Wang et al. 2018
CM22	-	R - MR	Unknown[z]	Cruppe et al. (this report)
CM49	-	R - MR	Unknown	Cruppe et al. (this report)
CM52	-	MR -I	Unknown	Cruppe et al. (this report)
CM61	-	MR -I	Unknown	Cruppe et al. (this report)

[x] alternative wheat accessions with similar reaction
[y] based on inoculations with highly aggressive MoT isolates (B-71 and/or 008)
[z] it is unknown if the CIMMYT genotypes carry the same resistance genes

Appendix B - Supplementary Figures and Tables from Chapter 2

Appendix B. Figures and Tables



Supplementary Figure B1. Percentage of missing loci for the significant SNPs identified in the full GWAS with MoT isolates T-25 and the SNPs with the highest *p* values with isolate B-71**.**

Supplementary Table B1. Pairwise comparison of significant SNPs identified in the full GWAS with MoT isolate T-25.

QTL 1	QTL 2	Difference (%)	*p* value	Significance[a]
1B	1B + 1D	5.04	2.6E-01	ns
1B	1B + 1D + 2A	22.52	4.1E-02	*
1B	1B + 2A	29.07	6.7E-04	***
1B	1D	7.2	7.1E-02	ns
1B	1D + 2A	21.97	1.3E-02	*
1B	2A	25.84	6.9E-05	****
1B	None	-15.46	5.3E-04	***
1D	1D + 2A	14.77	5.1E-02	ns
1D	2A	18.64	1.7E-03	**
1D	None	-22.66	1.8E-07	****
2A	None	-41.3	6.2E-09	****
1B + 1D	1B + 1D + 2A	17.48	1.2E-01	ns
1B + 1D	1B + 2A	24.03	7.7E-03	**
1B + 1D	1D	2.16	7.1E-01	ns
1B + 1D	1D + 2A	16.93	6.3E-02	ns
1B + 1D	2A	20.8	3.6E-03	**
1B + 1D	None	-20.5	1.1E-04	***
1B + 2A	1D	-21.87	4.3E-03	**
1B + 2A	1D + 2A	-7.1	5.6E-01	ns
1B + 2A	2A	-3.23	6.6E-01	ns
1B + 2A	None	-44.53	2.3E-06	****
1D + 2A	2A	3.87	8.1E-01	ns
1D + 2A	None	-37.43	8.6E-05	****
1B + 1D + 2A	1B + 2A	6.55	7.8E-01	ns
1B + 1D + 2A	1D	-15.32	1.0E-01	ns
1B + 1D + 2A	1D + 2A	-0.55	9.0E-01	ns
1B + 1D + 2A	2A	3.32	9.9E-01	ns
1B + 1D + 2A	None	-37.98	1.9E-03	**

[a] ns non-significant, * significant at $p<0.05$, ** $p<0.01$, *** $p<0.001$, and **** $p<0.0001$