

Limits of detection of *Magnaporthe oryzae* Triticum pathotype in wheat seed: implications for pathogen dissemination

by

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Abstract

Wheat blast is an emerging disease on wheat, caused by the seedborne fungal pathogen *Magnaporthe oryzae* Triticum pathotype (MoT). The first objective of this research was to establish the detection threshold for MoT in infected wheat seeds as a function of sensitivity and specificity of a modified quantitative and conventional PCR assay. Accurate amount mix ratios of MoT colonized ground seeds / non-MoT colonized ground seeds were established with different MoT severity and incidence gradient ratios. Disease severity and incidence gradients were verified by end-point PCR and q-PCR. To further verify sensitivity and specificity in the presence of potentially interfering DNAs, gradients based on ten-fold serial dilutions of MoT DNA were established with genomic *M. oryzae* populations DNA and wheat seed DNA. The detection threshold from the end-point PCR was 0.1% of MoT colonized seeds in a seed sample as a function of disease incidence. The detection threshold varied as a function of disease severity for MoT-colonized seeds; the minimum MoT detectable by end-point PCR was 3×10^3 MoT cells/seed. This study verified the specificity to discriminate the Triticum pathotype from other *M. oryzae* pathotypes as well as demonstrated no interference from wheat seed DNA. This study provides evidence that to establish pathogen detection thresholds in plant seed, both disease incidence and severity must be evaluated to reduce the probabilities of false negatives.

The second objective was to develop a preliminary risk assessment tool as a guide to minimize the probability of disseminating MoT-infected seeds. A field survey of commercial wheat production farms in Bolivia was conducted during a MoT epidemic year. The effects of planting dates and cultivar susceptibility on disease severity were determined. The preliminary risk assessment tool was based on weather conditions during an epidemic and non-epidemic year, previously published studies on MoT infection of wheat seeds during epidemics, detection

thresholds by Blotter seed test and PCR tests for infected seed detection and practical field implications. Planting date and cultivar susceptibility significantly ($P = <0.0001$) affected disease incidence and severity. Late planting of a resistant wheat genotype resulted in ~90% less disease in the field with and consequently higher yield and seed quality. The combination of high rainfall accumulation (67.7 mm) and prolonged high relative humidity (>80% for 18 hrs.) during the heading stage was associated with epidemic blast development. A previously published study provided strong evidence of a positive correlation between disease incidence in the field and infection of wheat seeds. Given a uniform infected seeds distribution and fully efficient sampling method, the PCR detection threshold of 0.1% of MoT infected seeds in a seed sample represents up to 20 kg from a 20 metric tonnes harvest wagon will go undetected, equivalent to 6.66×10^5 infected seeds. The calculated threshold for the Blotter test was equivalent to 1600 kg from a 20 metric tonnes or 5.3×10^7 infected seeds will go undetected. Therefore, it is crucial to complement seed inspections with field assessments to decrease the likelihood of MoT dissemination through infected seeds to non-MoT established areas.

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Dedication

To all persons that believed in me.

Chapter 1 - Literature review

Importance of the host: wheat

Food security is one of the most important challenges for this century. The population is increasing considerably decade by decade and by 2050, it is calculated to be around 10 billion (~ 37% increases in population) (Tilman et al. 2011). On the other hand, increase land availability will be harder from now on. Therefore, it is imperative to protect our current food production and be more productive on less land. Given this scenario, wheat plays a critical role in this challenge. It is one of the most important crops and essential for food security globally. Since 1961, it is the most widely grown crop in the world and by 2014 wheat production increased by over 200% (FAO 2014). Wheat production was over 700 million tons globally, with China, India, USA, Russia, and France the top 5 producers in the world (FAO 2014). Economically, wheat represents around 46.8 billion US\$ in export value worldwide with the US as the top exporter, followed by France, Australia, Canada and Russia (FAO 2014). Wheat is also considered one of the most important components in human nutrition, providing almost 20% of the calories consumed and 17% of the protein required in the human diet (FAO 2014). Therefore, wheat constitutes a crucial crop economically and socially worldwide.

Pathogen: Wheat Blast

Wheat Blast is a disease caused by the fungal pathogen *Magnaporthe oryzae* Triticum pathotype (MoT) (synonymous with *Pyricularia oryzae*) (Couch et al. 2005). *Magnaporthe oryzae* is divided into distinct sub-specific groups based on host preference; e.g., Triticum pathotype (wheat), *Oryza* pathotype (rice) and the *Lolium* pathotype (ryegrass) (Couch et al. 2005; Farman 2002; Heath et al. 1990; Tosa et al. 2004). However, *Magnaporthe oryzae*

strains have the ability to infect non-primary hosts usually causing less disease (Farman 2002; Kohli et al. 2011; Tosa et al. 2004). It is an aggressive pathogen under conducive conditions of high temperature (25-30 °C), high humidity (>80% RH) and prolonged leaf wetness (Cruz and Valent 2017; Goulart et al. 2007; Cardoso et al. 2008). Inefficient control practices can lead to 100% losses in susceptible cultivars (Cardoso et al. 2008; Cruz and Valent 2017; Goulart et al. 2007; Urashima et al. 2009; Andersen et al. 1947). Wheat blast epidemics vary considerably based on the combination of weather conditions, cultivar susceptibility and the time of infection relative to the grain development stage (Cruz and Valent 2017; Goulart et al. 2007). Observations on the epidemiology of wheat blast indicate epidemics may initiate from hotspots within the field at an early stage; conidia from senescent leaves in the lower canopy on some wheat cultivars may be an important factor for initial epidemic development (Cruz et al. 2015; Rios et al. 2016). Wheat blast symptoms occur on all above-ground parts of the plant, each capable of sporulation. The symptoms can appear on leaves and less commonly on the stem, showing elliptical lesions with a grey center during sporulation (Igarashi et al. 1986; Cruz and Valent 2017).

Depending on the time of infection at the grain development stage, it could affect considerably the quality of grain and seeds, making them shriveled, small and low test-weight (Goulart and Paiva 1992, 2000; Urashima et al. 2009). The most characteristic and devastating symptom on the crop is the blasted spike, which could be partial or total depending on the point of infection on the spike (Cruz and Valent 2017). Once the pathogen infects the spike, the disease develops from the point of infection and above, blocking the passage of nutrients required for grain filling (Cruz and Valent 2017). There is a direct relationship between the incidence and severity of MoT in a field and MoT incidence in seeds or gains collected from that field (Goulart et al., 1995), suggesting a high probability for

pathogen spread and introduction to new areas by seedborne. Since its first report in 1985 at Parana, Brazil (Igarashi et al. 1986; Nunes Maciel 2011), wheat blast spread to neighboring countries, Bolivia in 1996 (Toledo and Barea 1996), Paraguay in 2002 (Viedma 2005) and northeastern Argentina in 2007 (Cabrera and Gutierrez 2007). However, wheat blast was reported outside of South America for the first time in 2016, in Bangladesh, (Callaway 2016; Malaker et al. 2016).

Wheat is one of the most important crops in Bangladesh. Increasing demand for local consumption and level domestic wheat production (including poor wheat production during crop seasons during this period), required Bangladesh in the need to import wheat from other countries (Sadat and Choi 2017). For farmers in Bangladesh, wheat seed availability for planting was insufficient, resulting in farmers planting their own grain produced in prior seasons (Sadat and Choi 2017). Phylogenetic studies confirmed that MoT isolates sampled in Bangladesh were closely related to South American lineage, suggesting that the wheat blast pathogen was introduced on contaminated seeds/grains (Islam et al. 2016; Malaker et al. 2016). Considering wheat blast is seed-borne (Goulart and Paiva 1990; Cruz and Valent 2017) and often symptomless (Urashima et al. 2009), that is a reasonable explanation based on the epidemiology of the disease; that in one year, the outbreak occurred in several districts of wheat production of Bangladesh.

For disease management and control

Genetic resistance appears to be the most effective strategy to control wheat blast, however it has been challenging to identify many sources of resistance (Cruz and Valent 2017). In 2016, the 2NS translocation was identified as a new source of resistance (Cruz et al. 2016). It is a segment of chromosome identified from *Aegilops ventricosa*, a wild wheat

relative (Cruz et al. 2016). Many studies were conducted, and the most planted resistant cultivars carry the 2NS translocation (Cruppe et al. 2019). Another effective control practiced in South America is to delay planting dates in order to reduce disease (de Oliveira Coelho et al. 2016). This practice is to avoid the conditions (high temperature, high humidity, and precipitations) that favor infection during the grain development stage (Mehta et al. 1992). Deep plowing to eliminate sources of inoculum was proven to also be effective (Urashima et al. 2009), however it is a less common practice today in the widely implemented no-till systems. Fungicide applications under highly conducive conditions for wheat blast have shown very poor control (Goulart et al. 2007; Urashima et al. 2009). In addition, it is suggested that controlling the basal inoculum in the early stages might decrease the disease on heads (Cruz et al. 2015). Also, seed treatments with specific fungicides have shown a decrease in disease (Toledo 2015). It is recommended to treat seed lots that were produced from fields highly affected by wheat blast (Toledo 2015).

Diagnostic methods for *Magnaporthe oryzae* Triticum pathotype

Increasing global trade of plant commodities increases the risk to introduce plant pathogens unintentionally (Stack et al. 2006; Rossman 2001; Palm 1999). In history, there are many pathogen introduction examples with terrible consequences, such as disease outbreaks of Chestnut blight in the US (Anagnostakis 1987) or Dutch elm disease in the US and Europe (Schlarbaum et al. 1997; Hubbes 1999). The recent outbreak of wheat blast in Bangladesh adds a risk of spread to top wheat producer countries in Asia (Sadat and Choi 2017) such as China and India. Spread from Bangladesh throughout Asia could compromise food security globally. Therefore, it is imperative to take preventive actions, like the

development of reliable and accurate diagnostic and detection technologies, due to the fact of the importance of its potential economic, political and ecologic impacts (Stack et al. 2014).

Traditional diagnostic methods for plant diseases are often based on symptomatology, culturing and serological-based assays, e.g., enzyme-linked immunosorbent assay (ELISA); they are still the main methods for detection for many plant pathogens in many labs (Konstantinova et al. 2002; Berg et al. 2005). However, traditional methods may be time consuming and inadequate in some cases, such as complex symptoms caused by two or more pathogens, inability to detect physical structures of the pathogen in certain tissues (Konstantinova et al. 2002; Berg et al. 2005). The development of the Polymerase Chain Reaction (PCR) in the last century and its application to diagnostics, allowed more accurate and rapid identification of plant pathogens; it provides high sensitivity and specificity when primers (a specific sequence of DNA from a determined specie) and annealing temperatures are well designed (Kerkoud et al. 2002; Barros et al. 2001; Harmon et al. 2003). The sensitivity, specificity, speed, and wide utility make this molecular technology adaptable for many targets in plant pathology (Henson and French 1993). PCR is a molecular technique that amplifies target DNA to millions of copies of a specific sequence of DNA. PCR consists of three main steps: 1) Melting or denaturation to separate the two strands of the DNA fragment, 2) Annealing the two designed primers (Forward and Reward), each primer annealing its complimentary DNA strand and finally 3) Extension of the primer by DNA Polymerase enzyme; the result being two copies of synthetized DNA (Henson and French 1993). These 3 steps comprise a cycle, and the PCR technique can amplify exponentially up to 50 cycles depending on the organism, creating billions of copies of a specific DNA sequence (Henson and French 1993).

At present, there is no effective protocol for the detection and identification of MoT within and on wheat seed or grain that industries can use to screen during seed and grain movement. Classical diagnostic methods for MoT are based on visual assessment for symptoms or signs of sporulation of *M. oryzae* on incubated infected seeds (Pieck et al. 2017). PCR-based diagnostic methods were designed for *Magnaporthe oryzae* species identification (Pieck et al. 2017). The multi-locus PoT 2 transposon, and other transposable elements, such as MGR583 and MoTeR, were determined to be present in all *Magnaporthe oryzae* pathotypes and differentiated from other fungal species (Farman et al. 1996; Farman 2002; Pieck et al. 2017; George et al. 1998). However, PoT2 was not able to distinguish pathotypes (Pieck et al. 2017; Yasuhara-Bell et al. 2018). Several diagnostic assays with high sensitivity and specificity to detect *M. oryzae* Triticum pathotype were developed based on a partial DNA sequence, designated MoT3, a single copy within a retinol dehydrogenase gene (Pieck et al. 2017; Yasuhara-Bell et al. 2018). A controversy occurred when it was reported the MoT 3 locus did not distinguish Triticum from Oryza pathotypes (Gupta et al. 2019). However, additional research by Yasuhara-Bell et. al. (2019) demonstrated the reliability of the MoT3 marker to discriminate Triticum from Oryza pathotypes, as well as from other pathotypes. Recently, a new molecular diagnostic assay was developed capable to discriminate the Triticum pathotype from another *M. oryzae* subpopulations (Thierry et al. 2019).

The importance to understand the limits of detection

The development of nucleic acid-based assays for plant pathogen diagnostics has increased the accuracy and decreased the time required for pathogen identification, often to sub-specific levels of taxonomic discrimination. PCR (end-point and real-time) and

isothermal (Loop-mediated isothermal amplification) based molecular assays using the MoT3 specific primers are very sensitive and reliable tools able to distinguish the Triticum pathotype from other *M. oryzae* pathotypes (Pieck et al. 2017; Yasuhara-Bell et al. 2018). However, all these efficient and sensitive diagnostic tools might not be effective if the plant derived sample is not representative of the area or location of interest. Many PCR seed detection assays are established solely on genomic DNA. It is known that some plant tissues release some compounds that inhibit partially or totally the PCR reaction for the DNA target, driving to a false negative of the infected plant sample (Mavrodieva et al. 2004; Louws et al. 1999). DNA extracted from some bacterial infected plant tissue could release compounds that inhibit Taq polymerase affecting PCR amplification, degradation of the DNA target sequence or PCR reagent interaction problems (Louws et al. 1999), as same as might occur in fungal infected plant tissue. All these parameters are important for a reliable diagnostic that derives for actions whether the sample results in a true positive or negative (Stack et al. 2014). In addition, mostly the PCR assays validated on infected seed samples, solely considered mix gradient incidence ratios without including the severity variability on the infected seeds for detection sensitivity. Nowadays, in the actual plant derived trade system worldwide, several plant pathogens introduction to new areas occurred through infested derived plant commodities (Fletcher et al. 2006; Kim et al. 2003; Williamson et al. 2002). Seed-borne pathogens are one of the most common introduction into new areas due to diseased symptomless seeds and deficient detection system (Garcia et al. 2008; Gaige 2016). Due to this fact, it is important to take account many aspects on the pre-harvest stage, such as incidence, severity, and distribution of infected plants to define the sampling method with the number of samples required to make an efficient representation of the area where is tested (Madden and Hughes 1999). It is crucial to understand the diagnostic tools and sampling

strategy limitations, in order to define the sensitivity for false positive or false negative, due to the fact either situations could cause economic, social and environmental impacts (Stack et al. 2014). A false positive is when a given diagnostic declares a positive presence of the pathogen, when in fact it does not; carrying with it mainly economic consequences like unnecessary mitigation measures deployed or trade stopped unnecessarily (Stack et al. 2014). On the other hand, a false negative is when a given diagnostic declares a negative presence of the pathogen, when in fact it does; causing mainly economic, social and environmental consequences like spread a pathogen in a new area (i.e. wheat blast outbreak in Bangladesh risking the food security for one of the most important crops in the world) (Stack et al. 2014).

The objectives of the present study were to: 1) define and understand the sensitivity and specificity to detect MoT within and on wheat seeds, and 2) build up a preliminary risk assessment to develop some guidelines to minimize the probabilities of moving MoT infected seeds.

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Chapter 2 - Limits of Detection of *Magnaporthe oryzae* Triticum in Wheat Seed: Implications for Pathogen Dissemination

Abstract

Wheat blast is an emerging disease on wheat, caused by the seedborne fungal pathogen *Magnaporthe oryzae* Triticum pathotype (MoT). Dissemination of MoT-infected seed is likely responsible for the spread of the wheat blast pathogen within South America and from South America to southern Asia. The objective of this research was to establish the detection threshold (conventional and real time PCR) for MoT in infected wheat seeds as a function of incidence (DI) and severity (DS) of infected seed. Wheat seed lots were established with known MoT incidence (percentage of seed infected) and severity (MoT biomass per seed). Incidence and severity levels were verified by end-point PCR and q-PCR, respectively. Sensitivity and specificity in the presence of potentially interfering DNAs was verified using DNA gradients based on ten-fold serial dilutions of MoT DNA with genomic DNA from rice and ryegrass *M. oryzae* populations and wheat seed DNA. The detection threshold with end-point PCR was 0.1% of MoT colonized seeds in a seed lot. The detection threshold varied as a function of MoT biomass in colonized seeds; the minimum MoT detectable by end-point PCR was 3×10^3 MoT cells/seed. This study verified the specificity to discriminate the Triticum pathotype from other *M. oryzae* pathotypes as well as demonstrated no interference from wheat seed DNA. This study provides evidence that to establish pathogen detection thresholds in plant seed, both disease incidence and severity must be evaluated to reduce the probabilities of false negatives.

Introduction

Wheat blast is caused by the haploid ascomycete *Magnaporthe oryzae* Triticum pathotype (MoT) (synonymous with *Pyricularia oryzae*) (Couch et al. 2005). *Magnaporthe oryzae* is sub-divided by primary host-specialized populations. In addition to the Triticum pathotype that causes wheat blast, it includes the *Oryza* pathotype (MoO) and the *Lolium* pathotype (MoL) that cause rice blast and gray leaf spot on ryegrass, respectively (Couch et al. 2005; Farman 2002; Heath et al. 1990; Tosa et al. 2004). However, *M. oryzae* host-specialized populations have the capability to infect non-primary hosts with variable pathogenicity (Farman 2002; Kohli et al. 2011; Tosa et al. 2004). MoT is an aggressive pathogen and under high levels of disease pressure, yield losses can be as great as 100% in individual fields (Cruz and Valent 2017). Conducive conditions for wheat blast development include moderate to high temperatures (25 to 30°C) and prolonged high relative humidity (>80%), along with excessive precipitation period and prolonged leaf wetness (Cruz and Valent 2017; Urashima et al. 2009; Goulart et al. 2007). Symptoms occur on most above ground parts of the plant with elliptical lesions with a grey center where sporulation takes place (Cruz and Valent 2017). However, the greatest yield losses occur when infections occur on the neck or panicle during the early stages of grain development (Cruz and Valent 2017). Seeds or grains produced from infected spikes are often small, shriveled, deformed with low test weight (Cruz and Valent 2017; Urashima et al. 2009). MoT is seed-borne; infected seeds can be asymptomatic. Seeds or grains from symptomatic or asymptomatic spikes can have similar levels of MoT infection, probably caused by the moment of infection (Urashima et al. 2009).

MoT was first reported in Brazil in 1985 (Igarashi et al. 1986; Nunes Maciel 2011). Since then, it has spread to neighboring wheat producing countries such as Bolivia in 1996 (Toledo and Barea 1996), Paraguay 2002 (Viedma 2005) and Argentina in 2007 (Cabrera and Gutierrez 2007). In 2016, it was confirmed for the first time outside of South America, in Bangladesh (Callaway 2016; Malaker et al. 2016). Considering wheat blast is seed-borne and the epidemic occurred in many districts of Bangladesh, the hypothesis is that MoT was introduced in contaminated seeds/grains.

With global trade increasing, it is critical to establish an effective pathogen detection system to prevent or minimize the movement of exotic pathogens into new areas. Since plant pathogens can be present at low levels and asymptomatic in seeds and grains, detection of plant pathogens can be challenging. An effective detection assay must be highly sensitive and specific (Konstantinova et al. 2002). Traditional diagnostic methods are based on visual symptomatology, culturing and/or serological tests such as ELISA (Konstantinova et al. 2002; Berg et al. 2005; Chilvers et al. 2007). However, difficulties with these methods, such as asymptomatic, infected commodities, the inability of pathogens to produce reproductive structures for proper identification, the presence of two or more pathogens, and time-consuming procedures can lead to an incorrect diagnosis (Konstantinova et al. 2002; Berg et al. 2005; Chilvers et al. 2007). Advanced molecular-based detection methods, e.g., Polymerase Chain Reaction (PCR), contribute to an efficient biosecurity system. PCR allows reliable and rapid plant pathogen identification with high sensitivity and specificity when primers are well designed (Kerkoud et al. 2002; Barros et al. 2001; Harmon et al. 2003). For seed or grain trade, many phytosanitary certification tests for seed-borne pathogens are inefficient. Currently, detection of MoT within and on seeds is based on conventional diagnostic methods such as visual symptomatology, blotter test, and culturing methods by

confirmation of the conidia of *Magnaporthe oryzae*. However, in some cases MoT infected seeds are asymptomatic, mostly caused by a late infection on spikes (Urashima et al. 2009). In the Blotter and culturing methods, MoT may be overgrown by other organisms, making difficult and time consuming the proper identification of MoT structures. Additionally, these methods are limited to identification at the species level and not at the pathotype level, which is very important for the identification of *M. oryzae* host-specialized populations (Pieck et al. 2017; Yasuhara-Bell et al. 2018). Therefore, a PCR molecular diagnostic tool has the potential to achieve a rapid, sensitive, and specific detection for MoT within and on seeds. However, studies report some plant tissues could release some compounds that could affect or inhibit the amplification on the target in the PCR, resulting a false negative diagnoses (Mavrodieva et al. 2004; Louws et al. 1999). Therefore, it is essential to test and validate the detection sensitivity of the assay using real samples to understand the limits of detection for wheat seeds. A false negative diagnosis could result in the introduction of an aggressive plant pathogen into a new area (Stack et al. 2014), causing economic, social and environmental consequences; e.g., the introduction of MoT into Bangladesh in 2016 (Malaker et al. 2016). The sequence of the MoT 3 locus of the *M. oryzae* within the retinol dehydrogenase gene was identified to be specific for MoT detection (Pieck et al. 2017); multiple diagnostic assays were performed validating its specificity and sensitivity for wheat blast detection (Pieck et al. 2017; Yasuhara-Bell et al. 2018, 2019). This achievement is essential for the proper identification and detection of the blast, considering its symptoms similarities with Fusarium Head Blight (FHB), caused by the fungal pathogen *Fusarium graminearum* (Cruz and Valent 2017). Even though a controversy occurred when it was reported the MoT 3 primer was not specific to differentiate the Triticum from the Oryza pathotype (Gupta et al. 2019), it was demonstrated with strong evidence the MoT 3 locus assay was highly efficient and specific

(Yasuhara-Bell et al. 2019). Recently, a new molecular detection assay was developed capable to discriminate the aggressive Triticum pathotype from others *M. oryzae* sub-populations (Thierry et al. 2019). Additionally, is worth to mention that several molecular MoT detection tools were developed using the MoT 3 locus by Pieck et al. (2017) and Yasuhara-bell et al. (2018) showing similar high MoT detection specificity. Furthermore, the capacity to differentiate MoT from other *Magnaporthe oryzae* pathotypes is important to avoid unnecessary mitigation measures that could affect or interrupt the economy in a regional commodity trade (Pieck et al. 2017; Stack et al. 2014). The main objective of this study was to determine the limits of detection, i.e., assay and sampling sensitivities and specificity, for a modified MoT-specific PCR-based assay (Pieck et al. 2017) as a function of MoT incidence and MoT cell number in wheat seed.

Material and methods

Fungal and plant material

All laboratory and growth chamber experiments with *M. oryzae* Triticum were performed at the Biosecurity Research Institute (BRI) at Kansas State University in a biosafety level 3 (BSL-3) laboratory in compliance with USDA APHIS PPQ526 permit conditions (P526P-19-02185). *Magnaporthe oryzae* (B.C. Couch) Triticum isolate B-71 used in this study was collected in 2012 from a diseased wheat plant in Quirusillas, Bolivia; *Magnaporthe oryzae* (B.C. Couch) Triticum isolate T-25 used in this study was collected in 1988 from a diseased wheat plant in Parana, Brazil (Yasuhara-Bell et al. 2018). Both MoT isolates were imported under USDA APHIS PPQ permit P526P-09-01917 and stored in the BSL-3 laboratory at BRI. *Magnaporthe oryzae* (B.C. Couch) Lolium strain G-239 used in this study was collected by M. Kennelly (*personal communication*) in 2007 from infected

ryegrass in Marysville, Kansas; it is a pathogen of ryegrass (*Lolium spp.*). *Magnaporthe oryzae* (B.C. Couch) Oryza strain Guy-11 used in this study was collected in 1998 from a diseased rice plant in French Guyana; Guy-11 DNA was provided by B. Valent lab. Wheat seed, *Triticum aestivum* L. cultivar Everest (hard red winter wheat), used in this study was obtained from the Kansas State University Agronomy Farm in Manhattan, Kansas. In lab tests at BRI, wheat cultivar Everest was rated susceptible to wheat blast at the heading stage of development (Cruppe et al. 2019; Cruz et al. 2012). Preliminary experiments for methods development and validation were conducted in a BSL 2 lab using the MoL isolate G-239. For MoT seed colonization experiments the MoT isolate B-71 was used. For MoT detection sensitivity and specificity assays, the genomic DNAs of MoT isolates B-71 and T-25, MoL isolate G-239, MoO isolate Guy-11 and wheat seed were used.

DNA extraction and quantification

Five grams of ground seed were prepared from all seed samples placed into a tube with 10 ml of sterile deionized water, vortexed for 25-30 seconds and filtered through 4 layers cheesecloth. A 200 ul sample was taken for DNA extraction using the Quick-DNA Fungal/Bacterial Miniprep Kit (ZYMO, USA). All MoT DNA extractions were performed within a biosafety cabinet inside the BSL-3 lab at BRI.

To avoid cross contamination among replicates and treatments, individual blender containers and changeable blades were washed and disinfested after each use. To disinfest, blades were submerged in undiluted Cavicide (Diisobutylphenoxyethoxyethyl Dimethyl Benzyl Ammonium Chloride, Isopropanol) for a minimum of 2 minutes and then washed with sterile deionized water (1 minute). The blade unit was then placed and run in the blender apparatus with the following sequential treatments: water and detergent (15 seconds), bleach (10% solution for 15 seconds), sterile deionized water (15 seconds), and finally rinsed in

ethanol (75% for 30 seconds). The sterile blades were then dried inside the biosafety cabinet. Samples from the residual of every disinfestation step was plated onto oatmeal agar to confirm the absence of viable MoT. DNA extracted from non-inoculated seeds was confirmed as MoT-free by PCR and all PCR test results reported.

End-point and real-time PCR

For detection, the modified End-point PCR was performed in a 25 μ l reaction, containing 5 μ l of template DNA, 1 μ M each of Forward (MoT3-F) and Reverse (MoT3-R) primers (Pieck et al. 2017), 12.5 μ l of GoTaq® G2 Green master mix (Promega, Madison, US) and 2.5 μ l of nuclease free water. A Gradient Thermocycler (BIO RAD, US) was used with 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. Amplified PCR products were resolved by electrophoresis on a 2% agarose gel (90 V for 60 min). The DNA bands were visualized by ethidium bromide staining and ultraviolet illumination. All experiments had three biological replicates, each replicate with two subsamples performed for each seed sample in End-point PCR.

For quantification, a modified real-time PCR was performed in a 20 μ l reaction containing 1 μ l of template DNA, 10 μ l SsoAdvanced Universal Probes Supermix (BIO RAD), 0.4 μ M each of Forward (MoT3-1F) and Reverse (MoT3-1R) primers and 0.1 μ M probe (MoT3 Probe; 6'-carboxyfluorescein [FAM] at the 5' end and black hole quencher – 1 at the 3' end) (Pieck et al. 2017). The modified real-time PCR quantification was performed using the CFX96 Real-Time System (BIO RAD, Hercules, CA, USA) with 3 min for polymerase activation and DNA denaturation at 95°C followed by 40 cycles at 95°C for 15 s and 60°C for 30 s. Real-time PCR reactions were performed in duplicate and three

biological replicates were performed for each real-time PCR seed sample. Average Ct values with calculated standard deviation are reported.

Methods Development using an MoT surrogate

Determination of the detection thresholds for MoT in wheat seed required MoT-infected seeds. A protocol was developed to generate wheat seed lots with 100% seed infection. To circumvent the limitations associated with working within a BSL-3 laboratory environment, a surrogate pathogen was used in a BSL-2 laboratory to facilitate protocol development and optimization. Preliminary experiments were performed using the *M. oryzae* Lolium pathotype (MoL) isolate G-239 as the surrogate pathogen. Experiments with MoT inoculation on seeds were conducted in the BSL3 lab at BRI. Based on phylogenetic studies, the Lolium pathotype is the closest related host-specialized population to the Triticum pathotype (Farman et al. 2017; Gladieux et al. 2018).

The Lolium pathotype of *M. oryzae* is established in Kansas and over a large area within the U.S. Given that MoL is indigenous to Kansas, its use within a laboratory environment poses little risk for environmental, social and economic impact. All experiments with MoL G-239 were performed in a BSL-2 laboratory. For seed inoculation, MoL G-239 was cultured on oat meal agar medium (OMA) (Valent et al. 1991) for 8-10 days at 25 °C with 24 hours of light. Conidial suspensions were prepared by adding sterile deionized water to OMA cultures, dislodging the conidia from conidiophores using a sterile disposable loop, and filtering through four layers of cheesecloth (Cruz et al. 2015). Conidial concentrations were determined using a Neubauer chamber and adjusted using sterile deionized water. To avoid germination, wheat seeds (hard red winter wheat cultivar Everest, highly susceptible to MoT (Cruppe et al 2019, Cruz et al 2012) were heat treated at 65°C for 1 hour followed by drying within a biosafety cabinet for 24 hours. Experiments were designed to determine

inoculation method, incubation times, colonization periods, seed surface disinfection methods and seed processing for DNA extraction.

Surface disinfestation of seed

Hypochlorite solution. A method for surface disinfestation of seed was developed using bleach at different concentrations, different exposure periods and different rinsing periods. The conidial concentration for seed inoculations was 7.65×10^5 conidia/ml. After inoculation, the seeds were immediately surface disinfested followed by rinsing with sterile deionized water for 30 seconds twice. The experimental design had 10 treatments with 3 replications per treatment; each replication was 10 gr seeds (ca. 334 seeds). The seed surface disinfestation treatments were: T1: 5% bleach (sodium hypochlorite 0.31%) for 1 minute; T2: 5% bleach (sodium hypochlorite 0.31%) for 5 minutes; T3: 5% bleach (sodium hypochlorite 0.31%) for 10 minutes; T4: 5% bleach (sodium hypochlorite 0.31%) for 20 minutes; T5: 10% bleach (sodium hypochlorite 0.62%) for 1 minute; T6: 10% Bleach (sodium hypochlorite 0.62%) for 5 minutes; T7: 10% Bleach (sodium hypochlorite 0.62%) for 10 minutes; T8: 10% Bleach (sodium hypochlorite 0.62%) for 20 minutes; T9: no surface disinfestation and T10: non-inoculated seeds without surface disinfestation. After the surface disinfestation treatment, 20 seeds were randomly selected from each replication/treatment and plated on OMA in a 24 well cell culture plate and placed at 25°C with 24 hours light for 9 days. The seeds were then observed through a dissecting microscope for the presence of MoL sporulation. .

Hypochlorite solution plus ethanol. This experiment was conducted twice, with the first experiment using 5×10^5 conidia/ml and the second experiment with 7×10^5 conidia/ml inoculum concentrations for the MoL colonized seeds. The experimental design was based on 6 treatments, with 3 replications each treatment. Each replication was based on 10 gr

seeds. The objective of this experiment was to determine a more effective surface disinfection procedure. The treatments were: Treatment 1 (Colonized seeds without surface disinfection), Treatment 2 (Non-colonized seeds), Treatment 3 (Bleach 10% or equivalent to Sodium hypochlorite 0.62% concentration rinsed for 1 minute followed by rinsed with sterile deionized water for 30 seconds twice), Treatment 4 (Bleach 15% or equivalent to Sodium hypochlorite 0.92% concentration rinsed for 1 minute followed by rinsed with sterile deionized water for 30 seconds twice), Treatment 5 (Ethanol 75% rinsed for 30 seconds, then Bleach 10% or equivalent to Sodium hypochlorite 0.62% concentration rinsed for 1 minute followed by rinsed with sterile deionized water for 30 seconds twice) and Treatment 6 (Ethanol 75% rinsed for 30 seconds, then Bleach 15% or equivalent to Sodium hypochlorite 0.92% concentration rinsed for 1 minute followed by rinsed with sterile deionized water for 30 seconds twice). After the surface disinfection process, from each replication/treatment randomly were chosen 20 colonized seeds and plated in OMA 24 well cell culture cluster plate and incubated in a grower chamber at 25°C and 24 hours light for 9 days. After the incubation period, the seeds were evaluated through a dissecting microscope for incidence based on the MoL sporulation on the seeds.

***M. oryzae* infected seed lots**

MoL biomass gradient established by blending with non-infected seed - sodium hypochlorite disinfection. The experimental objective was to generate MoL-infected seed lots with varying amounts of MoL biomass per seed in order to simulate wheat seed lots from fields with different levels of blast severity. Wheat seed (60 gr) were inoculated with a conidial suspension (7.65×10^5 conidia/ml) of MoL G-239 and placed in a moist chamber (Aluminum chamber dimension of 28.8 cm x 23.6 cm 6.5 cm with 3 layers of sterile paper towel moistened with 50 ml of sterile deionized water and covered with clear plastic wrap)

at 25C for 48 hours. After the incubation period, the colonized seeds were dried inside a biosafety cabinet for 24 hours. Subsequently, the colonized seeds were surface disinfested with 10% bleach solution (sodium hypochlorite 0.62%) for 1 minute, and then rinsed with sterile deionized water twice for 30 seconds each. The experimental design was based on 6 treatments, with 3 replications per treatment; each replication was the equivalent of 100 MoL colonized seeds. The treatments were: Treatment 1: 0% MoL-colonized seeds (100 seeds non-inoculated); Treatment 2: 5% MoL-colonized seed (5 MoL colonized seeds + 95 non-colonized seeds); Treatment 3: 10% MoL-colonized seed (10 MoL colonized seeds + 90 non-colonized seeds); Treatment 4: 25% MoL-colonized seed (25 MoL colonized seeds + 75 non-colonized seeds); Treatment 5: 50% MoL-colonized seed (50 MoL colonized seeds + 50 non-colonized seeds); and Treatment 6: 75% MoL-colonized seed (75 MoL colonized seeds + 25 non-colonized seeds). The treated seeds were plated on OMA in 24-well cell culture plates and placed at 25°C with 24 hours light for 9 days. After incubation, the seeds were observed through a dissecting microscope for the presence of MoL sporulation.

MoL biomass gradient established by varying the post-inoculation colonization period - sodium hypochlorite disinfestation. The objective of this experiment was to generate a MoL fungal biomass gradient among seed lots by varying the post-inoculation colonization period. Wheat seed were inoculated with a conidial suspension (5×10^5 conidia/ml) of MoL G-239 and placed in a moist chamber (aluminum chamber dimension with 28.8 cm x 23.6 cm 6.5 cm with 3 layers of sterile paper towel moisten with 50 ml of sterile deionized water and covered with a clear plastic wrap) for varying post-inoculation incubation periods. After the incubation period, the colonized seeds were dried inside a biosafety cabinet for 24 hours followed by surface disinfestation with 0.62% sodium hypochlorite (10% bleach solution) for 1 minute, and then rinsed with sterile water twice for 30 seconds each. The experimental

design was based on 60 gr seed/lot and 7 treatments, with 3 replications per treatment. There was no validated molecular assay available for quantification of MoL DNA. For the molecular detection, a validated highly sensitive and specific isothermal amplification (LAMP) assay was used (Yasuhara-Bell et al. 2018). LAMP is generally used to determine the presence or absence of a target sequence; in this study, the the ct times were used to infer approximate values for fungal biomass. The treatments were: Treatment 1 (post-inoculation colonization period = 0 hrs, plus surface disinfection), Treatment 2 (post-inoculation colonization period = 12 hrs, plus surface disinfection), Treatment 3 (post-inoculation colonization period = 24 hrs, plus surface disinfection), Treatment 4 (post-inoculation colonization period = 48 hrs, plus surface disinfection), Treatment 5 (post-inoculation colonization period = 72 hrs, plus surface disinfection), Treatment 6 (post-inoculation colonization period = 0 hrs, no surface disinfection), Treatment 7 (non-inoculated seed, no surface disinfection). For treatment 1, immediately after inoculation, seed were dried inside a biosafety cabinet for 24 hours. For each treatment, one portion of the colonized seeds was used for culturing and another portion was ground for DNA extraction and subsequent detection by the LAMP assay. For each replication/treatment, 20 seeds were randomly chosen and plated onto OMA in a 24 well cell culture plate and incubated in a grower chamber at 25°C with 24 hours light for 9 days. After the incubation, the seeds were evaluated (dissecting microscope) for MoL sporulation and incidence calculated.

MoL biomass gradient established by varying inoculum concentration and varying the post-inoculation colonization period – ethanol plus sodium hypochlorite disinfection.

The objective of this experiment was to generate a MoL fungal biomass gradient among seed lots by varying the MoL inoculum concentration as well as varying the post-inoculation colonization period. Wheat seed were inoculated with one of three conidial suspensions (7.5

x 10^5 conidia/ml, 7.5×10^4 conidia/ml, or 7.5×10^3 conidia/ml) of MoL G-239 and placed in a moist chamber (aluminum chamber dimension with 28.8 cm x 23.6 cm 6.5 cm with 3 layers of sterile paper towel moistened with 50 ml of sterile deionized water and covered with a clear plastic wrap) for varying post-inoculation incubation periods. After the incubation period, the colonized seeds were dried inside a biosafety cabinet for 24 hours. The dried seed were then surface disinfected with 75% ethanol for 30 seconds, followed by 0.62% sodium hypochlorite (10% bleach solution) for 1 minute, and then rinsed with sterile water twice for 30 seconds each. The experimental design was based on 13 treatments (60 gr seed/lot) with 3 replications per treatment. There was no validated molecular assay available for quantification of MoL DNA. For the molecular detection, a validated highly sensitive and specific isothermal amplification (LAMP) assay was used (Yasuhara-Bell et al. 2018). LAMP is generally used to determines the presence or absence of a target sequence; in this study, the ct times were used to infer approximate values for fungal biomass. The treatments were: Treatment 1 (inoculum concentration = 7.5×10^5 conidia/ml, post-inoculation incubation period = 0 days, no surface disinfestation), Treatment 2 (inoculum concentration = 7.5×10^4 conidia/ml, post-inoculation incubation period = 0 days, no surface disinfestation), Treatment 3 (inoculum concentration = 7.5×10^3 conidia/ml, post-inoculation incubation period = 0 days, no surface disinfestation), Treatment 4 (inoculum concentration = 7.5×10^5 conidia/ml, post-inoculation incubation period = 0 days, plus surface disinfestation), Treatment 5 (inoculum concentration = 7.5×10^4 conidia/ml, post-inoculation incubation period = 0 days, plus surface disinfestation), Treatment 6 (inoculum concentration = 7.5×10^3 conidia/ml, post-inoculation incubation period = 0 days, plus surface disinfestation), Treatment 7 (inoculum concentration = 7.5×10^5 conidia/ml, post-inoculation incubation period = 1 day, plus surface disinfestation), Treatment 8 (inoculum concentration

= 7.5×10^4 conidia/ml, post-inoculation incubation period = 1 day, plus surface disinfection), Treatment 9 (inoculum concentration = 7.5×10^3 conidia/ml, post-inoculation incubation period = 1 day, plus surface disinfection), Treatment 10 (inoculum concentration = 7.5×10^5 conidia/ml, post-inoculation incubation period = 6 days, plus surface disinfection), Treatment 11 (inoculum concentration = 7.5×10^4 conidia/ml, post-inoculation incubation period = 6 days, plus surface disinfection), Treatment 12 (inoculum concentration = 7.5×10^3 conidia/ml, post-inoculation incubation period = 6 days, plus surface disinfection), Treatment 13 (Non inoculated seeds, no surface disinfection). For treatments 1-6, the seeds were dried inside a biosafety cabinet for 24 hours immediately after inoculation. For each treatment (1-13), one portion of the colonized seeds was used for culturing and another portion was ground to flour for DNA extraction and subsequent detection by the LAMP assay. For each replication/treatment, 20 seeds were randomly chosen and plated onto OMA in a 24 well cell culture plate and incubated in a grower chamber at 25°C with 24 hours light for 9 days. After incubation, the seeds were evaluated (dissecting microscope) for MoL sporulation and the incidence calculated. For each replication/treatment, the LAMP assay (Yasuhara-Bell et al. 2018) was performed on the DNA extracted.

MoT biomass gradient established by varying inoculum concentration and varying the post-inoculation colonization period. The experimental objective was to establish seed lots with varying amounts of MoT biomass per seed. Based on the inoculation protocol established from the preliminary experiments with MoL, MoT seed colonization experiments were conducted in the BSL-3 lab at BRI. For inoculum preparation, MoT was cultured on oat meal agar medium (OMA) (Valent et al. 1991) for 8-10 days at 22-23 °C with 24 hours of light. Conidial suspensions were prepared by adding sterile deionized water to OMA cultures,

dislodging the conidia from the conidiophores using a sterile disposable loop, and filtering through four layers of cheesecloth (Cruz et al. 2015). The conidial concentration was adjusted to 8×10^4 conidia/ml or 6.5×10^5 conidia/ml using sterile deionized water.

Wheat seeds were heat treated at 65°C for one hour to prevent germination, dried for 24 hours, and subsequently inoculated with the MoT conidial suspension. The experimental design included nine treatments, three replications/treatment and two subsamples for each replication; the experiment was conducted twice. Non-MoT colonized seed were included as a negative control treatment. For each treatment (i.e., seed lot), 60 g of wheat seeds were inoculated in 100 ml of an MoT conidia suspension and placed on a rotary shaker for 10 minutes at 250 RPM and 25°C . Inoculated seeds were collected on 4 layers cheesecloth and placed in moist chambers (aluminum chamber dimension with 28.8 cm x 23.6 cm 6.5 cm with 3 layers of sterile paper towel moisten with 50 ml of sterile deionized water, and covered with a clear plastic wrap) for varying times in order to establish an MoT biomass gradient (i.e., MoT cells per seed).

The treatments were: treatment 1 (inoculum concentration = 8×10^4 conidia/ml, post-inoculation incubation period = 0 days), treatment 2 (inoculum concentration = 8×10^4 conidia/ml, post-inoculation incubation period = 1 day), treatment 3 (inoculum concentration = 8×10^4 conidia/ml, post-inoculation incubation period = 2 days), treatment 4 (inoculum concentration = 8×10^4 conidia/ml, post-inoculation incubation period = 3 days), treatment 5 (inoculum concentration = 8×10^4 conidia/ml, post-inoculation incubation period = 4 days), treatment 6 (inoculum concentration = 8×10^4 conidia/ml, post-inoculation incubation period = 5 days), treatment 7 (inoculum concentration = 8×10^4 conidia/ml, post-inoculation incubation period = 6 days), treatment 8 (inoculum concentration = 6.5×10^5 conidia/ml, post-inoculation incubation period = 6 days) and finally treatment 9 (non-inoculated seed).

After the colonization period, seed were dried for 24 hours, followed by surface disinfection with 75% ethanol for 30 sec, followed by 0.62% hypochlorite (10% bleach solution) for 60 sec, and then rinsed with sterile deionized water twice for 30 sec each. After surface disinfection, seed were dried for another 24 hours.

For each treatment, average seed weight was determined. Seed were then ground to flour in a modified blender apparatus prior to DNA extraction using the Quick-DNA Fungal/Bacterial Miniprep Kit (ZYMO, USA). MoT fungal biomass concentration (MoT cells per seed) was determined for each seed lot using a modified q-PCR protocol previously established (Pieck et al. 2017). Based on the total DNA per seed lot and the average seed weight per treatment, the MoT fungal biomass concentration per seed was calculated.

Establishing disease incidence and severity gradients for sampling sensitivity determination. The experimental objective was to establish a matrix of seed samples that represented gradients in disease incidence (i.e., the proportion of seed per seed lot infected with MoT) and disease severity (i.e., the amount of MoT biomass per seed). Ground MoT-colonized seeds (described previously) with known MoT biomass/seed levels (established previously) were blended with ground non-colonized wheat seeds in the following ratios: 100%-0%, 10%-90%, 1%-99%, 0.1%-99.9%, and 0%-100%. Blends were made with flour from the colonized seed lots described previously. The result was a matrix of seed lots that ranged in incidence from 100% to 0% and severity from 10^2 to 10^5 MoT cells per seed. The experimental design was 25 treatments with 3 replications per treatment and 2 subsamples for each replication. The experiment was conducted twice. DNA was extracted and tested for detection by the modified End-point PCR (Pieck et al 2017).

Detection sensitivity and specificity

Sensitivity and specificity of the End-point PCR MoT detection assay were verified using genomic DNA preparations. This experiment was conducted twice, each with 3 replications, with 2 sub-samples per replication. Genomic MoT DNA was blended *in vitro* with genomic DNA of *Magnaporthe oryzae* Oryza pathotype (MoO), *M. oryzae* Lolium pathotype (MoL), or wheat seed DNA. Ten-fold serial dilutions of genomic MoT DNA blended with MoO, MoL or seed DNA in the following ratios: 100-0 ng, 10-90 ng, 1-99 ng, 0.1-99.9 ng, 0.01-99.99 ng, 0.001-99.999 ng, and 0-100 ng were made. For genomic MoT DNA isolate B71 was used, for genomic MoL DNA isolate G-239 was used, for genomic MoO DNA isolate Guy 11 was used and for genomic seed DNA non-infected seed from cultivar Everest (hard red winter wheat) was used. DNA concentrations were determined using a NANODROP 2000C spectrophotometer (NanoDrop Technologies, Wilmington, DE) and/or a Qubit 3.0 fluorometer (Life Technologies, Oslo). Detection sensitivity and specificity for MoT in the blended DNA samples was determined by the modified End-point PCR (Pieck et al. 2017). MoT strain T 25 DNA was used as a positive control and sterile deionized water used as a negative control.

Data Analysis

The number of MoT cells per seed was calculated based on a single copy of the MoT3 locus per genome and the MoT genome size of 41.03 Mb (Figure 2.2 & 2.3). Based on those calculations, the minimum number of MoT cells per seed detectable by End-point was quantified by q-PCR. MoT cells/seed values (qPCR) were log transformed for statistical analysis.

The MoT seed colonization experiments were conducted twice; results from the two experiments were combined and analyzed by standard ANOVA, using SAS PROC MIX procedure. The statistical analysis was based on the relationship between fungal biomass concentration and disease severity. In addition, a regression analysis was performed to test the best fit model for the observed vs predicted data using SAS PROC REG procedure. The sampling sensitivity experiment was conducted twice; results from the two experiments were combined and analyzed by standard ANOVA, using PROC MIX procedure. The statistical analysis for sampling sensitivity was based on the relationship between the seed samples and severity. Similar to the MoT colonization experiment, a regression analysis was performed for the sampling sensitivity experiment to test the best fit of the model from the observed vs predicted data using SAS PROC REG procedure. All means were back-transformed to the original scale for graphs and tables for this paper.

Results

Surface disinfestation of seeds

Sodium hypochlorite: The objective was to develop an efficient surface disinfestation treatment. All 0.62% sodium hypochlorite treatments substantially reduced MoL incidence on inoculated seeds; sodium hypochlorite at 0.62% was slightly more effective than sodium hypochlorite at 0.31% (Table 2.1). There were no meaningful differences among 1, 5, 15, and 20 minutes treatment times (Table 2.1). Inoculated seeds without surface disinfestation had 100% MoL incidence while the non-inoculated seeds without surface disinfestation had 0% MoL incidence (Table 2.1). Although not completely effective, from these results, the following surface disinfestation procedure was determined to be the most effective: Bleach 10% or the equivalent sodium hypochlorite 0.62% (10% bleach solution) for 1 minute.

Ethanol plus sodium hypochlorite: Sodium hypochlorite alone was not completely effective. Treatment with 75% ethanol for 30 seconds prior to the 0.62% sodium hypochlorite solution resulted in 0% MoL incidence on inoculated seeds; it was completely effective in eliminating MoL from the surface of treated seeds (Table 2.4). Adding ethanol in the surface disinfection procedure is reported to enhance the penetrability of disinfectant to narrower part of the wheat seeds (Sauer and Burroughs 1986). In addition, ethanol is known to have some disinfection effect for plant pathogens that might contribute to decreasing the MoL incidence on the inoculated seeds. It was important to establish an efficient surface disinfection protocol to decrease the variability of fungal biomass in the seeds between colonized seed lots.

MoL Biomass Gradient

Incidence: There was a high correlation between the observed and expected incidence (Table 2.2) in samples from the established ratios of inoculated and non-inoculated seeds (0%, 5%, 10%, 25%, 50% and 75% MoL colonized seeds). There was no evidence of cross contamination nor inhibition of sporulation between or among seeds indicating that this method could be used efficiently for determining sampling sensitivity as a function of incidence of infected seeds.

Severity: In preliminary experiments to establish a biomass gradient among seed lots, it was evident that the surface disinfection procedure was inadequate. Although there was suggestive evidence of a correlation across treatments, the inoculated seeds with no post-inoculation colonization period (treatment 1) followed by surface disinfection yielded high MoL incidence (Table 2.3). These results indicated the need for a more effective surface disinfection procedure.

The improved surface disinfection procedure with 75% ethanol and 0.62% sodium hypochlorite was completely effective as evidenced by no growth from inoculated treatments with no colonization period (Table 2.5). By varying the inoculum concentration (7.5×10^5 or 7.5×10^3 conidia/ml) and the post-inoculation colonization period (1 or 6 days), incidence and severity gradients were established (Table 2.5). The LAMP reactions yielded threshold cycles of 9.21 (7.5×10^5 conidia/ml, 6 days incubation), 11.78 (7.5×10^5 conidia/ml, 1 day incubation), 19.77 (7.5×10^3 conidia/ml, 1 day incubation) and ND (non-detectable for the non-inoculated controls) reflective of decreasing concentrations of starting target sequence which was a function of MoL cell number. Culturing on OMA medium resulted in a similar gradient of 93.3% (7.5×10^5 conidia/ml, 6 days incubation), 50% (7.5×10^5 conidia/ml, 1 day incubation), 1.7% (7.5×10^3 conidia/ml, 1 day incubation), and 0% (non-detectable for the non-inoculated controls) respectively (Table 2.5). These results demonstrated a direct correlation between the results from the LAMP assay and the results with the culturing test; the greater the MoL incidence in the culturing test, the faster the reaction time in the LAMP assay indicative of a higher concentration of fungal biomass. As further evidence of the correlation, comparison of Treatment 7 (high inoculum concentration, short colonization period) and Treatment 12 (low inoculum concentration, long colonization period) yielded similar LAMP reaction cycles, 11.78 and 11.37, respectively (Table 2.5), and similar culturing results, 50% and 55%, respectively (Table 2.5). These results demonstrated that fungal biomass in the colonized seeds varied as a function of the inoculum concentration and colonization period.

Final protocol for MoT colonization of wheat seed

Based on the results from the experiments with the surrogate strain MoL G-239, the following conditions were established for the MoT inoculation protocol:

- 8×10^4 conidia/ml for 0 – 6 days (low inoculum concentration, variable colonization periods)
- 7.5×10^5 conidia/ml for 6 days (high inoculum concentration, long colonization period)
- surface disinfestation with ethanol 75% + bleach 10% + sterile deionized water (twice)

The high inoculum concentration with long colonization period was to establish a seed lot that represented the worst-case scenario. To increase accuracy and precision of incidence and severity gradients, colonized seeds were ground to flour, and specific amounts of infected flour were blended with specific weights of MoT-free flour to create the desired ratios.

Quantification of MoT DNA

The sensitivity of the MoT3 locus-based PCR assay was confirmed using a 10-fold serial dilution of MoT genomic DNA, from 100 ng to 1 pg DNA corresponding to $\sim 2.29\text{E}+06$ MoT cells to $\sim 2.29\text{E}+01$ MoT cells, respectively. The sensitivity was determined to be between 10 pg and 1 pg, approximately, $2.3\text{E}+01$ - $2.3\text{E}+02$ MoT cells) (Figure 2.1). Although detection at 1 pg was demonstrated in some replicates, it was not consistent across all replicates and experiments. Similar sensitivity was reported by Pieck et. al (2017) and Yasuhara-bell et. al (2018) for the MoT3 locus-based assays.

MoT Seed Colonization

The objective of the seed colonization experiment was to establish seed lots with varying MoT fungal biomass per seed; the experiment was conducted twice. For analysis, the two experiments were combined and seed lots as a function of disease severity based on

colonization period were considered for modeling. There was a positive correlation between colonization period (1 to 6 days) and fungal biomass concentration per seed when seed lots were inoculated with 8×10^4 conidia/ml (Figure 2.4). However, the statistical model ($y = -0.2254x^2 + 2.6964x + 1.8722$) indicated that at a certain point beyond 6 days, MoT fungal biomass concentration per seed would no longer increase; the coefficient for the fitting model of the observed vs predicted data was $R^2 = 0.59$, indicating an acceptable model for analysis. The variance associated with the random effects (experiments, replications and sub-samples) was analyzed using SAS Proc Mixed. The replications and sub-samples random effect had a low variance coefficient, 0.06167 and 0.003563, respectively. The random effect for experiments had a variance coefficient of 4.6193. Variance may have been caused by experimental variabilities, including, different seed lots between experiments, temperature, and moisture within chambers.

Similar to the experiments with MoL G-239, the concentration of MoT biomass in the seeds was a function of the duration of the colonization period (Table 2.6 and Figure 2.5). The surface disinfestation procedure proved completely effective with MoT inoculum (8×10^4 conidia/ml) as evidenced in the no colonization period treatment; there was no MoT biomass detected in the seeds (Table 2.6 and Figure 2.5). In the two separate experiments, MoT biomass per seed varied with the initial inoculum concentration (8×10^4 conidia/ml or 6.5×10^5 conidia/ml) and the duration of the subsequent colonization period (1 – 6 days); the higher the initial inoculum concentration and the longer the subsequent colonization period, the greater the MoT biomass per seed (Table 2.6 and Figure 2.5). Although the specific biomass concentrations varied among experiments, the correlation between MoT biomass per seed and inoculum concentration and colonization period was the same. The seed lot with the lowest detectable MoT biomass per seed (1.54×10^2 MoT cells per seed) was obtained

with the lowest inoculum concentration (8×10^4 conidia/ml) and the shortest colonization period (1 day) (Table 2.6 and Figure 2.5). The seed lot with the highest MoT biomass per seed (2.64×10^5 MoT cells per seed) was obtained with the highest inoculum concentration (6.5×10^5 conidia/ml) and the longest colonization period (6 days) (Table 2.6 and Figure 2.5). In the second experiment, MoT biomass, 5.03×10^1 MoT cells per seed (treatment 2) and 2.59×10^5 MoT cells per seed, varied as a function of inoculum concentration, 8×10^4 conidia/ml or 6.5×10^5 conidia/ml, respectively, and colonization period, 1 or 6 days, respectively, (Table 2.6 and Figure 2.5). MoT was not detected in any non-inoculated seeds in any experiments indicating no cross contamination among seed lots or during assays (Table 2.6 and Figure 2.5).

To determine the detection threshold for MoT in colonized seed lots, MoT incidence and severity gradients were established among wheat seed lots that were created under the following conditions: 8×10^4 conidia/ml with 1 day colonization, 8×10^4 conidia/ml with 2 days colonization, 8×10^4 conidia/ml, 3 days colonization, 8×10^4 conidia/ml with 4 days colonization, and 6.5×10^5 conidia/ml with 6 days colonization.

MoT Detection Threshold

The objective was to determine the end-point PCR detection threshold for MoT in seed samples as a function of disease incidence (proportion of MoT seed colonized) and severity (concentration of MoT biomass per seed). The detection threshold was defined as that level of MoT in a seed lot that was detected in 100% of all replications and sub-samples of seed lots where MoT was known to be present. To determine the detection threshold, a matrix of wheat seed lots that varied with respect to incidence (proportion of seed colonized) and severity (amount of MoT biomass per seed) of MoT was created. Disease incidence

values across seed lots were 100%, 10%, 1%, and 0.1% MoT-colonized seed. For each disease incidence level, there was range of disease severity (MoT biomass per seed) levels.

For endpoint PCR, the detection threshold from both experiments was approximately 3×10^3 MoT cells per seed (Figures 2.7 and 2.8). In the first experiment, the detection threshold was a seed lot with 0.1% MoT-colonized seeds having an average 3.06×10^3 MoT cells per seed (Figure 2.7 and Table 2.7), while the detection threshold in the second experiment was a seed lot with 10% MoT-colonized seeds having an average 1.11×10^3 MoT cells per seed (Figure 2.8 and Table 2.8). In Figures 2.7 and 2.8, each chart represents one seed lot with established disease incidence and severity values; the y-axis indicates the incidence gradient (proportion of infected seeds) and the x-axis indicates the severity gradient (MoT biomass per seed). The border color for each chart indicates detection by the end-point PCR assay; a blue border indicates MoT was detected in 100% of all replications and sub-samples, while a red border indicates MoT was either not detected at all or was not detected in 100% of all replications and sub-samples. The number inside each chart indicates the average MoT biomass (cells/seed) in the seed lot. The results of the two experiments clearly indicate that detection is a function of both MoT incidence and severity; i.e., the higher the proportion and severity of infected seeds in a seed lot, the higher is the likelihood of detection (Figure 2.7 & 2.8).

In these two experiments, disease incidence and disease severity were correlated; as incidence increased, severity increased. At the lowest disease incidence, MoT was rarely detected by end-point PCR, although present (Figures 2.9 & 2.10). At the highest disease incidence, MoT was detected most often by end-point PCR, but not always in 100% of samples (Figures 2.9 & 2.10). For seed lots with 0.1% to 10% disease incidence and/or low

to moderate average MoT biomass per seed, MoT was detected only approximately 50% of the time using the validated by end-point PCR assay (Figure 2.9).

This experiment was conducted twice and the two experiments were combined for analysis. The model indicated that MoT fungal biomass in a seed sample increased with increasing incidence of MoT colonized seeds in a seed lot, for each MoT severity level (Figure 2.6). In addition, the intercepts for the MoT biomass models varied with MoT severity in a seed lot (Figure 2.6). The coefficient for model fit of observed vs predicted data was $R^2 = 0.74$, indicating an acceptable model for statistical analysis. Random effects (experiments, replications and sub-samples) variance was analyzed using SAS Proc Mixed. The variance coefficients for experiments, replications, and sub-samples were 2.381, 0.06502, and 0, respectively. The high variance for experiments was due to the use of two sources of colonized seeds with different severity levels to create the seed samples (Figure 2.5).

Detection sensitivity and specificity

The results from these experiments confirmed the high specificity and sensitivity of the MoT3 locus-based endpoint PCR assay for the detection of *M. oryzae* Triticum pathotype as previously reported (Pieck et al. 2017; Yasuhara-Bell et al. 2018, 2019). To test the sensitivity and specificity of the MoT 3 primers (Forward and Reverse), genomic MoT DNA (B-71) was blended in vitro with potentially interfering DNAs (e.g. wheat seed DNA, DNA of the *Lolium* and *Oryza* populations of *M. oryzae*) keeping the total DNA concentration constant at 100 ng genomic DNA for PCR reaction. This experiment was conducted twice. The sensitivity was constant at 0.1 ng of genomic MoT DNA among all mixtures with genomic MoL, MoO, or wheat seed DNA (Gel line 4 from Figure 2.11). In every test, positive (T-25 MoT DNA) and negative (sterile deionized water) controls were included. No

unexpected amplicons were displayed with any non-MoT DNA sample (Gel line 7), nor with the positive (Gel line 8) or negative (Gel line 9) controls (Figure 2.11). Even in the presence of 1000 times higher concentration of potentially interfering DNA (genomic MoL, MoO, and seed DNA), no amplicons were observed with any of the potentially cross-reacting DNAs (genomic MoL and MoO DNA) demonstrating high assay specificity to discriminate the *Triticum* pathotype from the other *M. oryzae* species (Figure 2.11). The predicted MoT biomass equivalent of the endpoint PCR assay sensitivity of 0.1 ng DNA was calculated to be 2.29×10^3 MoT DNA cells/PCR reaction. That is within the range of and consistent with the measured MoT detection threshold from seed in this study (1.1×10^3 to 3.1×10^3 MoT cells/seed).

Discussion

Wheat blast is an aggressive disease able to cause up to 100% losses based on a combination of conducive weather conditions, wheat cultivar susceptibility, infection point on the wheat spike and time of infection relative to wheat maturity; the earlier the infection occurs, the higher the yield losses (Cruz and Valent 2017). Given the uncertainties associated with climate change, the intensive movement of seeds via international trade and the recent outbreak of wheat blast in Bangladesh, it is imperative to develop accurate and sensitive detection tools for the wheat blast pathogen, *Magnaporthe oryzae* *Triticum* to prevent the spread and introduction of MoT into new geographic areas. Equally important is the lack of statistically validated seed sampling and processing protocols to maximize the detection of MoT-infested seed lots. At present, there are no established protocols for the detection and identification of MoT within and on wheat seed or grain that industries can use to screen seed prior to grain movement.

Classical diagnostic methods for MoT are based on visual assessments of symptoms or signs of sporulation of *M. oryzae* on infected seeds, often based on some version of the blotter test (Goulart et al 1990, Pieck et al. 2017). However, traditional methods for MoT detection in wheat seeds are time consuming to perform, take several days to get a final diagnosis, often complicated by the presence of other faster growing pathogens on the seeds, and the inability to distinguish the Triticum pathotype from other *M. oryzae* pathotypes, e.g., the Lolium pathotype on seeds.

In the seed colonization experiments reported here, the duration of the colonization period determined the amount of MoT biomass in the seeds. This relationship suggested that earlier infections at the spike stage in the field will likely lead to greater amounts of fungal biomass in seeds produced from the infected spikes when the environmental conditions remain favorable. This study suggests the potential importance of considering conditions in wheat blast affected fields to complement interpretation of detection assays for MoT in infected seeds. This is essential for determining MoT detection thresholds when using molecular diagnostic assays with infected wheat seeds. In this study, the detection threshold for MoT in colonized seed lots using a validated endpoint PCR assay (Pieck et al 2017) varied considerably as a function of the incidence (proportion of MoT colonized seeds in a seed lot) and severity (amount of MoT fungal biomass in the colonized seeds). When disease incidence and/or severity were low, MoT was not detected 100% of the time from known colonized seeds, consequently providing a false negative result.

There are many challenges associated with seed sampling to optimize detection of a plant pathogen. Many PCR-based assays for detection of pathogens in infected seeds are developed relying solely on genomic plant pathogen DNA, and rarely are they validated with diseased seeds and consider a seed sampling strategy and sampling protocol. Consequently,

detection thresholds for pathogens in infected seed are rarely accurate. This study showed evidence of the impact of variation in disease incidence and severity of MoT-infected seeds in a seed lot on the detection threshold. It is critical in establishing pathogen detection thresholds in plant seeds, to consider both disease incidence and severity.

Some organisms release compounds that partially or totally inhibit the PCR reaction, potentially leading to false negative results (Mavrodieva et al. 2004; Louws et al. 1999). Some compounds inhibit the Taq polymerase affecting PCR amplification, degrade the DNA target sequence or interfere with PCR reagents (Louws et al. 1999). In the present study, assay sensitivity was not affected by the presence of possible interfering DNA (e.g. wheat seed DNA, and *Lolium* and *Oryza* populations of *M. oryzae*), even in the presence of 1000 times higher concentrations. The results from the present study verified the specificity to discriminate the *Triticum* pathotype from other *M. Oryzae* pathotypes as well as demonstrated non-interference from the host DNA. In agreement with previous reports (Pieck et al 2017, Yasuhara-Bell et al 2019), this study provides additional evidence of the specificity of the MoT3 locus-based assay in contrast to a report that the MoT3 locus-based assay does not differentiate the *Triticum* pathotype from the *Oryza* pathotype (Gupta et al. 2019). In diagnostics, it is important to follow the complete protocol, not just using the same primers.

In addition, the MoT3 locus-based assay has been used successfully in multiple labs in the U.S. and Bangladesh. One complication with *M. oryzae* is that host of isolation is not always indicative of pathotype. One example of this was the single wheat spike with blast symptoms discovered in Kentucky in 2011 (Farman et al. 2017) that was shown to be due to cross-infection by a *Lolium* pathotype during a period of conducive environment conditions. Consequently, without proper identification, inaccurate conclusions can be drawn from assay

results (Farman et al. 2017; Pieck et al. 2017; Yasuhara-Bell et al. 2018). Recently a new PCR MoT detection assay was developed, in which the MoT 3 locus was used for comparison (Thierry et al. 2019). To date, ~500 *M. oryzae* strains collected from many grass hosts from many countries worldwide were tested to validate its high specificity (99.6% efficacy) to discriminate the Triticum pathotype from other *M. oryzae* pathotypes (Pieck et al. 2017; Yasuhara-Bell et al. 2018; Thierry et al. 2019).

Given current international trade, seed movement is one of the most common ways to introduce aggressive pathogens into new areas (Gauge 2016; Garcia et al. 2008). The first wheat blast outbreak in Bangladesh in 2016 (Callaway 2016; Malaker et al. 2016) was a serious alert for the possible spread of MoT to top wheat producers like India or China, potentially jeopardizing food security worldwide. The need for an accurate detection protocol that discriminates the Triticum pathotype from other *M. oryzae* host populations and based on a solid understanding of the detection threshold with respect to sampling limitations is critical. This study should contribute to the development of more effective detection systems for wheat blast within and on seeds, to inform effective prevention measures when required, and enhance preparedness for an eventual introduction of the wheat blast pathogen into the U.S or any other MoT-free wheat producing country.

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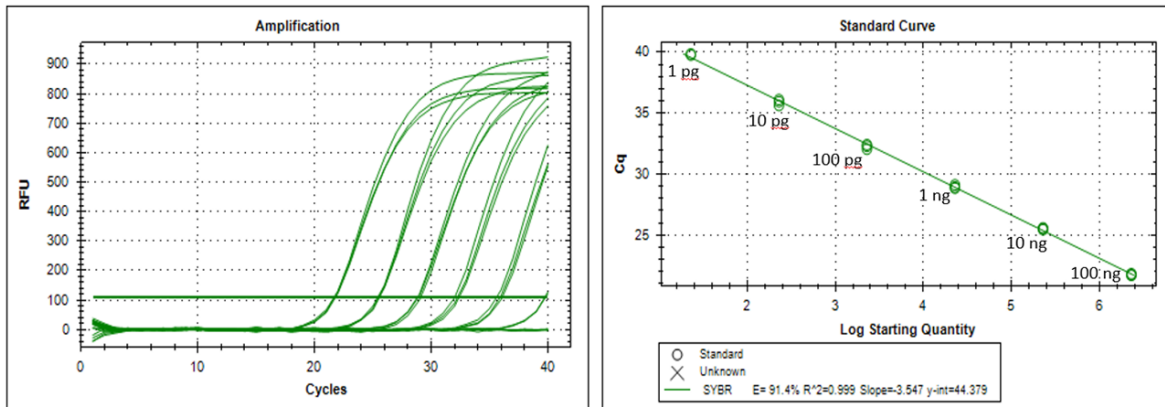
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Figures

Figure 2.1 q-PCR amplification curves and derived standard curve based on a ten-fold concentration gradient of genomic MoT DNA using MoT3-specific primers and probe previously established (Pieck et al. 2017). Calculation of MoT DNA (ng) to MoT cells.



Calculation to convert MoT DNA from ng to MoT cells

1 MoT genome	= 41.03 Mb
1 Mb	= 1 000 000 bp
1 bp	= ~640 Daltons
1 MoT genome	= 2.63E+10 Daltons
1 ng	= 6.02E+14 Daltons
1 copy of MoT-3 locus	= 1 MoT genome
1 copy of MoT-3 locus	= 1 MoT cell
Therefore, 1 MoT cell represents 4.36E-05 ng	

Figure 2.2 Quantification of MoT fungal biomass concentration (MoT cells/seed) from colonized seed lots using the MoT 3 primers specific from a previous established q-PCR protocol (Pieck et al. 2017). For fungal biomass concentration, 1µl DNA sample is quantified by the q-PCR. Followed by conversion to X number of MoT cells/seed.

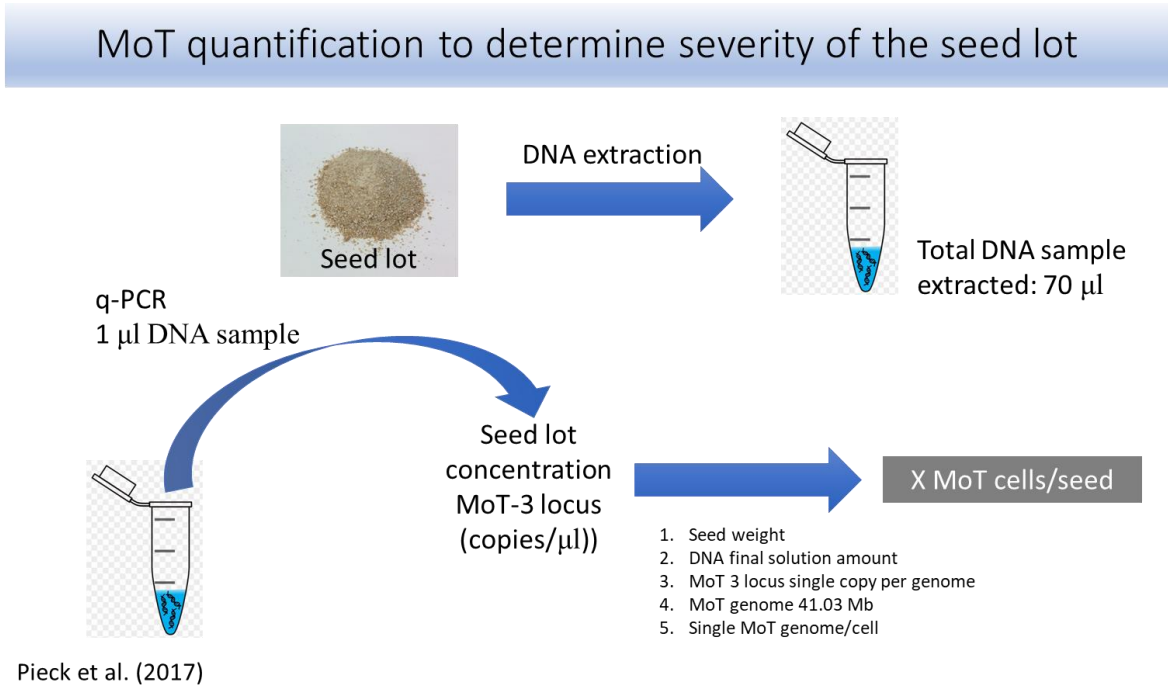


Figure 2.3 MoT detection and quantification of fungal biomass concentration (MoT cells/seed) from seed samples using the MoT 3 primers specific from a previous established q-PCR protocol (Pieck et al. 2017). First, initial concentration from extracted DNA sample was determined from 1 μ l DNA sample. Second, 5 μ l of DNA sampled was tested for detection (end-point PCR) and fungal biomass concentration (MoT cells/seed) was determined.

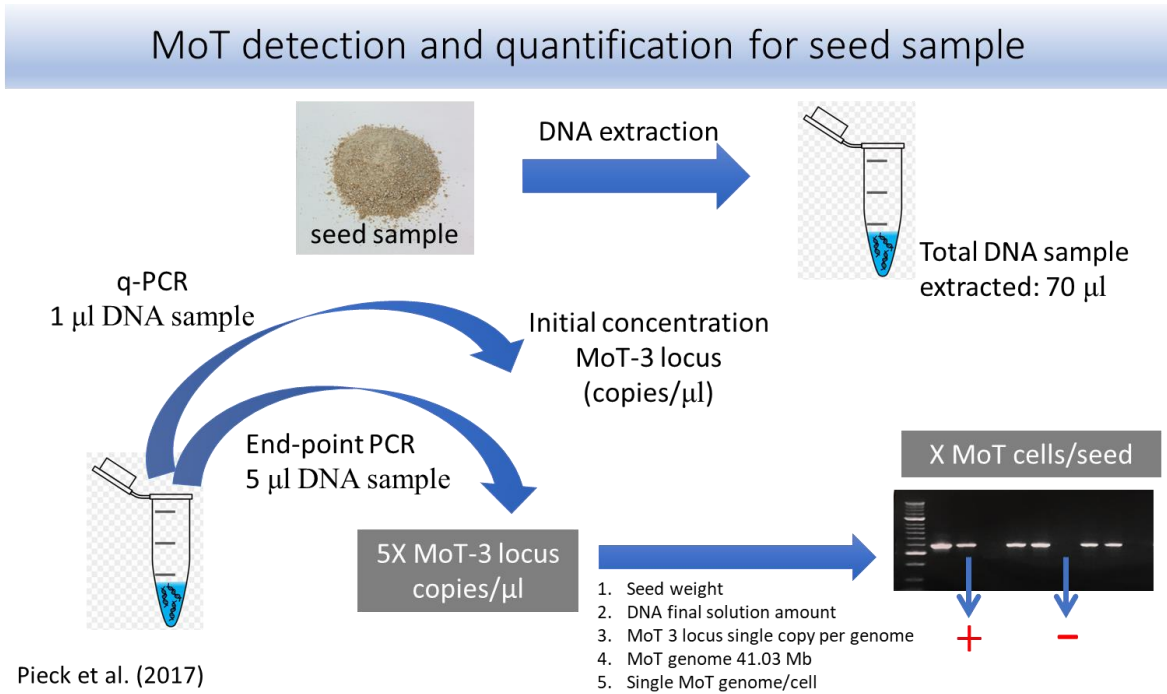


Figure 2.4 Relationship of the MoT fungal biomass concentration as a function of seeds MoT colonization was modeled. Seed lots inoculated with 8×10^4 conidia/ml concentration and incubated from 1-6 days in a moist chamber. Graph represented by the first and second experiment.

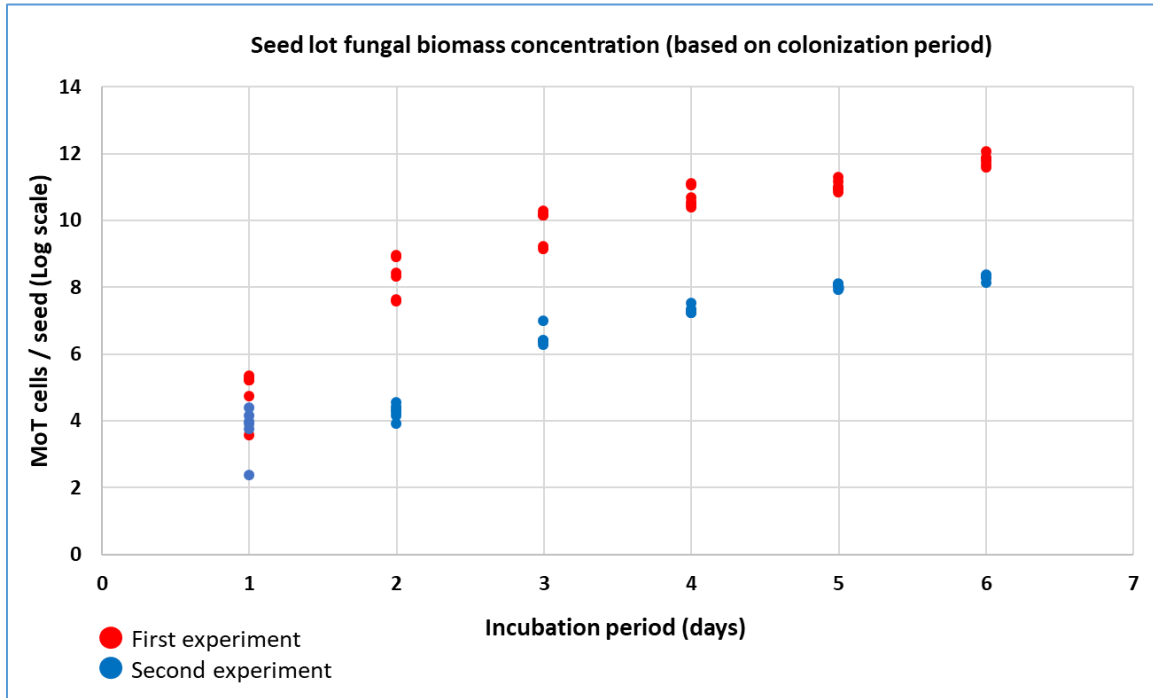


Figure 2.5 MoT fungal biomass quantification of MoT inoculated seed lots as a function of seed colonization period. Experiment conducted twice.

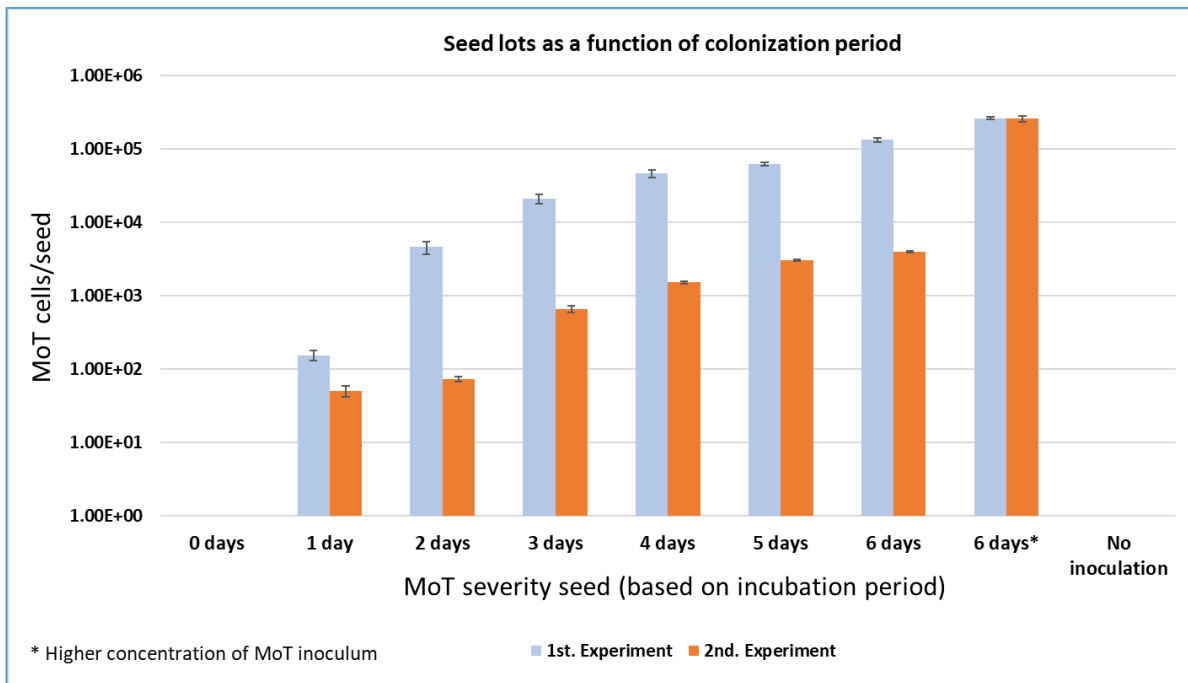


Figure 2.6 Relationship of the MoT fungal biomass concentration as a function of disease incidence and severity of the MoT mix gradient seed samples was modeled. Disease incidence was represented by the ratio (%) of infected seeds in the seed sample. Disease severity was represented by seed lots with different MoT fungal biomass concentration based on colonization period used in the blending (represented by colors). The graph is represented from the first and second experiments.

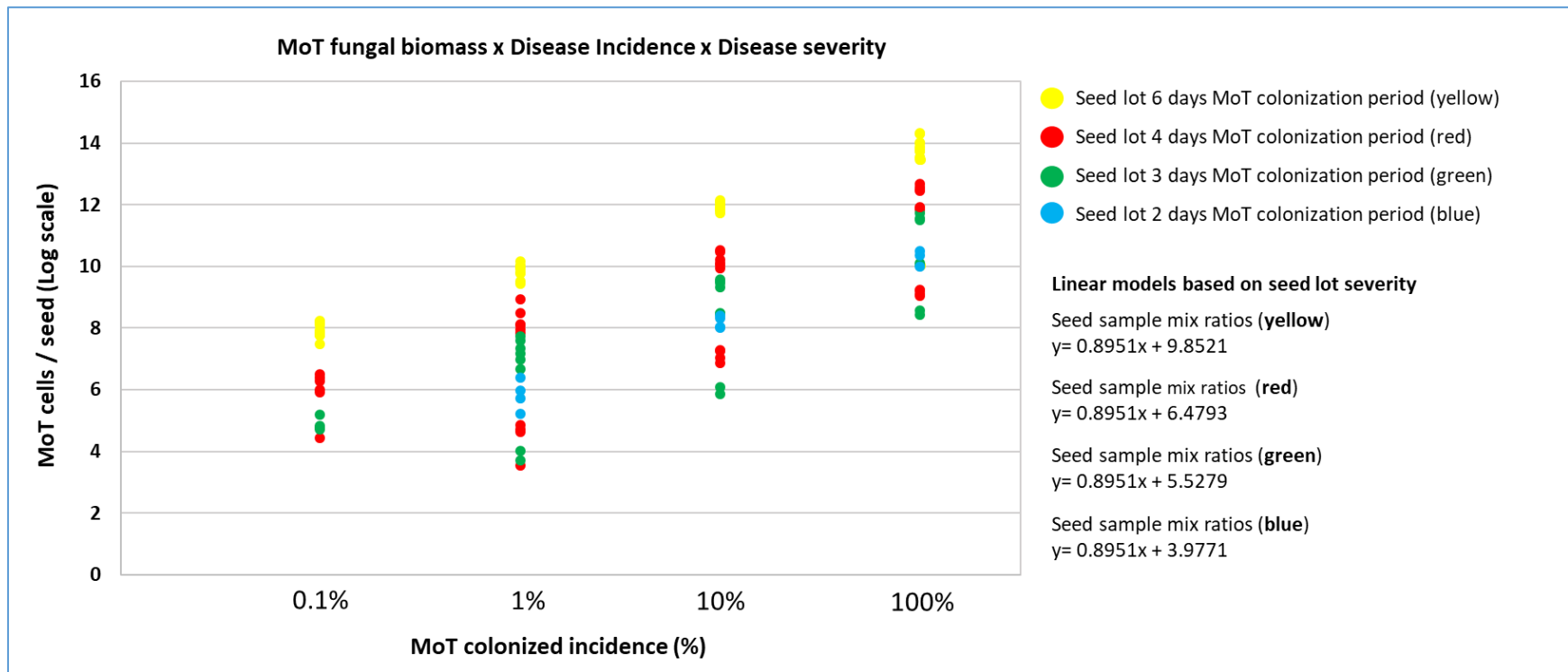


Figure 2.7 MoT detection (end-point PCR) and quantification (q-PCR) as a function of disease incidence and severity in a seed sample was determined. Chart represent a seed sample with the vertical line indicating the MoT colonized seeds incidence ratio and horizontal line indicating seed lot with different MoT fungal biomass concentration based on colonization period used for the mix ratio. Each seed sample was tested for detection and quantification with 3 replications, each replication 2 sub-sample. For detection: a blue line box represents a seed sample detectable 100% of the times tested; red line box represents a seed sample either not detectable (ND) or detectable but not 100% of the times tested. For quantification, fungal biomass concentration for each seed sample was determined by MoT cell/seed. First experiment.

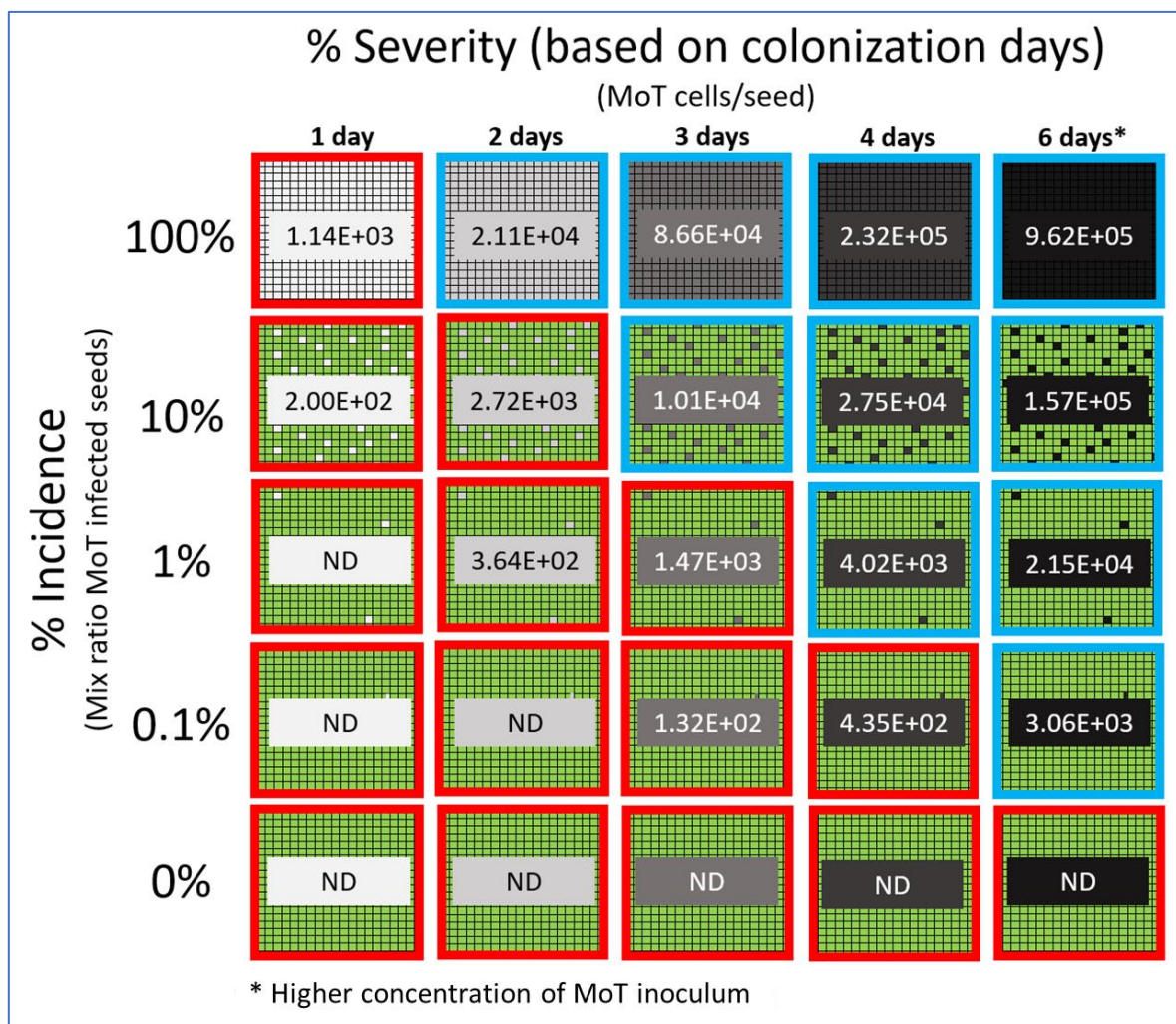


Figure 2.8 MoT detection (end-point PCR) and quantification (q-PCR) as a function of disease incidence and severity in a seed sample was determined. Chart represent a seed sample with the vertical line indicating the MoT colonized seeds incidence ratio and horizontal line indicating seed lot with different MoT fungal biomass concentration based on colonization period used for the mix ratio. Each seed sample was tested for detection and quantification with 3 replications, each replication 2 sub-sample. For detection: a blue line box represents a seed sample detectable 100% of the times tested; red line box represents a seed sample either not detectable (ND) or detectable but not 100% of the times tested. For quantification, fungal biomass concentration for each seed sample was determined by MoT cell/seed. Second experiment.

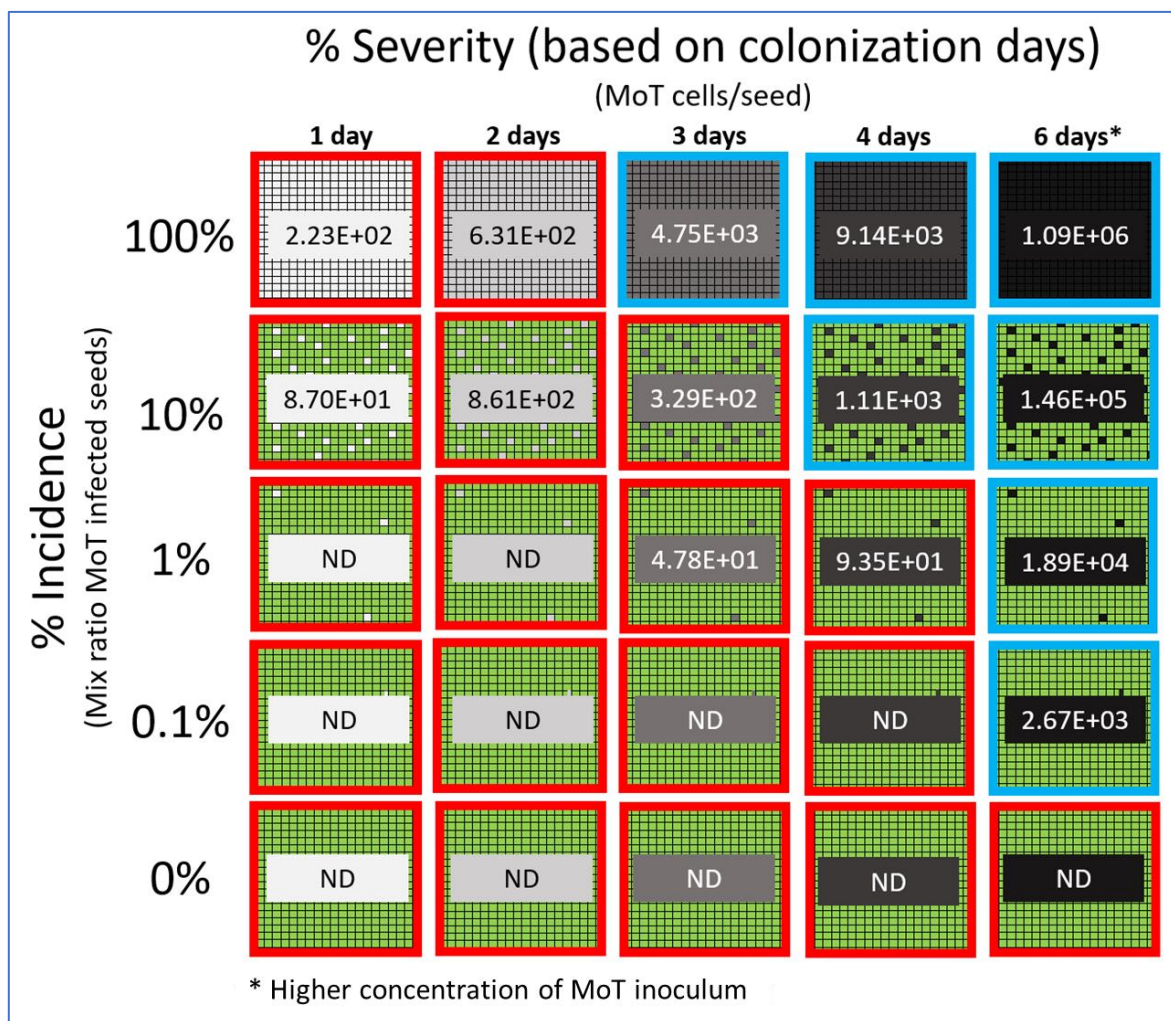


Figure 2.9 Seed samples from the two experiments tested for MoT detection (end-point PCR) and quantification (q-PCR) represented by fungal biomass concentration (MoT cell/seed) as a function of disease incidence, regardless the disease severity.

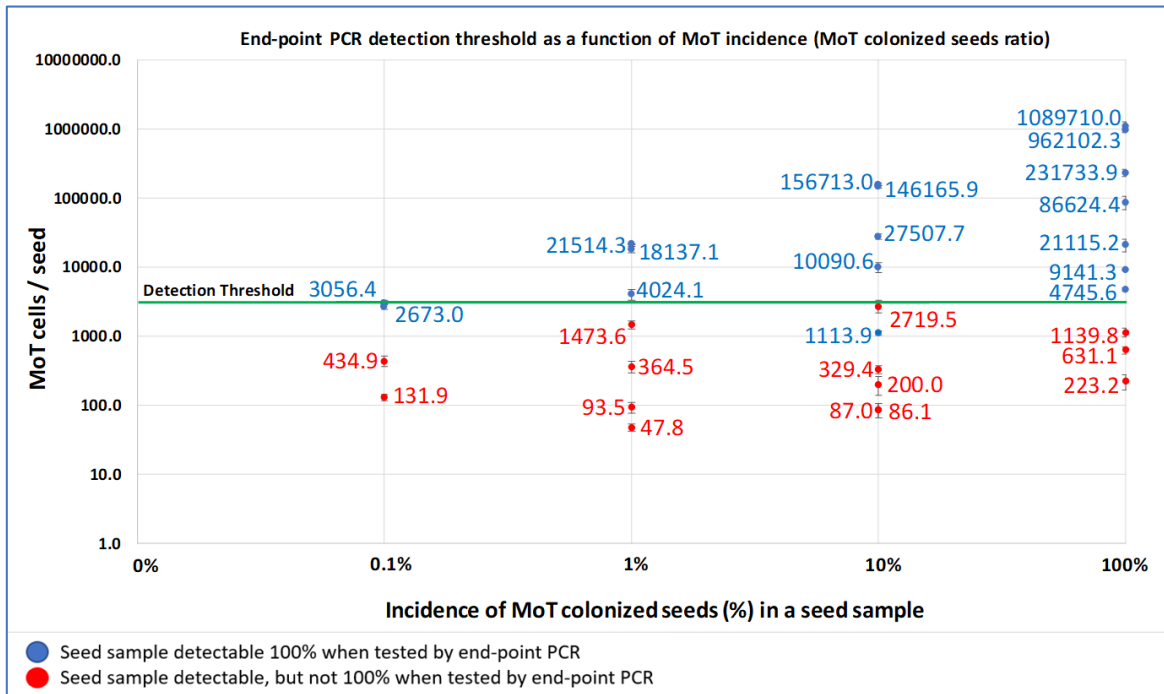


Figure 2.10 Seed samples from the two experiments tested for MoT detection (end-point PCR) and quantification (q-PCR) represented by fungal biomass concentration (MoT cell/seed) as a function of disease severity, regardless the disease incidence.

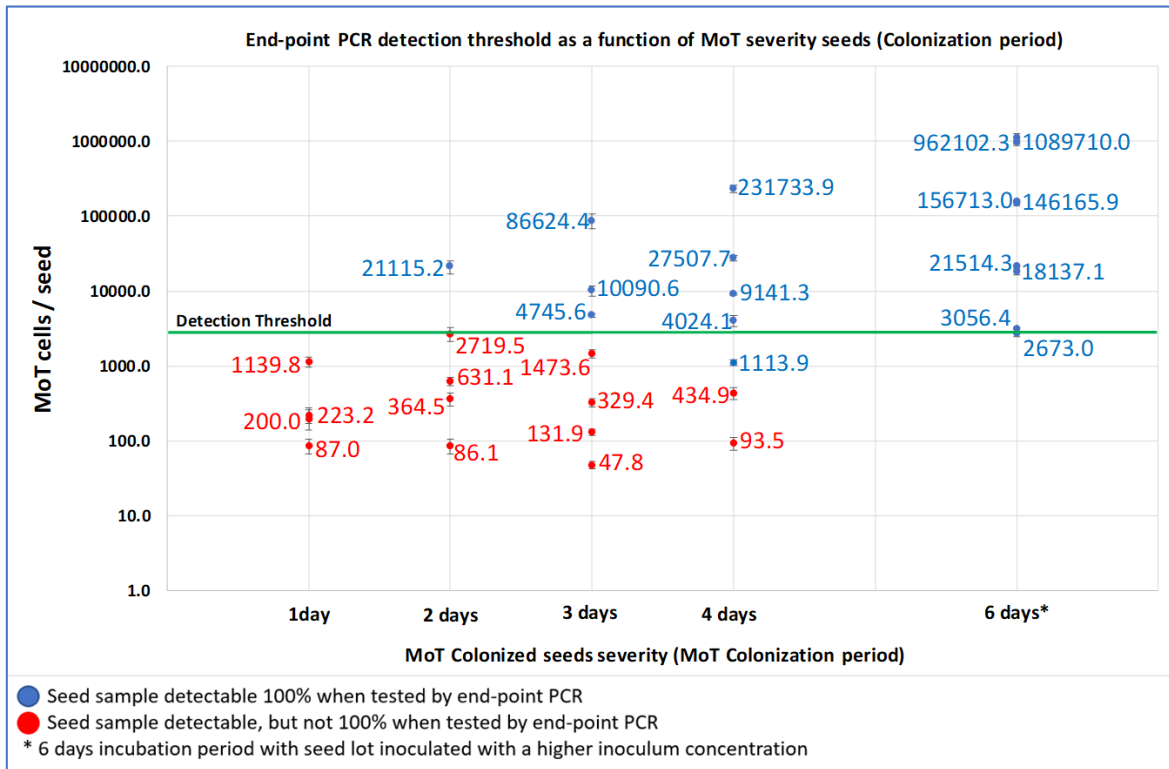
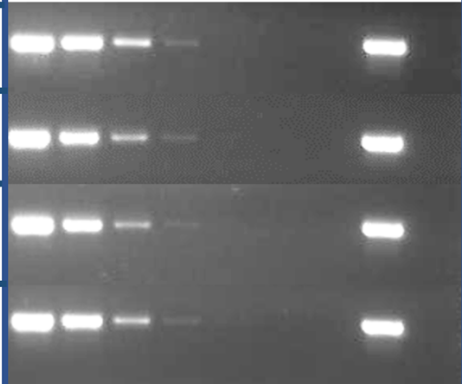
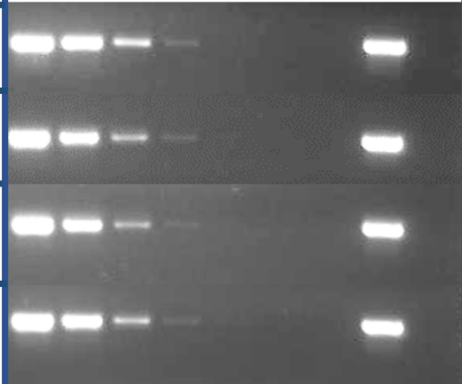
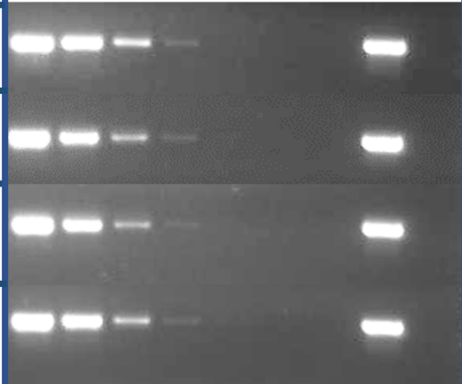
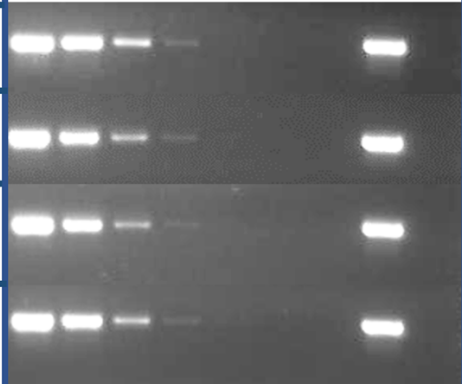
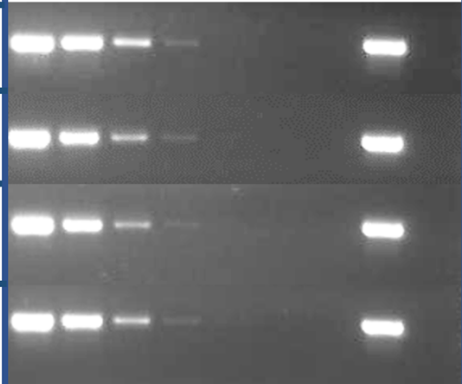


Figure 2.11 The potential for plant host DNA or DNA from rice and ryegrass populations of *Magnaporthe oryzae* to impact PCR assay sensitivity and specificity was determined. Ten-fold serial dilutions of *M. oryzae* Triticum (MoT) DNA were made with increasing concentrations of potentially interfering DNA to hold total DNA concentration constant. DNA from other sources did not affect sensitivity or specificity for the detection of MoT.

DNA mix	1 2 3 4 5 6 7 8 9									Gel line	DNA (ng / PCR reaction)	
											MoT	Other ^a
MoT										1	1.00E+02	0.00E+00
MoT & MoL										2	1.00E+01	9.00E+01
MoT & MoO										3	1.00E+00	9.90E+01
MoT & Seed										4	1.00E-01	9.99E+01
										5	1.00E-02	1.00E+02
MoT & Seed										6	1.00E-03	1.00E+02
										7	0.00E+00	1.00E+02
										8	MoT DNA ^b	
										9	Sterile reagent-grade water	

^a Either genomic MoL, MoO or host DNA

^b Genomic MoT DNA isolate T-25

Tables

Table 2.1 Preliminary experiment. Disease incidence score in inoculated seed lots based on surface disinfection procedures using Bleach (Sodium hypochlorite) in different concentration and time rinsing period. The surrogate pathogen *Magnaporthe oryzae* Lolium pathotype (MoL) was used for seed inoculation.

Seed lot ^a	Bleach concentration	Disinfection rinsing period (minutes) ^b	MoL incidence (%) in seeds ^c	Std. dev.
1	5%	1	2.22	0.91
2	5%	5	5.56	3.27
3	5%	10	1.11	0.91
4	5%	20	3.33	1.57
5	10%	1	0.00	0.00
6	10%	5	1.11	0.91
7	10%	10	0.00	0.00
8	10%	20	0.00	0.00
9	NA	NA	100.00	0.00
10	NA	NA	0.00	0.00

^a Seed lot (60 gr seeds each) inoculated with 7.65×10^5 MoL conidial suspension, except seed lot 10.

^b After rinsed with Bleach followed by rinsed with sterile deionized water twice for 30 seconds each, except seed lot 9 and 10.

^c Each seed lot tested with 3 replications, each replication plated 20 seeds in OMA plate.

^{NA} Not applicable.

Table 2.2 Preliminary experiment. Disease incidence score in seed samples with different incidence of MoL inoculated seeds. The surrogate pathogen *Magnaporthe oryzae* Lolium pathotype (MoL) was used for seed inoculation.

Seed sample	Mix gradient ratio		MoL incidence (%) in seeds ^b	Std. dev.
	MoL colonized seeds ^a	Non-colonized seeds		
1	0	100	0.00	0.00
2	5	95	4.67	0.27
3	10	90	10.00	0.00
4	25	75	24.00	0.00
5	50	50	48.67	0.72
6	75	25	71.33	0.27

^a MoL colonized seeds with 7.65×10^5 conidial suspension concentration and incubated in moist chamber for 2 days.

^b Seed sample tested in Oatmeal agar for incidence with 3 replications

Table 2.3 Preliminary experiment. Disease incidence score and molecular test using a previously established LAMP (Loop mediated isothermal amplification) in MoL inoculated seed lots as a function of colonization period. The surrogate pathogen *Magnaporthe oryzae* Lolium pathotype (MoL) was used for seed inoculation.

Seed lot^a	Moist chamber incubation period^b	LAMP Cycle^c	Std. dev.	MoL Incidence % in seeds (Plating)^d	Std. dev.
1	0 hours	9.14	0.19	78.33	4.91
2	12 hours	11.37	0.71	88.33	5.93
3	24 hours	9.22	0.17	95.00	2.36
4	48 hours	8.51	0.09	100.00	0.00
5	72 hours	7.59	0.10	100.00	0.00
6	Non-incubated	7.48	0.08	100.00	0.00
7	Non-inoculated	0.00	0.00	0.00	0.00

^a Inoculated seed lots (60 gr seeds) except seed lot 7. Seed lots inoculated with MoL conidial suspension with 5×10^5 conidia/ml concentration.

^b After incubation period, colonized seeds left for drying 24 hrs. Seed lots surface disinfected with Bleach 10% (60 seconds) followed by rinsed with deionized sterile water twice (30 seconds each) except seed lots 6 & 7.

^c Lamp assay (Yasuhara-Bell et al. 2018) with 3 replicates for each seed lot; Genomic MoL DNA G-239 isolate used as Positive control (Lamp cycle 7.12; std. error 0.08); Sterile deionized water used as negative control (no amplification).

^d Each seed lot tested for MoL incidence with 3 replications, each replication with 20 seed plated in Oatmeal agar.

Table 2.4 Preliminary experiment. Disease incidence score in inoculated seed lots based on surface disinfection procedures using Bleach (Sodium hypochlorite) in different concentration combined with Ethanol. The surrogate pathogen *Magnaporthe oryzae* Lolium pathotype (MoL) was used for seed inoculation.

Seed lot ^a	Surface disinfection procedure ^b	500 000 conidia/ml		700 000 conidia/ml	
		MoL Incidence (%) ^c	Std. dev.	MoL Incidence (%) ^c	Std. dev.
1	Non-surface disinfection	100.00	0.00	100.00	0.00
2	Non-surface disinfection	0.00	0.00	0.00	0.00
3	Bleach 10% - ddH2O (Rinsed twice)	73.33	6.80	83.33	1.36
4	Bleach 15% - ddH2O (Rinsed twice)	68.33	4.91	88.33	1.36
5	Ethanol 75% - Bleach 10% - ddH2O (Rinsed twice)	0.00	0.00	0.00	0.00
6	Ethanol 75% - Bleach 15% - ddH2O (Rinsed twice)	1.67	1.36	0.00	0.00

^a Seed lots inoculated with MoL conidial spore suspension and then dried for 24 hours, except seed lot 2

^b Ethanol rinsed for 30 seconds, Bleach rinsed for 60 seconds, ddH2O (deionized sterile water) rinsed for 30 secs

^c Each seed lot tested for MoL incidence with 3 replications, each replication with 20 seed plated in Oatmeal agar.

Table 2.5 Preliminary experiment. Inoculated seed lots were tested for disease incidence and detection determined by plating test and LAMP molecular detection tool respectively. Relationship between plating and LAMP test were inferred to estimate the fungal biomass concentration in the seed lots. The surrogate pathogen *Magnaporthe oryzae* Lolium pathotype (MoL) was used for seed inoculation.

Seed lot ^a	MoL Inoculum (spores/ml)	Moist Chamber incubation	Surface disinfection ^b	MoL incidence % (Plating) ^c	Std. dev.	LAMP cycle ^d	Std. dev.
1	7.50E+05	0 days	NO	100.00	0.00	NT	NA
2	7.50E+04	0 days	NO	88.33	6.24	NT	NA
3	7.50E+03	0 days	NO	91.67	6.24	NT	NA
4	7.50E+05	0 days	YES	3.33	2.36	NT	NA
5	7.50E+04	0 days	YES	0.00	0.00	NT	NA
6	7.50E+03	0 days	YES	0.00	0.00	NT	NA
7	7.50E+05	1 day	YES	50.00	4.08	11.78	0.20
8	7.50E+04	1 day	YES	0.00	0.00	NT	NA
9	7.50E+03	1 day	YES	1.67	2.36	19.77	0.03
10	7.50E+05	6 days	YES	93.33	2.36	9.21	0.31
11	7.50E+04	6 days	YES	70.00	17.80	NT	NA
12	7.50E+03	6 days	YES	55.00	10.80	11.37	0.05
13	NA	NA	NO	0.00	0.00	0.00	0.00

^a Seed lots inoculated except seed lot 13. Seed lot based on 60 gr wheat seeds (previously heat treated).

^b After incubation of inoculated seeds, seeds were dried and followed by surface disinfection of ethanol 75% (30 seconds), Bleach 10% (160 seconds) and rinsed with dionezed sterile water twice (30 seconds each).

^c Each seed lot tested for MoL incidence with 3 replications, each replication with 20 seed plated in Oatmeal agar.

^d Based on previous established LAMP protocol (Yasuhara-Bell et al. 2018). Seed lot tested by LAMP test with 3 replications.

NT Not tested

NA Not applicable

Table 2.6 MoT fungal biomass quantification as a function of seed colonization period. Experiment conducted twice.

Seed lot ^a	Inoculum conc. MoT conidia/ml	MoT colonized seed incubation period ^b	1st. Experiment		2nd. Experiment	
			MoT cells / seed	Std. dev.	MoT cells / seed	Std. dev.
1	8x10 ⁴	0 days	ND	NA	ND	NA
2	8x10 ⁴	1 day	1.54E+02	2.51E+01	5.03E+01	8.69E+00
3	8x10 ⁴	2days	4.59E+03	9.17E+02	7.26E+01	5.63E+00
4	8x10 ⁴	3 days	2.10E+04	3.29E+03	6.61E+02	7.58E+01
5	8x10 ⁴	4 days	4.61E+04	5.41E+03	1.51E+03	6.42E+01
6	8x10 ⁴	5 days	6.24E+04	3.94E+03	3.08E+03	7.98E+01
7	8x10 ⁴	6 days	1.34E+05	8.80E+03	3.99E+03	1.13E+02
8	6.5x10 ⁵	6 days	2.64E+05	9.03E+03	2.59E+05	2.62E+04
9	NA	NA	ND	NA	ND	NA

^a MoT inoculated seed lots except seed lot 9.

^b After incubation period, seed lots were surface disinfected with ethanol 75% (30 seconds) - bleach 10% (60 seconds) - rinsed with deionized sterile water twice (30seconds each), except seed lot 9.

ND No detectable

^{NA} Not applicable

Table 2.7 End-point PCR detection threshold as a function of disease incidence and severity of MoT colonized seeds in a seed sample. First experiment.

Seed sample ^a	Seed lot severity source (based on MoT colonization period) ^b	MoT colonized seeds ratio	1st. experiment MoT Incidence-severity mix ratio seed sample		
			MoT cells / seed concentration ^c	Std. dev.	End-point PCR detection ^d
1	1 day	100.0%	1.14E+03	1.78E+02	+/-
2	1 day	10.0%	2.00E+02	6.01E+01	-
3	1 day	1.0%	ND	NA	-
4	1 day	0.1%	ND	NA	-
5	1 day	0.0%	ND	NA	-
6	2 days	100.0%	2.11E+04	4.34E+03	+
7	2 days	10.0%	2.72E+03	5.61E+02	+/-
8	2 days	1.0%	3.64E+02	7.41E+01	-
9	2 days	0.1%	ND	NA	-
10	2 days	0.0%	ND	NA	-
11	3 days	100.0%	8.66E+04	1.94E+04	+
12	3 days	10.0%	1.01E+04	1.61E+03	+
13	3 days	1.0%	1.47E+03	2.05E+02	-
14	3 days	0.1%	1.32E+02	1.36E+01	-
15	3 days	0.0%	ND	NA	-

16	4 days	100.0%	2.32E+05	2.74E+04	+
17	4 days	10.0%	2.75E+04	2.46E+03	+
18	4 days	1.0%	4.02E+03	6.82E+02	+
19	4 days	0.1%	4.35E+02	7.66E+01	-
20	4 days	0.0%	ND	NA	-
21	6 days	100.0%	9.62E+05	7.63E+04	+
22	6 days	10.0%	1.57E+05	6.92E+03	+
23	6 days	1.0%	2.15E+04	2.99E+02	+
24	6 days	0.1%	3.06E+03	2.35E+02	+
25	6 days	0.0%	ND	NA	-

^a Seed lot tested for detection by the End-point PCR and quantified by q-PCR with 3 replications, each replication with 2 sub-samples

^b MoT colonized seed lots based on period colonization, inoculated with 8×10^4 MoT conidia/ml concentration, except seed lot 6 days colonization which was inoculated with 6.5×10^5 MoT conidia/ml concentration

^c Seed sample quantified by q-PCR from previous established protocol (Pieck et al. 2017)

^d "+" Seed sample detectable 100% when tested; "+/-" seed sample detectable but not 100% when tested; "-" seed sample no detectable when tested

ND Not detectable

^{NA} Not applicable

Table 2.8 End-point PCR detection threshold as a function of disease incidence and severity of MoT colonized seeds in a seed sample. Second experiment.

Seed sample ^a	Seed lot severity source (based on MoT colonization period) ^b	MoT colonized seeds ratio	2nd. experiment MoT Incidence-severity mix ratio seed sample		
			MoT cells / seed concentration ^c	Std. dev.	End-point PCR detection ^d
1	1 day	100.0%	2.23E+02	5.40E+01	-
2	1 day	10.0%	8.70E+01	2.03E+01	-
3	1 day	1.0%	ND	NA	-
4	1 day	0.1%	ND	NA	-
5	1 day	0.0%	ND	NA	-
6	2 days	100.0%	6.31E+02	7.69E+01	+/-
7	2 days	10.0%	8.61E+01	1.95E+01	-
8	2 days	1.0%	ND	NA	-
9	2 days	0.1%	ND	NA	-
10	2 days	0.0%	ND	NA	-
11	3 days	100.0%	4.75E+03	2.66E+02	+
12	3 days	10.0%	3.29E+02	3.97E+01	+/-
13	3 days	1.0%	4.78E+01	5.21E+00	-
14	3 days	0.1%	ND	NA	-
15	3 days	0.0%	ND	NA	-

16	4 days	100.0%	9.14E+03	3.25E+02	+
17	4 days	10.0%	1.11E+03	9.50E+01	+
18	4 days	1.0%	9.35E+01	1.78E+01	-
19	4 days	0.1%	ND	NA	-
20	4 days	0.0%	ND	NA	-
21	6 days	100.0%	1.09E+06	1.57E+05	+
22	6 days	10.0%	1.46E+05	7.42E+03	+
23	6 days	1.0%	1.81E+04	1.87E+03	+
24	6 days	0.1%	2.67E+03	2.41E+02	+
25	6 days	0.0%	ND	NA	-

^a Seed lot tested for detection by the End-point PCR and quantified by q-PCR with 3 replications, each replication with 2 sub-samples

^b MoT colonized seed lots based on period colonization, inoculated with 8×10^4 MoT conidia/ml concentration, except seed lot 6 days colonization which was inoculated with 6.5×10^5 MoT conidia/ml concentration

^c Seed sample quantified by q-PCR from previous established protocol (Pieck et al. 2017)

^d "+" Seed sample detectable 100% when tested; "+/-" seed sample detectable but not 100% when tested; "-" seed sample no detectable when tested

ND Not detectable

^{NA} Not applicable

Chapter 3 - Estimating Risk for Seed Dissemination of *Magnaporthe oryzae* Triticum: A Weather X Detection Threshold Approach

Abstract

Wheat blast is an emerging disease caused by the Triticum pathotype *Magnaporthe oryzae* (MoT). This study developed a preliminary risk assessment tool to estimate the likelihood of disseminating MoT in wheat seed based on weather parameters that favor wheat blast development and limits of MoT detection using commercial sampling methods and PCR-based diagnostic assays. A field survey of commercial wheat production farms was conducted in Bolivia during 2013, a wheat blast epidemic year. The effect of planting dates and cultivar susceptibility on disease severity were determined. The preliminary risk assessment tool was based on weather conditions during epidemic and non-epidemic years, previously published studies on MoT infection of wheat seeds during epidemics, a relative analysis of the Blotter seed test with PCR assays for MoT detection in seed and practical field implications. Planting date and cultivar susceptibility significantly ($P = <0.0001$) affected disease incidence and severity. Late planting of a resistant wheat genotype resulted in ~90% less disease in the field with and consequently higher yield and seed quality. The combination of high rainfall accumulation (67.7 mm) and prolonged high relative humidity (>80% for 18 hrs.) during the heading stage was associated with epidemic blast development. A previously published study provided evidence of a positive correlation between wheat blast disease (spike incidence %) in the field and the incidence of infected seeds. Given a hypothetical uniform infected seeds distribution and fully efficient sampling method, the PCR detection threshold of 0.1% of MoT infected seeds in a seed sample represent up to 20 kg from a 20 metric tonnes harvest wagon will go undetected, equivalent to 6.66×10^5 infected seeds. In similar hypothetical condition, the calculated threshold for the Blotter test was equivalent up to

1600 kg from a 20 metric tonnes or 5.3×10^7 infected seeds will go undetected. Consequently, to minimize the risk of pathogen dissemination, seed from MoT endemic areas should not be shipped to non-MoT infested areas during MoT epidemic years. It is crucial to complement seed inspections with field assessments to decrease the likelihood of MoT dissemination through movement of infected seeds to non-MoT established areas.

Introduction

Wheat is one of the main crops for food security worldwide. This cereal is the most widely grown crop globally and second for protein and calories provided for human diet requirement (FAO 2014). By the increasing population, it is crucial to assure and increase food production to satisfy the global demand, where by 2050 will be ~9 billion habitants (Tilman et al. 2011). The introduction of plant pathogens into new areas could cause plant disease outbreak that could affect considerably the ecosystem and food crop production on determined regions (Palm 1999; Pimentel et al. 2000; Stack et al. 2006). Sometimes the introduction of these new pathogens could be overlooked because they do not get to survive into the new environment, however sometimes they adapt and disease outbreaks occur with significant consequences (Rossman 2001). The recent introduction of *Magnaporthe oryzae* Triticum pathotype (MoT) to Bangladesh, causing wheat blast on wheat, is a good example of a new plant pathogen introduction into a new area with terrible consequences and the potential risk to spread to top wheat producers like India and China (Sadat and Choi 2017).

Wheat blast is caused by fungal pathogen ascomycetous *Magnaporthe oryzae* Triticum pathotype (MoT) (synonymous with *Pyricularia oryzae*) (Couch et al. 2005). *Magnaporthe oryzae* species are divided by sub-species called pathotypes, which is basically determined by the primary host-specificity infection (Couch et al. 2005; Farman 2002; Heath et al. 1990; Tosa et al. 2004).

However, some *Magnaporthe oryzae* species are reported to infect secondary hosts in a reduced pathogenicity level (Farman 2002; Kohli et al. 2011; Tosa et al. 2004). The MoT was reported for the first time in Brazil in 1985, and since then was spread to the neighboring countries (Nunes Maciel 2011; Cabrera et al. 2007; Toledo and Barea 1996; Viedma 2005). MoT was restricted to South America until 2016, when the cereal pathogen was reported in Bangladesh for the first time (Callaway 2016; Malaker et al. 2016). A phylogenetic analysis made from an MoT strain isolated from an infected tissue collected in Bangladesh suggested a close relationship with the South America MoT lineage (Islam et al. 2016; Malaker et al. 2016).

The most important symptom of wheat blast is the blasted spike, which is caused by the infection on the rachis and blocking passage of nutrients from the point the infection and above (Cruz and Valent 2017). However, MoT is able to infect mostly aerial part of the plant including stem, leaves, seeds, glumes and awns, forming elliptical lesions with a grey center where sporulation occurs (Cruz and Valent 2017). The optimal weather conditions for wheat blast epidemic are the combination of high temperature between 25-30 °C, excessive rainfall, high humidity and prolonged tissue wetness (Goulart et al. 2007; Cardoso et al. 2008). Only the combination of high temperature and prolonged spike wetness could cause an MoT epidemic (Cruz and Valent 2017). Once the MoT conidium land to the plant, it produces a solution called spore tip mucilage which function is to attach to the tissue surface (Hamer et al. 1988). After the conidium germinates, eventually the germ tube swells and appressorium is formed for penetration, therefore the infection occurs (Cruz and Valent 2017). For infection, it is critical the presence of standing water on the surface, such as spike wetness, to activate the appressorium to produce a high turgor pressure (80 times atmospheric pressure) to allow penetration and consequently, tissue colonization (Howard et al. 1991; Cruz and Valent 2017). MoT is seedborne and able to

disseminate from infected spike to seed, and from infected seed to seedling (Cruz and Valent 2017). The high losses arise when the infection occurs early in the spike stage between the heading and flowering stage (Goulart et al. 2007). The most infected seeds could get small, shriveled and low-test weight (Goulart et al. 2007; Cruz and Valent 2017). As early the infection occurs at the wheat spike stage, the most infected seeds or grains would be discarded by the combine at the harvest (Goulart et al. 2007). Based on the first MoT epidemic occurred in Bangladesh, where within a single crop season, many districts reported fields with the typical spike blasted symptoms, it is believed the MoT was introduced by contaminated seeds (Islam et al. 2016; Malaker et al. 2016; Sadat and Choi 2017).

In temperate regions, studies suggest freezing temperatures as one of the limiting factors for *Magnaporthe oryzae* species reproduction and infection (Kapoor and Singh, 1978; Latin and Harmon, 2003). Severe low temperatures might affect MoT overwintering survival (Fisher 2016). On the other hand, tropical and sub-tropical regions like South America's countries and Bangladesh, moisture is likely the most limiting factor for MoT epidemic (Fernandes et al. 2017). For MoT control management in countries where the MoT is established, the use of resistant cultivars is one of most effective method to mitigate MoT infection (Cruz and Valent 2017). A strategy based on delaying the planting date to avoid matching with raining periods and high relative humidity to favor MoT epidemic showed a considerable MoT infection decline (Mehta et al. 1992). Also is suggested that late maturity cultivars could mitigate MoT infection delaying its heading stage that avoids humid periods that favors MoT infection (Goulart et al. 2007). MoT control through fungicides application showed inconsistent under high conducive weather conditions (Goulart et al. 2007; Urashima et al. 2009; Cruz and Valent 2017).

A reliable diagnostic method with high sensitivity and specificity detection level is necessary due to the fact that any final diagnoses (either positive or negative diagnose) could derive for actions that could generate economic, political and ecologic impacts in determined regions (Stack et al. 2014). Today, there are many plant disease diagnostic laboratories that still rely on the traditional method of the Blotter test (Neegard 1979) for MoT detection on seeds. The Blotter test defines the incidence of MoT infected seeds based on the presence of fungal reproductive structures (MoT sporulation) on the seed surface tested through a dissecting microscope. In Brazil, the MoT tolerance threshold established for certified seeds is 10% based in the Blotter test (Abrates, 1992; Goulart et al, 1995). However, the Blotter test for MoT infected seeds could present several limitations: it is time consuming and takes several days to release a result (Goulart et al, 1995); practically impossible distinguish between the *Triticum* pathotype from other *Magnaporthe oryzae* species; the MoT, like many other many fungi, needs to be alive. In addition, it is known the MoT lose viability in the infected seeds over the time (Goulart and Paiva 1993). MoT has a slow sporulation performance in the seed and could be easily overcome in presence of any other common fungal contaminant in the infected seed making difficult for MoT detection. The Polymerase Chain Reaction (PCR), is one of the most important technologies developed in the last century due to its multiple applications. The PCR is a molecular tool that rapidly was adapted for plant disease studies since its high sensitivity and specificity allows more accurate and rapid identification of plant pathogens (Kerkoud et al. 2002; Barros et al. 2001; Harmon et al. 2003). A recent (PCR)-based molecular diagnostic tool was developed with high sensitivity and specificity for MoT DNA detection (Pieck et al. 2017). In the present thesis (Chapter 2), it was used this PCR tool to optimize the MoT detection thresholds for MoT infected seeds. This study showed the PCR tool is able to detect as low as 0.1% incidence of MoT infected seeds in a seed

sample; however, it was shown the detection sensitivity could vary based on the severity extent in the MoT infected seed.

Based on the actual trade system worldwide, several introductions of plant pathogens to new areas occurred through contaminated derived plant commodities (Fletcher et al. 2006; Kim et al. 2003; Williamson et al. 2002). One of the most common ways of plant pathogens introduction is through contaminated seeds where shown symptomless, and established detection systems are not able to detect them (Gaije 2016; Garcia et al. 2008). Nowadays, Bangladesh is the only country outside of South America where a MoT outbreak is officially reported (Callaway 2016; Malaker et al. 2016). Considering wheat is the most grown crop in the world (FAO 2014) and key for food security worldwide, the recent wheat blast outbreak in Asia turned on the concern about the MoT to continue the spread worldwide and reaching some of the top wheat producers like China, India, USA, Russia, and France.

Today there is not established parameters to move MoT infected seeds to a non-MoT established area. Therefore, in case of some seed test for MoT is made, it is basically based on the presence or absence of the pathogen, which if it results negative, seeds could be moved, otherwise should be rejected. It is critical to understand the practical implications of the likelihood of MoT dissemination through infected seeds. The goal of the present study was to build up a preliminary risk assessment to develop some guidelines to minimize the probabilities of moving MoT infected seeds.

Material and methods

Field survey for MoT infection

Field surveys were conducted during the winter growing season (April – September) in 2013 in the department of Santa Cruz, Bolivia. Santa Cruz is the main wheat producer department

in Bolivia (Figure 3.1). The field surveys were conducted on commercial wheat production farms in Okinawa 1 county, located 80 km from Santa Cruz de la Sierra (Figure 3.1). Okinawa 1 is considered a natural “hotspot” for wheat blast due to high temperatures and high humidity during the wheat growing season. In total, 17 commercial wheat fields were surveyed. Each field was approximately 20-30 ha. Cultural management decisions (i.e. cultivar selection, planting date, fertilization, herbicide, insecticide and fungicide applications, etc.) for each commercial field were made by the landowner farmer. None of the surveyed fields were irrigated; 100% of the water required for crop growth was provided by natural rainfall. The cultivars planted on the surveyed commercial fields were 110-120 days for maturity. The surveyed fields were classified into two groups based on their planting dates. The “Early planting date” group comprised fields planted between 04-16-2013 and 04-30-2013 (15 days planting window). The “Late planting date” group comprised fields planted between 05-11-2013 and 05-25-2013 (15 days planting window).

All surveyed fields were evaluated for disease incidence, disease severity and yield. Disease incidence (%) and severity (%) was recorded on infected spikes at two moments at the grain filling stage: soft dough stage and before maturity stage (ripening stage). The first incidence and severity evaluation (soft dough stage) was made in 6 sample sites from each surveyed commercial field. The second incidence and severity evaluation (ripening stage) was made in 4 sample sites from each surveyed commercial field, as the same number of points for harvest for yield and quality seed evaluation. Disease incidence was determined by the average number of infected spikes with blast symptoms divided by the total number of spikes evaluated. Disease severity was determined by the proportion of the spike with blast symptoms. A spike with no blast symptoms was considered 0% spike severity, an infected spike with $\frac{1}{4}$ blasted, was considered 25% spike severity; an infected spike with $\frac{1}{2}$ blasted, was considered 50% spike severity; an

infected spike with $\frac{3}{4}$ blasted, was considered 75% spike severity; an infected spike with all the spike blasted, was considered 100% spike severity. For yield and seed quality evaluations, seeds were harvested in a 2x2 meter quadrat at each sample site during the last disease evaluation for each surveyed field. Average yield (metric tons/hectare), 1000 seeds weight (grams) and test weight (%) were determined. Test weight was calculated as the weight (grams) of seeds in a volume of 1 liter (1000 ml); e.g. if the seeds weight in a volume of 1 liter is 800 gr, the test weight will be 80%.

Weather conditions

Weather data was collected from a single weather station located at the CAICO (Integral Cooperative Agricultural Colonies of Okinawa) experimental research station located in Okinawa 1; data from the CAICO weather station was representative of all the surveyed commercial fields. The distance was considered between the location of each surveyed commercial field and the weather station. The weather parameters analyzed for each planting date group, were those considered favorable for MoT infection: rainfall accumulation (millimeters), temperature (average number of hours/day between 25-30°C) and relative humidity (average number of hours/day >80%) during the heading stage of wheat development (most critical stage for MoT infection in wheat) (Goulart et al. 2007; Cardoso et al. 2008).

In addition, weather data at the heading stage for the same planting date groups (early and late) were analyzed for 2011, 2012 and 2014, non-MoT epidemic years. In Bolivia, similar to other MoT-established countries in South America (tropical region), spring wheat is planted during the crop winter season. The heading stage period was calculated for each planting date group for 2011, 2012 and 2014, using growing-degree accumulation days for spring wheat (NDAWN Wheat Growing Degree Days Help) (Bauer et al. 1984) and adapted to the cultivars planted on the

commercial fields for this study. A qualitative epidemic assessment was made based on disease incidence before the ripening stage. Fields affected by wheat blast based on the planting date (either early or late) was defined as a severe epidemic if there were fields with susceptible cultivars that reached an MoT spike incidence in the field >50% incidence and caused >50% yield reduction from the average yield based on the cultivar. Fields affected by wheat blast based on the planting date (either early or late) were defined as moderate epidemic if there were fields with susceptible cultivars that had MoT spike incidence in the field 20-50% and caused 20-50% yield reduction from the average yield based on the cultivar. Fields affected by wheat blast based on the planting date (either early or late) were defined as low epidemic if there were fields with susceptible cultivars that had MoT spike incidence in the field <20% and caused <20% yield reduction from the average yield based on the cultivar. Qualitative epidemic assessment for 2013 was based on quantitative data from the conducted field survey, for 2011, 2012 and 2014 were based on personal communication with field technicians from the cooperative CAICO and Fundacion Cetabol, located in Okinawa 1 county.

Relationship between wheat blast disease in the field and MoT infection in seeds – Goulart et al. 1995.

For this study, we relied on the paper “Relationship between MoT incidence in wheat spike and MoT incidence from collected seeds” previously published from Goulart et al, 1995 (Translated from “Relacao entre a incidencia da brusone em espigas de trigo e a presence de *Pyricularia grisea* nas sementes colhidas”). This study was conducted in Itapora county, from Matto Grosso state, Brazil. The field experiments were conducted in 1990 (two experiments) and 1991 (three experiments) in two planting dates each year, in total 5 field experiments. For each field experiment was tested between 16-20 wheat genotypes (between cultivars and breeding

lines). The experimental design for each field experiment was a randomized block design with 4 repetitions. The incidence was determined by the number of infected spikes average based on the rachis dark lesion, over the total number of spikes. The infection occurred naturally, and the spikes were harvested and threshed manually, which means that almost all seeds were collected and kept from each treatment, even the high affected seeds that looked shriveled, small with low test weight. The MoT incidence for infected seeds was determined by the Blotter test (Needegard 1979). The moist chamber was based on a petri plate with 3 layers sterilized filter paper and moisten with sterilized water. The water used for moisture, it was added a 0.02% of 2,4 D herbicide solution to avoid germination on seeds. For each genotype wheat was plated 400 seeds incubated for seven days at 22°C temperature and 12/12 light/dark hours environment. After 7 days incubation period, the seeds were evaluated based on MoT sporulation on seeds determining incidence (%) of the number of infected seeds with MoT presence over the total seeds.

Implications of the MoT detection threshold from the PCR and Blotter method for infected seeds in a seed sample

This study was based on the practical implications of the MoT detection threshold from the PCR and the Blotter test might represent in the field. The detection threshold for the PCR method was obtained from the present MS thesis, chapter 2 (2019). According to chapter 2 of the present study, the PCR detection threshold for MoT was 0.1% incidence of MoT infected seeds in a seed sample. The detection threshold for the Blotter method was based on the previous established study from Goulart et al. (1995). The Goulart et al. (1995) study established the Blotter method detection threshold for MoT was infected seeds coming from MoT affected field with 8.2% spike incidence in the field (i.e. MoT infected seeds produced from fields with less than 8.2% spike incidence, the Blotter method might not be able to detect it). Based on the Goulart et al. (1995) study, it was made

a hypothetical calculation, where it was assumed the same MoT spike incidence in the field, it might represent the same proportion of infected seeds produced from this field in a seed sample. Based on the detection threshold of the Blotter and PCR methods, it was calculated the number of infected seeds might be below the detection threshold.

Statistical analysis

Data analyses were performed using SAS version 9.2 (SAS Institute, Cary, NC, 2001). Analysis of field survey data for MoT infection included planting date, cultivar MoT susceptibility and the interaction of planting date X cultivar MoT susceptibility. Disease incidence (%) and disease severity (%) at the soft dough and at ripening stage, yield (tons/hectare), 1000-seed weight (grams), and test weight (%) were analyzed by standard ANOVA, using SAS GLIMMIX procedure.

Results

Field survey for MoT infection

Planting date, cultivar, and the interaction of planting date and cultivar each had effects on the parameters evaluated for the seventeen commercial wheat production fields (Table 3.1). Disease incidence and disease severity were different in fields planted early with a susceptible cultivar than fields planted late ($P < 0.0001$); in addition, the same trend followed with yield and seed quality ($P < 0.01$) (Table 3.1). For susceptible cultivars, the planting date effect was substantial at both evaluation times. At the soft-dough stage, disease incidence average was 53% and 4% for early and late-planted susceptible cultivars, while disease severity average was 44% and 3% for early and late-planted susceptible cultivars, respectively (Figure 3.2 and Table 3.2). At the ripening stage of wheat development, disease incidence average was 88% and 10% for early and late-planted susceptible cultivars, while disease severity average was 82% and 7% for early and late-

planted susceptible cultivars, respectively (Figure 3.3 and Table 3.3). The late planting date resulted in 90% less disease than the early planting date. The same trends were observed for both yield and seed quality with respect to effects of planting date on susceptible cultivars. Fields planted early with a susceptible cultivar had lower yield and seed quality average compared to fields planted late; yield = 1.0 ton/ha vs 3.4 tons/ha, 1000-seeds weight = 20.4 gr vs 33.8 gr, and test weight = 65.4% vs 79.8%, for early and late planting date, respectively (Figures 3.4, 3.5 & 3.6 and Table 3.4).

At both the soft dough and ripening stages of wheat development, resistant cultivars had ~90% less disease (spike incidence and severity) than susceptible cultivars when both were planted early (Table 3.2 & 3.3 and Figure 3.2 & 3.3). Disease incidence average (3% versus 5%) and disease severity average (2% versus 3%) remained low and were no difference in resistant cultivars at the soft dough and ripening stages ($P>0.05$), respectively (Table 1, 2 & 3 Figures 2 & 3).

Similarly, there was no evidence that planting date had an effect on any yield parameters for resistant cultivars ($P>0.05$). At the early planting date, yield and seed quality average for the resistant cultivars were different ($P<0.01$) than for the susceptible cultivars; yield = 1.0 ton/ha versus 2.7 tons/ha; 1000-seeds weight = 20.4 gr vs 36.8 gr; test weight = 65.4% vs 82.0%, respectively, for susceptible and resistant cultivars (Table 3.1 & 3.4 and Figures 3.4, 3.5 & 3.6).

There was no planting date effect with resistant cultivars ($P>0.05$) but there was a planting date effect with susceptible cultivars ($P<0.01$). The disease infection on the field, either in the soft dough or ripening stage, fields with a resistant cultivar planted late represented a ~98% less disease (spike incidence and severity) than fields with a susceptible cultivar planted early. In addition, the difference in yield and seed quality average between fields with a susceptible cultivar planted early was lower than fields with a resistant cultivar planted late ($P<0.01$) (yield of 1.0 ton/ha vs 3.0

tons/ha; 1000 seeds weight of 20.4 gr vs 38.2 gr; Test weight of 65.4% vs 79.8%) (Table 3.1 & 3.4 and Figures 3.4, 3.5, & 3.6).

There was no evidence of differences in disease, yield or seed quality average among fields with a susceptible cultivar planted late, fields with a resistant cultivar planted early, or fields with a resistant cultivar planted late ($P > 0.01$) (Table 1); all these fields had low disease incidence and severity, high yield, and high quality seeds (Figure 3.2, 3.3, 3.4, 3.5 & 3.6).

Weather conditions

The weather station was located in Okinawa 1 in CAICO research station ($17^{\circ}14'30.8''S$ $62^{\circ}53'18.3''W$). The distance from the weather station and the farthest surveyed commercial field was 9.05 miles, meanwhile the closest surveyed field distance was 0.98 miles. The distance average from the weather station and all commercial surveyed fields was 5.02 miles and the median distance was 6.21 miles. The weather conditions that favor wheat blast development during the heading stage included warm temperatures ($25-30^{\circ}C$), high humidity (several hours at $RH > 80\%$) and excessive rainfall accumulation (Goulart et al. 2007; Cardoso et al. 2008). The heading stage is the most critical stage for wheat blast development (Goulart et al. 2007). The parameters for this study were based on the rainfall accumulation (mm), temperature $25-30^{\circ}C$ (accumulation hours/day) and $>80\%$ RH (accumulation hours/day) during the heading stage period.

During the wheat growing season of 2013, a high MoT epidemic year, the heading stage period for the early planting date was between 06-21-2013 and 07-12-2013 (21 days) (Figure 3.9). During this period, rainfall accumulation was 67.7 mm, there were 2.7 hours/day average of MoT-conducive temperatures, and 18 hours/day average of MoT-conducive RH (Figure 3.11). The heading stage period for the late planting date was between 07-19-2013 and 08-08-2013 (20 days) (Figure 3.9). Rainfall accumulation in this period was 14.9 mm, 3.4 hours/day average of MoT-

conducive temperatures, and 7.0 hours/day average of MoT-conducive RH (Figure 3.11). The combination of high relative humidity and high rainfall favored MoT infection in those fields that were planted early with a susceptible cultivar. During the early and late heading stage period, similar temperatures occurred; the temperature was not a limiting factor for MoT infection (Figure 3.9 & 3.11).

Weather conditions and wheat blast development in 2013 were compared to the weather conditions and disease development in 2011, 2012 and 2014 (Figure 3.11). Field technicians from CAICO, a cooperative formed by local farmers, and from Fundacion Cetabol, indicated that 2011 from fields planted late, and 2012 and 2014 from fields planted early were considered moderate epidemic periods. Years in 2011 from fields planted early, and 2012 and 2014 from fields planted late were considered low epidemic periods.

Based on the accumulation of growing degree days (GDD) in 2011, the heading stage period for local cultivars and early planting date was between 06-18-2011 and 07-12-2011 (24 days) (Figure 3.7). During that period, the rainfall accumulation was 13.9 mm and the average hours/day for temperature and RH favorable to MoT was 2.7 and 5.4 hours, respectively (Figure 3.7 & 3.11). The heading stage period for the late planting date was between 07-18-2011 and 08-08-2011 (21 days) (Figure 3.7). During that period, the rainfall accumulation was 58.3 mm and the average hours/day for temperature and RH favorable to MoT was 3.0 and 8.9 hours, respectively (Figure 3.7 & 3.11).

Based on the accumulation of growing degree days (GDD) in 2012, the heading stage period for local cultivars and early planting date was between 06-22-2012 and 07-13-2012 (21 days) (Figure 3.8). During that period, the rainfall accumulation was 40.1 mm and the average hours/day for temperature and RH favorable to MoT was 3.8 and 12 hours, respectively (Figure

3.8 & 3.11). The heading stage period for the late planting date was between 07-21-2012 and 08-08-2012 (18 days) (Figure 3.8). During that period, the rainfall accumulation was 3.1 mm and the average hours/day for temperature and RH favorable to MoT was 4.8 and 5.8 hours, respectively (Figure 3.8 & 3.11).

Based on the accumulation of growing degree days (GDD) in 2014, the heading stage period for local cultivars and early planting date was between 06-26-2014 and 07-19-2014 (23 days) (Figure 3.10). During that period, the rainfall accumulation was 62.8 mm and the average hours/day for temperature and RH favorable to MoT was 4.2 and 11.1 hours, respectively (Figure 3.10 & 3.11). The heading stage period for the late planting date was between 07-28-2014 and 08-14-2014 (17 days) (Figure 3.10). During that period, the rainfall accumulation was 14.5 mm and the average hours/day for temperature and RH favorable to MoT was 4.9 and 9.8 hours, respectively (Figure 3.10 & 3.11).

Based on the 4-year weather conditions (2011, 2012, 2013 and 2014), the combination of high rainfall accumulation and prolonged RH >80% during the heading stage period was critical for high blast development in the field in 2013 when it was planted early with a susceptible cultivar. Rainfall accumulation during the late heading stage period of 2011 and the early heading stage period of 2012 and 2014 was adequate but the hours/day of RH >80% was insufficient to support a severe blast outbreak (Figure 3.11). Similarly, low rainfall accumulation and either low or intermediate hours/day RH >80% during the early heading stage periods of 2011 and the late heading stage periods of 2012 and 2014 were inadequate to support a severe MoT epidemic (Figure 3.11). In 2013, the late heading stage showed a low accumulation of rainfall and low RH accumulation hours/day that made a low epidemic of wheat blast. The temperature during the 4 years was similar, fluctuating between 2.7 and 4.8 hours/day at 25-30°C (Figure 3.11). This study

provides more evidence that humidity is a more limiting factor for a wheat blast epidemic than temperature in tropical regions like Santa Cruz-Bolivia, as stated in the publication of Fernandes et al. 2017.

**Relationship between wheat blast disease in the field and MoT seed infection
(data and results from Goulart et al. 1995)**

In the study of Goulart et al. (1995), there was a highly positive correlation ($R^2=0.88$) between wheat blast incidence in the field and the incidence of MoT infected seeds harvested from that field. All data from Goulart et al. (1995) was collected from naturally occurring wheat blast outbreaks. The seeds produced from the wheat genotypes were collected by hand, recovering almost 100% of the seeds produced, even those small and shriveled seeds collected from a highly MoT susceptible wheat materials. Based on the Blotter detection test from the Goulart et al. (1995) study, a high infection on the wheat genotype with >90% MoT spike incidence in the field, it represented between 13-27 % MoT incidence of infected seeds. Given the high MoT susceptible materials in Goulart et al. (1995) study were highly affected producing small and shriveled seeds, and almost total seeds were recovered from harvest, 13-27% MoT incidence of infected seeds could be considered low. On the other hand, wheat genotypes with at least 8.2% MoT spike incidence in the field, presented a 1.2% MoT incidence of infected seeds (Goulart et al. 1995). Given a uniform distribution of the disease in the field and a fully efficient sampling method, this study suggests the Blotter test might not detect MoT infected seed in a seed sample if the blast incidence in the field was less than 8%.

Implications of the MoT detection threshold from the PCR and Blotter method for infected seeds in a seed sample

This study was based on the practical implications of the MoT detection threshold from the PCR and the Blotter method might represent in the field. The practical implication was based on the results from the PCR test for MoT detection developed by Pieck et al. (2017) and optimized for seed detection in this study (Chapter 2, Kiyuna, MS thesis 2019) along with the results of the Blotter test reported by Goulart et al. (1995).

In order to understand the implication of these detection thresholds from the detection methods, it was considered the harvest moment, which a 20 metric tonnes harvest wagon is usually used to transport the harvested seeds. In the best case scenario of a uniform infected seeds distribution in the 20 metric tonnes harvest wagon combined with a fully efficient sampling method, the calculation of infected seeds going below the detection threshold was determined. The detection threshold of MoT infected seeds in a seed sample reported in this study (Chapter 2, Kiyuna, MS thesis 2019) was 0.1% of MoT infected seeds in a seed sample. The calculated estimate of the detection threshold for the Blotter method based on the Goulart et al. (1995) study was 8.2% of MoT infected seeds in a seed sample. The average seed weight determined in this study (Chapter 2, Kiyuna, MS thesis 2019) was 0.03 gr / seed. Given a hypothetical uniform infected seeds distribution and fully efficient sampling method, for the PCR method, the detection threshold of 0.1% incidence of infected seeds in a seed sample represents 20 kg or its equivalent of 6.6×10^5 infected seeds that might be undetectable from the 20 metric tonnes harvest wagon. For the same hypothetical condition, from the Blotter method, the detection threshold of the calculated estimate of 8.2% incidence of infected seeds in a seed sample represents 1600 kg or its equivalent of 5.3×10^7 infected seeds that might be undetectable. The main point is either the PCR

or the Blotter detection method for MoT infected seeds will fail for detection, and it is important to be aware the implications of these detection thresholds represent.

Preliminary risk assessment tool for MoT infected seeds detection and likelihood for dissemination

The risk assessment tool for MoT infected seed likelihood for dissemination was based on weather conditions during severe epidemic and low epidemic years, previously published study on MoT infection on wheat seeds during epidemics, the consequences of detection threshold from the Blotter and PCR test for infected seed and practical field implications.

The parameters for the risk assessment were based on: weather conditions either favorable or not favorable for MoT disease at the heading stage, cultivar susceptibility to MoT, planting date, the likelihood of MoT epidemic, correlation of wheat blast field infection and MoT infected seeds based on the MoT disease pressure on the field, the likelihood of MoT detection by the Blotter and PCR test and finally the likelihood for MoT infected seeds dissemination. The qualitative risk assessment tool was established in four case scenarios with the likelihood of field epidemic, seed infection and an adopted MoT infected seeds detection to determine the risk for MoT dissemination (Table 3.5):

1. Fields under favorable conditions for MoT disease at the heading stage planted with a susceptible cultivar. In case they were planted early, it might cause a high disease infection in the field (spike incidence and severity). Therefore, these fields might produce moderate seed infection (MoT seed incidence and severity). In case they were planted late, it might cause a moderate disease infection producing a moderately infected seed. This case scenario, either for fields planted early or late with a susceptible cultivar, it might represent a high probability for MoT infected seeds detection either for the Blotter

or PCR test. Therefore, the MoT infected seed lot might be stopped and not shipped, and it might represent a low likelihood of dissemination by MoT seed borne.

2. Fields under favorable conditions for MoT disease at the heading stage planted with a resistant cultivar. In case they were planted early or late, it might cause a low disease infection in the field (spike incidence and severity) and these fields might produce low seed infection (MoT seed incidence and severity). This case scenario, either for fields planted early or late, might represent low probability for detection by the Blotter test but highly detectable by the PCR test. Given this case, either from fields planted early or late with a resistant cultivar, infected seeds produced from this case scenario might be detectable at least by the PCR detection method. Therefore, the MoT infected seed lot might be stopped and not shipped. Similar to the first case scenario, it might represent a low likelihood of dissemination by MoT seed borne.

3. Fields under non-favorable conditions for MoT disease at the heading stage planted with a susceptible cultivar. In case they were planted early, it might cause a low-moderate disease infection in the field (spike incidence and severity). Therefore, these fields might produce low-moderate seed infection (MoT seed incidence and severity). In case they were planted late, it might cause a low disease infection producing a low infection in the seeds. This case scenario, either for fields planted early or late, it might represent an inconsistent detection for MoT infected seeds either for the Blotter or PCR test. This inconsistent detection for MoT infected seeds produced in this case scenario might be due to the possible high variability of incidence (MoT infected seeds ratio) and severity (MoT fungal biomass concentration in seeds). Given the high likelihood of false negatives from

the detection tests in this case scenario, it might represent a high likelihood of pathogen dissemination by MoT seed borne.

4. Fields under non-favorable conditions for MoT disease at the heading stage planted with a resistant cultivar. In case they were planted early or late, it might cause a low disease infection in the field (spike incidence and severity) and these fields might produce low seed infection (MoT seed incidence and severity). This case scenario, either from fields planted early or late, it might represent low probabilities for detection either for the Blotter or PCR test. Although the infected seeds produced from this case scenario might not be detectable either by the PCR or Blotter detection method, it might represent a low risk for dissemination by MoT seed borne due to the most likely low MoT infected seed incidence (MoT infected seeds ratio) and low severity seed (MoT fungal biomass concentration in seeds).

Based on the analyzed case scenarios, the third case scenario represent a high risk for MoT dissemination due to the high likelihood of false negative tested by either Blotter or PCR test on the infected seeds produced from this case scenario. This case scenario indicates that relying solely on the detection test is not enough for MoT infected seed detection. Therefore, is necessary to complement the detection system with field assessment. In addition, given the more accessibility to weather data through weather stations in several wheat producer regions, seeds produced from determined regions could be analyzed with an existent MoT disease weather forecasting to determine whether these regions were exposed to epidemic or non-epidemic weather conditions.

Discussion

Given the intense commodities trade worldwide, seed-borne pathogens are one of the most common ways for introduction into new areas. For pathogen invasion, a set of conditions have to

take place at the same time in order to occur: high frequency and concentration of pathogen introduction, conducive weather condition and host susceptibility (Stack et al. 2006). Today, there is not an established protocol for MoT detection on seeds or grain during seed or grain movement. A qualitative risk assessment and mitigation tool based on correlation analysis was developed to minimize disseminating MoT-infected seeds.

The present study on the field survey for MoT infection agrees with Fernandes et al. (2017) and Goulart (2007) that humidity is the most limiting factor for tropical countries and the most susceptible stage for the wheat head blast is the heading stage. The weather data from the field survey was obtained from a single weather station that represented the total surveyed area, and disease and yield data were collected from commercial fields. Although there might be a weather condition variation or different management variables across the surveyed fields, that does not change the final conclusion from the present study that highlights the importance of plant resistance and agronomic practices to minimize MoT infection in the field, so less MoT infected seed might be produced. For future studies, it would be essential to implement the same study under local weather conditions for each location and determine an experimental design to elaborate more accurate information that contributes to a better understanding of the plant resistance and planting date interaction for epidemiology.

It is crucial for detection methods to be highly specific and sensitive in order to decrease the occurrence of false positives or false negatives in seed inspections. Consequences for a false positive include unnecessary mitigation measures deployed or unnecessary trade interruption, while consequences for a false negative include the spread of a new pathogen in a new area (Stack et al. 2014). Today, many plant pathology labs, regulatory and inspection agencies still relying on the Blotter test for MoT detection on the seeds. The Blotter test is time consuming taking up to

seven days for a final result. It requires specific moisture condition, too much humidity in the moist chamber affects the MoT sporulation in the seeds. The pathogen has to be alive and might present high viability for sporulation. It is easily overcome by contaminant pathogens in the seeds like *Fusarium spp*, *Alternaria spp*, etc. It is almost impossible to distinguish the Triticum pathotype from other *Magnaporthe oryzae* pathotypes, very important specificity attribute considering the evidence of high sexual recombination on the MoT isolates collected lately or recent events where other *Magnaporthe oryzae* pathotypes infected wheat plant. On the other hand, a PCR method is proven to be more accurate, rapid and present wide utility for pathogen detection. However, one limitation of the PCR test is that detect DNA target whether the organism is alive or dead, ending up with a possible false positive. Consequently, you may take actions that are unnecessary. One solution for that might be culturing all positives; but again, that might be time-consuming. Therefore, every person must know and understand the advantages and limitations of the method that is working with, because no technology or approach is perfect. This study showed that both detection methods might miss MoT infected seeds for detection. It is critical to establish policies that promote efficient detection methods to avoid the introduction of MoT into new areas, as happened in Bangladesh.

High MoT-conducive conditions can cause up to 100% yield losses (Goulart and Paiva 1992, 2000) and the most affected seeds may be shriveled, small and low-test weight, which most probably might be discarded by the combine at the harvest (Goulart et al. 1995). Therefore, a low and intermediate MoT infected seeds might go to the harvest wagon. There are practical implications of the PCR detection threshold that need to be considered. The MoT detection threshold in seed by the PCR assay was 0.1% of MoT infected seeds in a seed sample. Given a disease uniform distribution combined with a fully efficient sampling method, if a 100-hectare

wheat field with MoT-infected plants produces 200 tons of seed/grain (2 tons/hectare average), approximately 200 kg of infected seeds might go undetected. This represents approximately 6.67×10^6 infected seeds from a single field that might be moved to another location and planted for the next crop season. This PCR detection threshold of 0.1% infected seeds in a seed sample was established in a worst-case scenario under high disease pressure and high MoT severity in infected seeds. Low or intermediate MoT-conducive weather conditions and/or a late infection in the field, might result in superficial infections in seed, therefore the detection threshold might vary increasing the likelihood of false negatives. Additionally, under epidemic conditions, most of the highly infected/diseased seeds (i.e., shriveled, small, low test weight) would be discarded by the combine during harvest (Goulart et al. 2007). These conditions may generate low or intermediate MoT severity in infected seeds may result in seed lots below the detection threshold. Both, incidence of MoT infected seed in a seed lot and the severity in those infected seeds (i.e., the degree of colonization and amount of MoT fungal biomass per seed) will impact the ability for the PCR detection.

Bangladesh, a country with an area of 56.977 square miles (similar to the Iowa state area) and ~164 million habitants, officially is the first country outside of South America affected by the wheat blast (Callaway 2016; Malaker et al. 2016). Given the static production of Bangladesh occurred during the last decade and the increasing domestic consumption, it was created the need to import wheat to meet the demand for feeding (Sadat and Choi 2017). It is likely that, given the shortage of seed, some farmers used the imported wheat kernels as seed for planting. In the year of the MoT outbreak, it is believed that Bangladesh imported wheat from several sources, including Argentina and Brazil (Sadat and Choi 2017). Considering MoT is seed-borne, a high probability based on the wheat blast epidemiology, that in one wheat crop season, the outbreak

occurred in several districts of wheat production of Bangladesh (Islam et al. 2016; Malaker et al. 2016). Phylogenetic studies suggest the MoT strain isolated from an MoT infected plant was clonal from an MoT lineage from South America (Islam et al. 2016; Malaker et al. 2016). Given the evidence of a high probability of the MoT introduction to Bangladesh occurred by MoT infected seeds, this represents a very deficient diseased seed detection system in the international commodity trade. Brazil grows wheat in ~2 Million hectares every year. Considering Brazil for the practical implication of the PCR detection threshold, estimating the number of MoT infected seeds that might be undetectable, is simply frightening. Consequently, to minimize the risk of pathogen dissemination: 1) Seed from MoT endemic areas should not be shipped to non-MoT infested areas during years with weather conditions that favor blast epidemic or blast disease development; 2) Test seed for MoT infection; 3) Use certified seed, do not use farm-saved seed; 4) Do not use grain for seed as seed. In addition, given the access to data from weather station in many wheat producer regions, weather conditions from the production fields might be analyzed by the wheat blast disease forecasting model for previous established study by Fernandez et al. (2017) to determine if seed lots produced from determined fields were exposed to MoT conducive conditions. It is crucial an MoT infected seed detection system is adopted and complemented with field and weather assessment to minimize the likelihood of MoT dissemination.

The present preliminary risk assessment for moving infected seeds studied the practical implications for MoT dissemination. It was established the detection threshold and probabilities of dissemination through MoT infected seeds. The critical follow-up to this study is setting the stage on the epidemiology prospective. Wheat blast, caused by a seedborne pathogen, is known as its capability for MoT transmission from infected seeds to seedlings (Cruz and Valent 2017). However, there is not accurate information about the transmission rate. The future work might be

critical to study and establish the transmission thresholds from infected seeds to the plant stage based on gradient incidence and severity MoT infected seeds in a seed sample. This information might give insights about the understanding the epidemiology of MoT introduction and establishment for MoT infected seeds planted in commercial field in a non-MoT established area.

Knowledments

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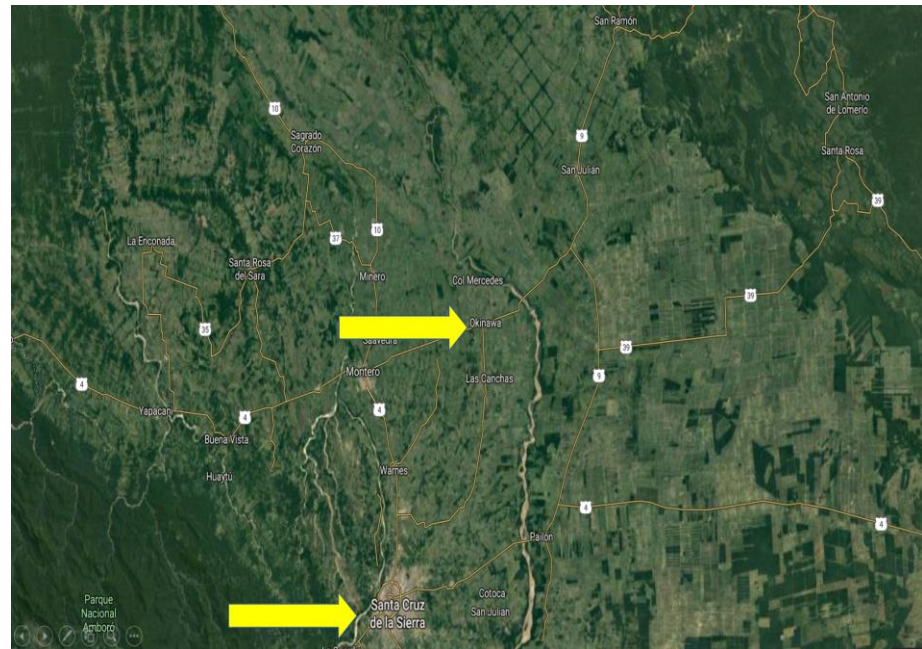
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Figures

Figure 3.1 On the left, a Bolivia map. The blue area indicates the main wheat production area from the department of Santa Cruz. On the right, arrows indicate the Santa Cruz de la Sierra city (main city in the department of Santa Cruz) and Okinawa 1 county (located at 80 kms from Santa Cruz de la Sierra city), location in which the field survey for wheat blast was conducted.



<https://www.mapsofworld.com/bolivia/> (left)



Google map (right)

Figure 3.2 Mean disease incidence and severity at the soft dough stage as a function of cultivar susceptibility and planting date from seventeen commercial wheat production fields. Medians are indicated by the horizontal lines. Means are indicated by the “X” marks.

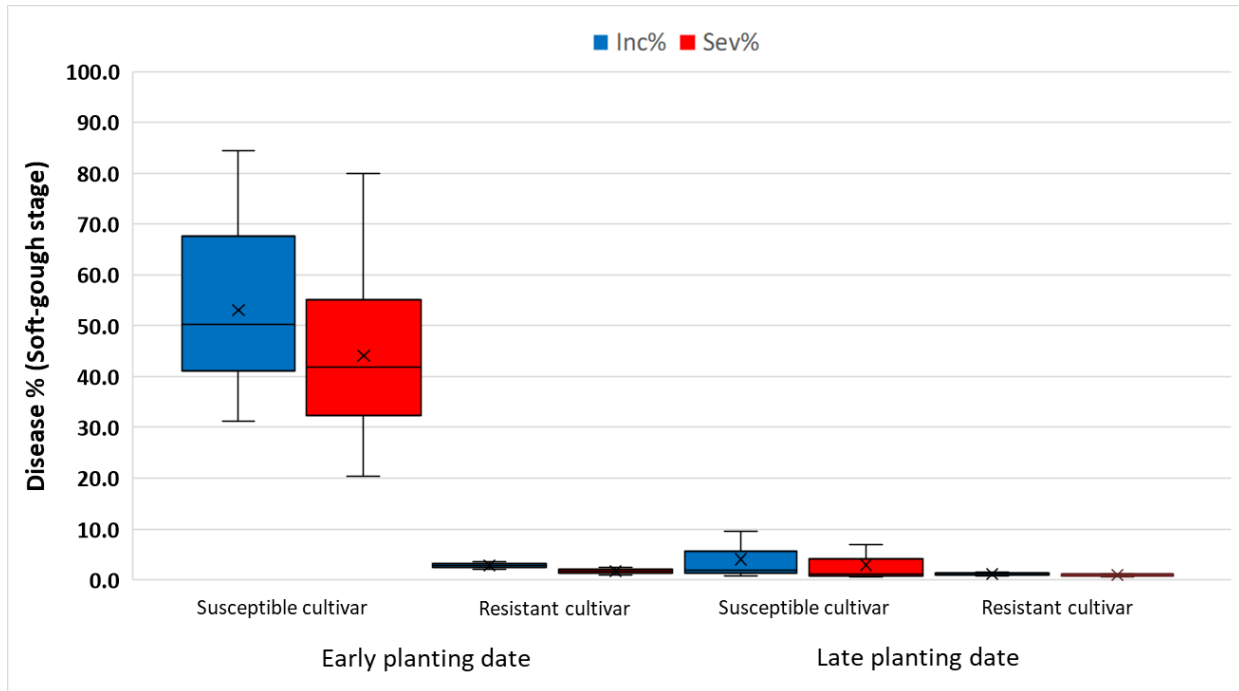


Figure 3.3 Mean disease incidence and severity at the ripening stage as a function of cultivar susceptibility and planting date from seventeen commercial wheat production fields. Medians are indicated by the horizontal lines. Means are indicated by the “X” marks.

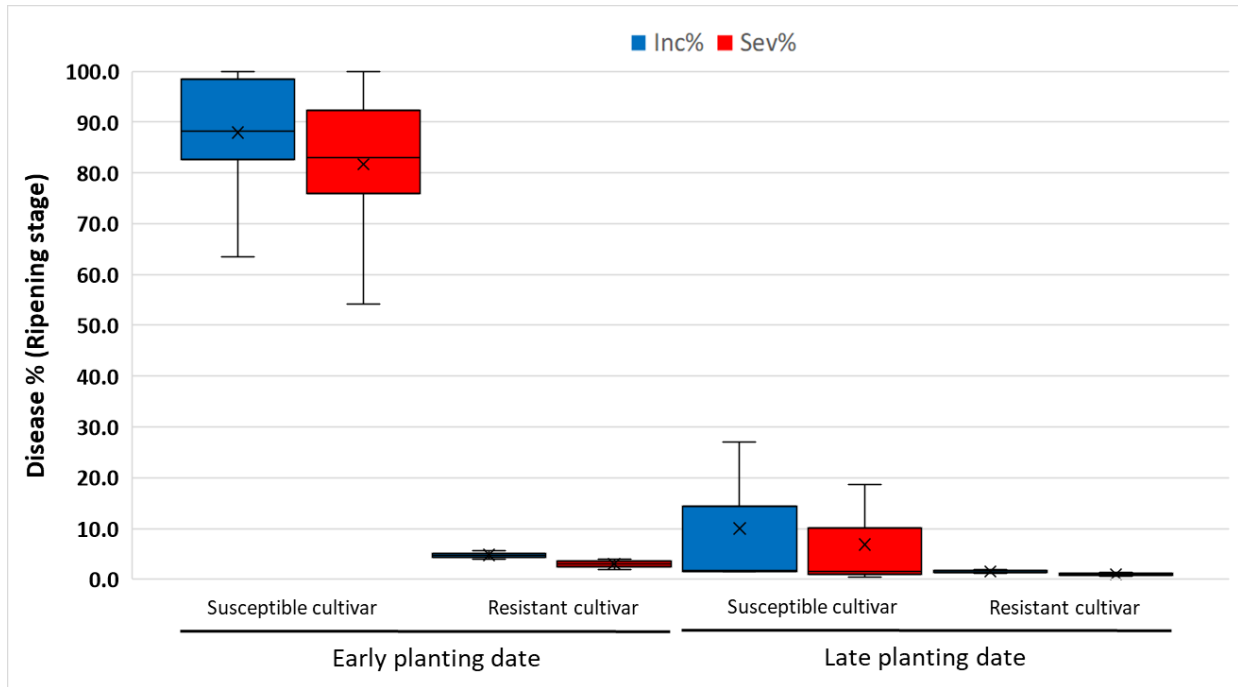


Figure 3.4 Mean yield (tonnes/hectare) as a function of cultivar susceptibility and planting date from seventeen commercial wheat production fields. Medians are indicated by the horizontal lines. Means are indicated by the “X” marks.

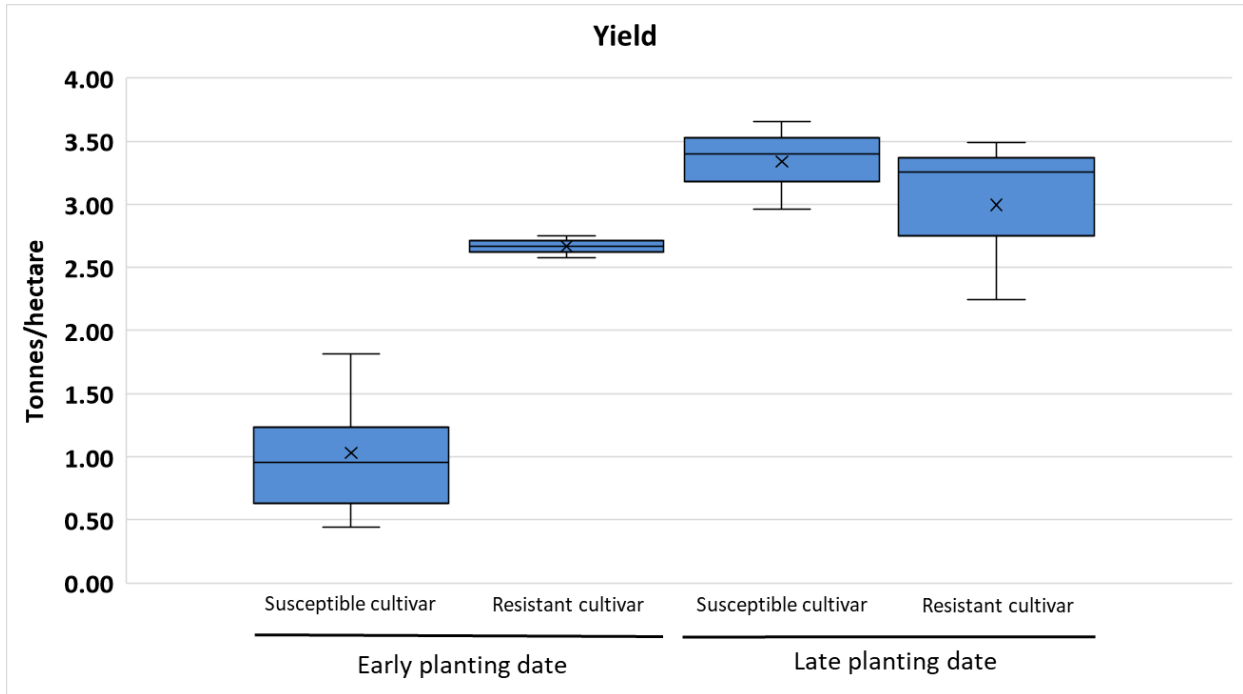


Figure 3.5 Mean 1000-seed weight (grams) as a function of cultivar susceptibility and planting date from seventeen commercial wheat production fields. Medians are indicated by the horizontal lines. Means are indicated by the “X” marks.

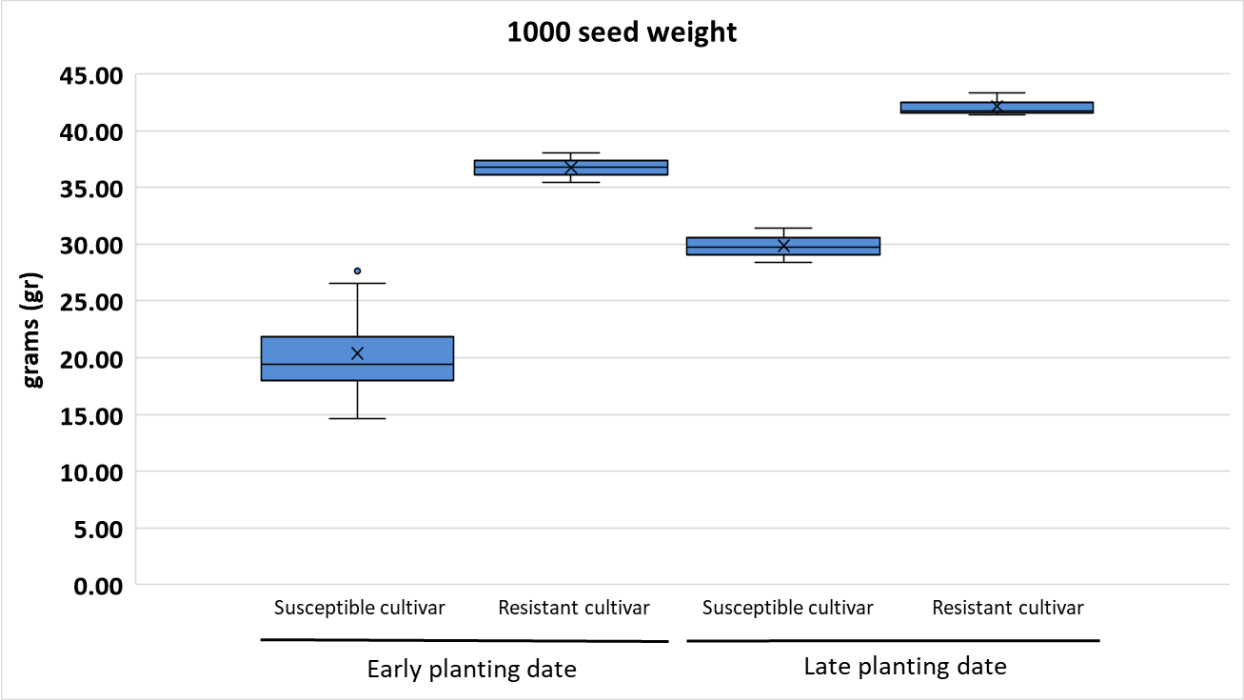


Figure 3.6 Mean test weight (%) as a function of cultivar susceptibility and planting date from seventeen commercial wheat production fields. Medians are indicated by the horizontal lines. Means are indicated by the “X” marks.

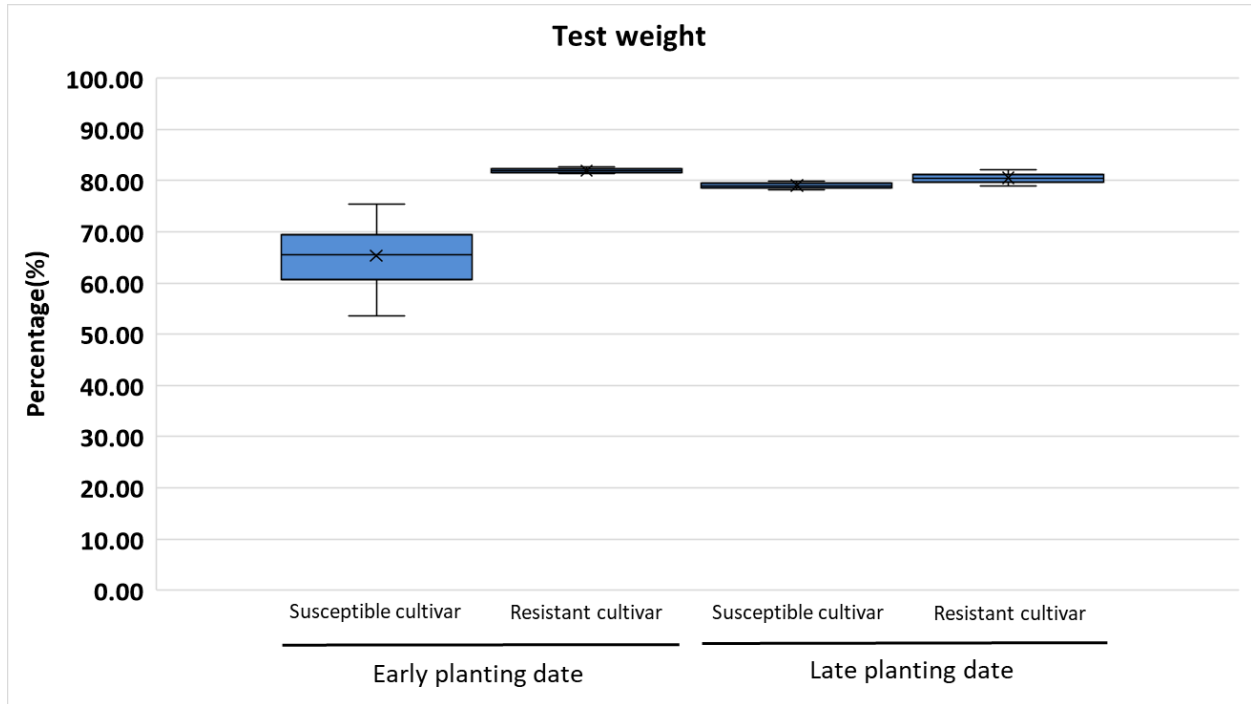


Figure 3.7 Weather conditions of rainfall accumulation, temperature between 25-30°C and relative humidity >80% (all conducive conditions for MoT disease) during early and late heading stage based on planting from the crop winter season in Santa Cruz-Bolivia 2011. Heading stage is the most critical stage for MoT infection (Goulart et al. 2007).

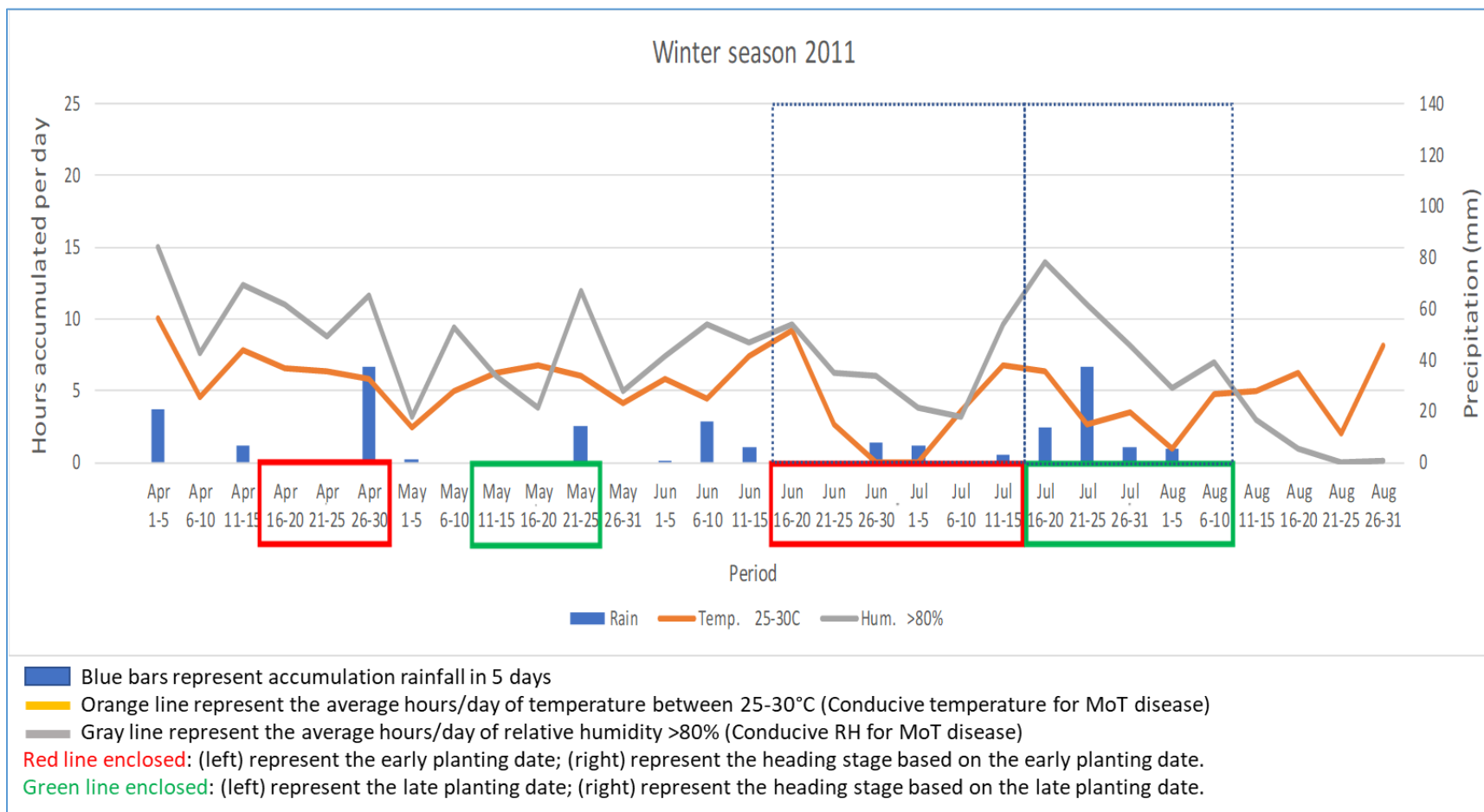


Figure 3.8 Weather conditions of rainfall accumulation, temperature between 25-30°C and relative humidity >80% (all conducive conditions for MoT disease) during early and late heading stage based on planting from the crop winter season in Santa Cruz-Bolivia 2012. Heading stage is the most critical stage for MoT infection (Goulart et al. 2007).

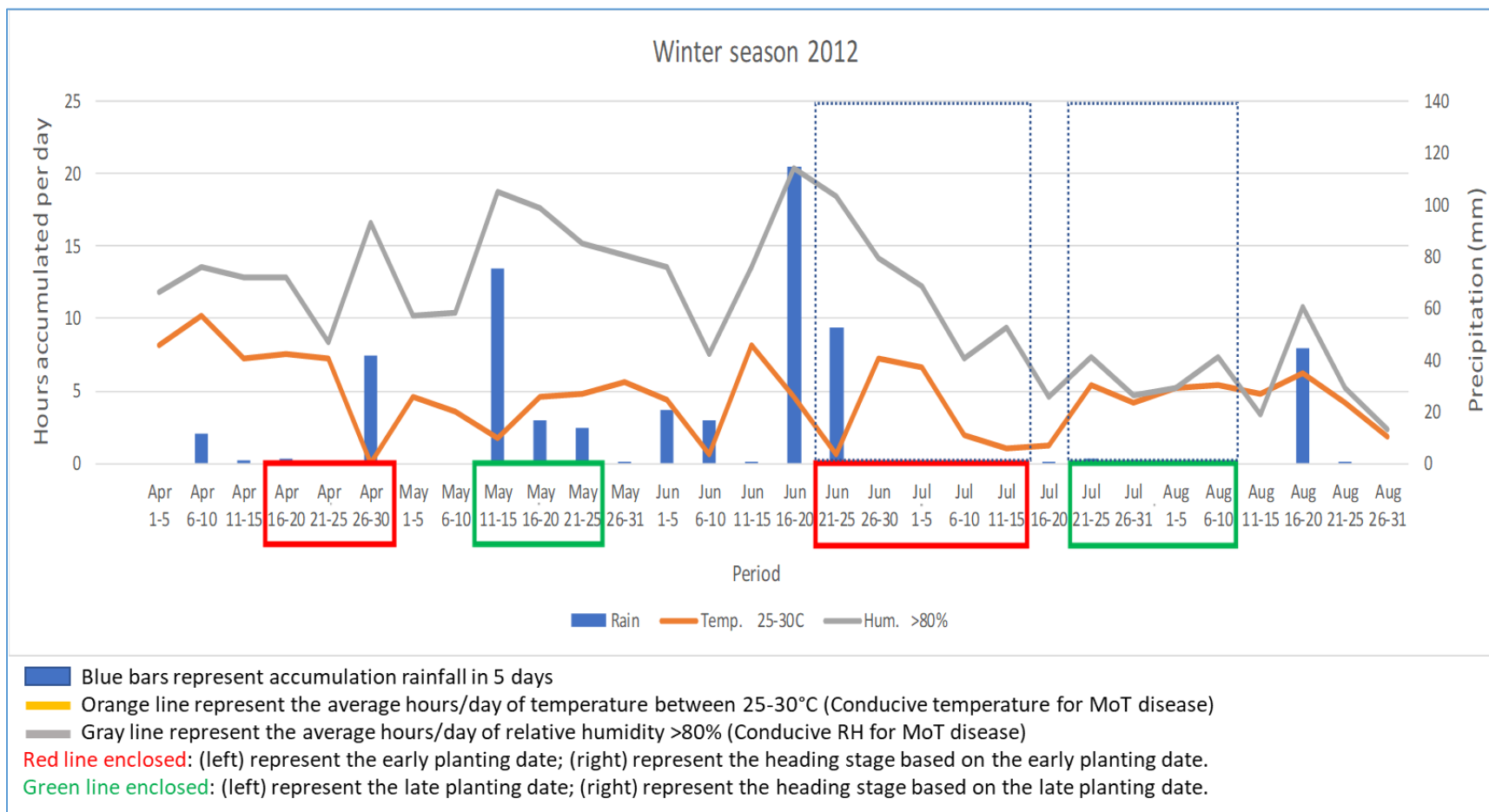


Figure 3.9 Weather conditions of rainfall accumulation, temperature between 25-30°C and relative humidity >80% (all conducive conditions for MoT disease) during early and late heading stage based on planting from the crop winter season in Santa Cruz-Bolivia 2013. Heading stage is the most critical stage for MoT infection (Goulart et al. 2007).

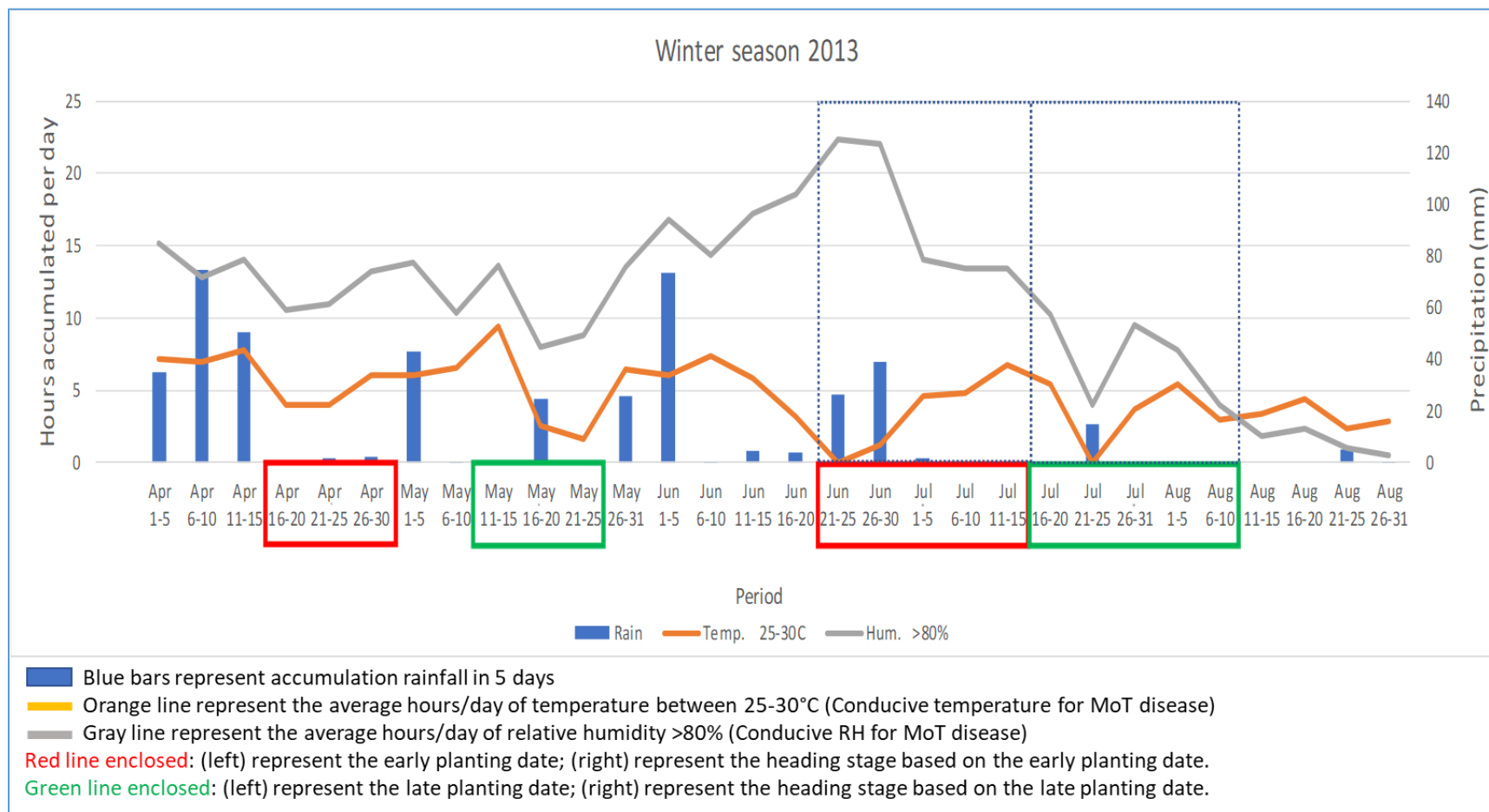


Figure 3.10 Weather conditions of rainfall accumulation, temperature between 25-30°C and relative humidity >80% (all conducive conditions for MoT disease) during early and late heading stage based on planting from the crop winter season in Santa Cruz-Bolivia 2014. Heading stage is the most critical stage for MoT infection (Goulart et al. 2007).

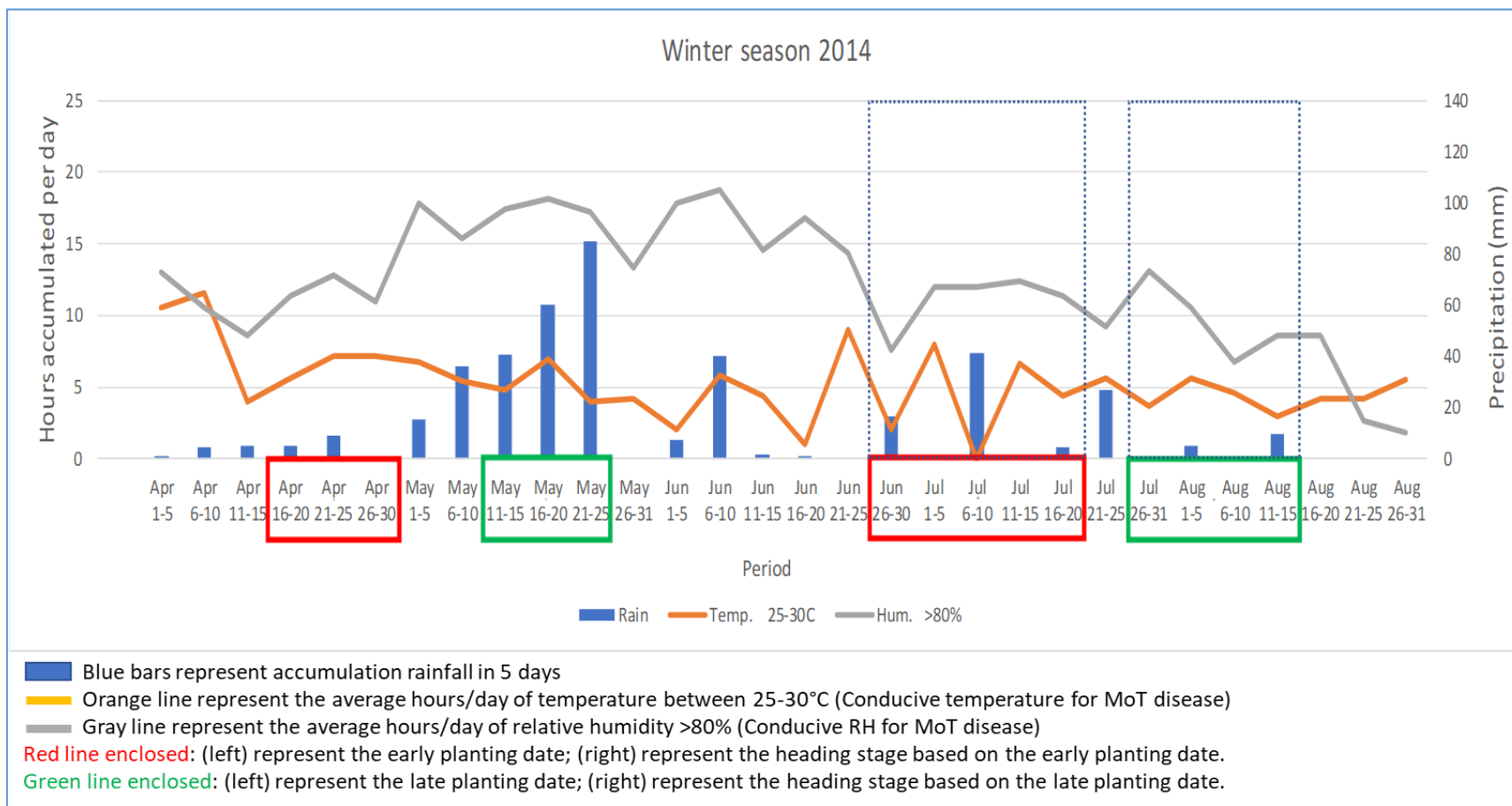
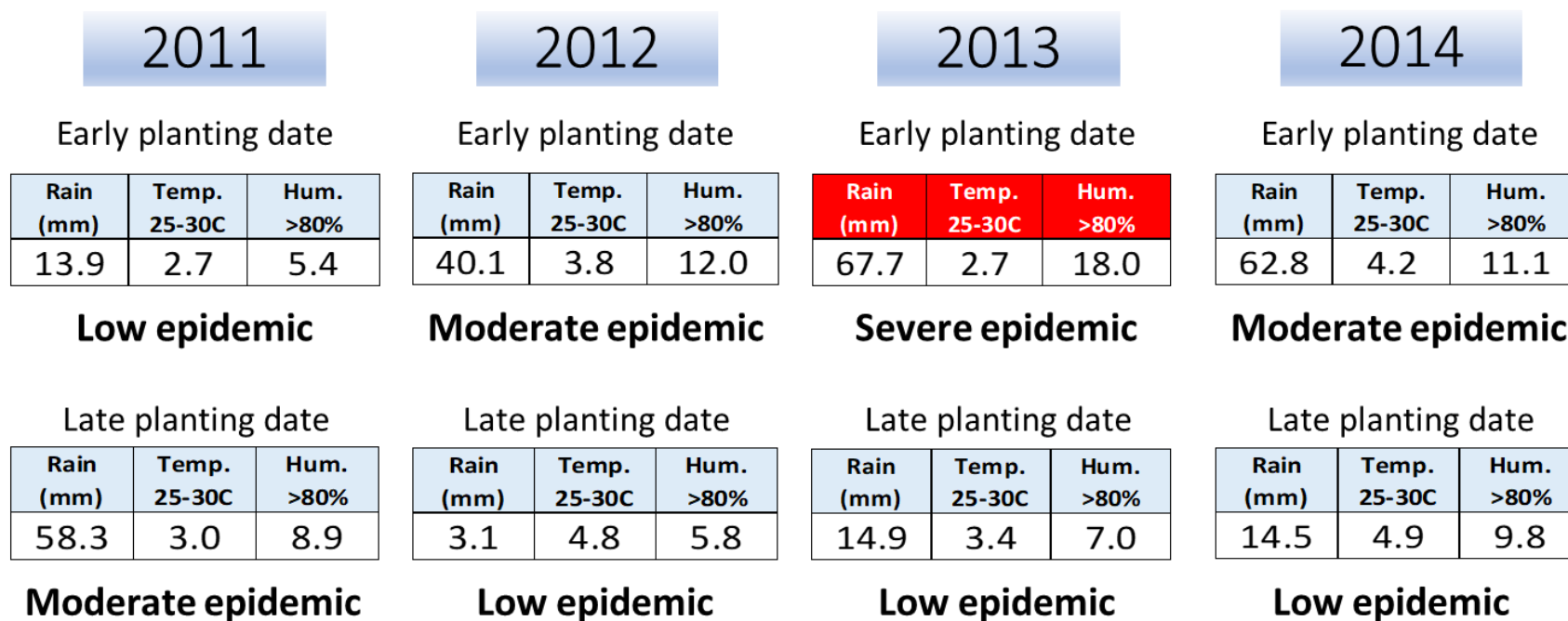


Figure 3.11 Summary of rainfall accumulation, temperature between 25-30°C and relative humidity >80% (all conducive conditions for MoT disease) during early and late heading stage based on planting date from 2011, 2012, 2013 and 2014. Heading stage is the most critical stage for MoT infection (Goulart et al. 2007).



Rain (mm) = Accumulation rainfall (mm).

Temp. 25-30°C = Accumulation hours/day average of conducive temperature condition for MoT disease between 25°-30° C.

Hum. >80% = Accumulation hours/day average of conducive relative humidity condition for MoT disease more than 80% RH.

Tables

Table 3.1 Analysis of variance on the planting date, cultivar MoT susceptibility and the interaction of planting date/cultivar MoT susceptibility for disease incidence and severity from surveyed commercial field, yield and grain quality performance.

Planting dates - Cultivar susceptibility	Soft-dough stage		Ripening stage		Yield and quality seed performance		
	Spike incidence (%)	Spike severity (%)	Spike incidence (%)	Spike severity (%)	Yield (tons / hectare)	1000 seeds weight (grams)	Test weight (%)
Early Pl. date - R cult. Vs Early Pl. date - S cult.	<0.0001	<0.0001	<0.0001	<0.0001	0.0051	0.0003	0.0037
Early Pl. date - R cult. Vs Late Pl. date - R cult.	0.0520	0.2294	0.0873	0.1450	0.6766	0.3149	0.8067
Early Pl. date - R cult. Vs Late Pl. date - S cult.	0.6778	0.5111	0.9437	0.8481	0.4180	0.1470	0.6278
Early Pl. date - S cult. Vs Late Pl. date - R cult.	<0.0001	<0.0001	<0.0001	<0.0001	0.0008	<0.0001	0.0024
Early Pl. date - S cult. Vs Late Pl. date - S cult.	<0.0001	<0.0001	<0.0001	<0.0001	0.0003	0.0029	0.0044
Late Pl. date - R cult. Vs Late Pl. date - S cult.	0.0155	0.0541	0.0562	0.0804	0.6468	0.0134	0.7870

Table 3.2 Wheat blast disease incidence and severity as a function of planting date and cultivar susceptibility at soft-dough stage for seventeen commercial wheat production fields.

Planting date	Field	Planting date	Cultivar	Susceptibility to MoT	Soft-dough stage			
					Incidence (%)	Std. dev.	Severity (%)	Std. dev.
Early planting date	1	4/17/2013	Atlax	S	84.42	3.49	80.01	3.24
	2	4/17/2013	Atlax	S	42.74	9.65	32.44	10.09
	3	4/20/2013	Atlax	S	31.13	4.68	20.31	4.16
	4	4/20/2013	Sausal	R	3.56	1.32	2.37	0.89
	5	4/23/2013	Arex	S	41.10	5.63	32.36	5.78
	6	4/23/2013	Atlax	S	52.04	10.54	45.08	11.85
	7	4/23/2013	Chané	S	50.27	4.51	41.80	4.56
	8	4/23/2013	Parapeti	S	67.65	3.04	55.09	2.11
	9	4/29/2013	Chané	S	39.89	4.53	26.39	3.87
	10	4/29/2013	Motacu	R	2.03	0.57	0.98	0.35
	11	4/29/2013	Ichilo	S	67.92	8.65	63.77	9.57
Late planting date	12	5/12/2013	Atlax	S	9.61	0.98	6.97	1.02
	13	5/14/2013	Atlax	S	1.81	0.36	1.13	0.26
	14	5/16/2013	Motacú	R	1.26	0.59	1.14	0.52
	15	5/16/2013	Motacú	R	0.73	0.51	0.55	0.43
	16	5/17/2013	Atlax	S	0.75	0.31	0.56	0.29
	17	5/17/2013	Motacú	R	1.52	0.24	1.13	0.22

Table 3.3 Wheat blast disease incidence and severity as a function of planting date and cultivar susceptibility at ripening stage of seventeen commercial wheat production fields.

Planting date	Field	Planting date	Cultivar	Susceptibility to MoT	Ripening stage			
					Incidence (%)	Std. dev.	Severity (%)	Std. dev.
Early planting date	1	4/17/2013	Atlax	S	98.53	0.42	97.89	0.43
	2	4/17/2013	Atlax	S	78.08	5.93	66.17	2.92
	3	4/20/2013	Atlax	S	63.46	12.23	54.24	14.71
	4	4/20/2013	Sausal	R	4.01	1.84	1.93	0.79
	5	4/23/2013	Arex	S	88.28	4.24	83.07	7.07
	6	4/23/2013	Atlax	S	97.56	1.53	92.28	3.20
	7	4/23/2013	Chané	S	83.47	0.73	77.39	3.89
	8	4/23/2013	Parapeti	S	99.27	0.46	88.22	5.97
	9	4/29/2013	Chané	S	82.55	9.76	75.96	10.97
	10	4/29/2013	Motacu	R	5.55	0.66	4.04	0.25
	11	4/29/2013	Ichilo	S	100.00	0.00	100.00	0.00
Late planting date	12	5/12/2013	Atlax	S	26.94	1.71	18.68	1.27
	13	5/14/2013	Atlax	S	1.54	0.53	0.45	0.17
	14	5/16/2013	Motacú	R	1.92	0.28	0.57	0.15
	15	5/16/2013	Motacú	R	1.43	1.01	1.34	0.93
	16	5/17/2013	Atlax	S	1.66	0.59	1.46	0.52
	17	5/17/2013	Motacú	R	1.17	0.04	1.03	0.16

Table 3.4 Mean yield and grain quality as a function of planting date and cultivar susceptibility to wheat blast for seventeen commercial wheat production fields.

Planting date	Field	Planting date	Cultivar	Susceptibility to MoT	Yield and seed quality performance					
					Yield Ton/ha	Std. dev.	1000 seeds weight (gr)	Std. dev.	Test weight (%)	Std. dev.
Early planting date	1	4/17/2013	Atlax	S	0.63	0.04	18.36	0.19	64.68	0.60
	2	4/17/2013	Atlax	S	1.82	0.05	27.64	1.86	75.35	0.15
	3	4/20/2013	Atlax	S	1.78	0.14	26.54	1.00	74.15	0.84
	4	4/20/2013	Sausal	R	2.58	0.17	38.06	0.17	82.66	0.08
	5	4/23/2013	Arex	S	0.95	0.07	18.57	1.04	60.95	1.91
	6	4/23/2013	Atlax	S	0.44	0.11	14.60	0.41	53.65	1.18
	7	4/23/2013	Chané	S	0.63	0.02	16.86	0.50	59.85	1.17
	8	4/23/2013	Parapeti	S	0.95	0.08	20.24	1.06	66.38	1.68
	9	4/29/2013	Chané	S	1.05	0.04	20.24	0.61	67.86	0.64
	10	4/29/2013	Motacu	R	2.75	0.24	35.46	0.80	81.31	0.49
	11	4/29/2013	Ichilo ^a	S	***	***	***	***	***	***
Late planting date	12	5/12/2013	Atlax	S	2.96	0.12	29.76	0.69	79.04	0.48
	13	5/14/2013	Atlax	S	3.66	0.02	28.36	0.38	78.24	0.15
	14	5/16/2013	Motacú	R	3.25	0.05	41.73	0.47	79.04	1.26
	15	5/16/2013	Motacú	R	2.25	0.16	41.44	0.45	80.46	0.14
	16	5/17/2013	Atlax	S	3.40	0.12	31.43	0.15	79.96	0.23
	17	5/17/2013	Motacú	R	3.49	0.10	43.36	0.15	82.05	0.13

^aMissing data

Table 3.5 Preliminary risk assessment tool for guidance to minimize the probability of disseminating MoT-infected seeds.

IF			THEN						
Field			Blasted field		MoT Seed Infection		MoT Detection		Risk
Weather	Cultivar	Planting date	DI^a	DS^b	DI^c	DS^d	Blotter	PCR	Dissemination
Favorable	Susceptible	Early	High	High	Mod.	Mod.	+	+	Low
		Late	Mod.	Mod.	Mod.	Mod.	+	+	Low
	Resistant	Early	Low	Low	Low	Low	-	+	Low
		Late	Low	Low	Low	Low	-	+	Low
Non-Favorable	Susceptible	Early	Low-Mod.	Low-Mod.	Low-Mod.	Low-Mod.	+/-	+/-	High
		Late	Low	Low	Low	Low	+/-	+/-	High
	Resistant	Early	Low	Low	Low	Low	-	-	Low
		Late	Low	Low	Low	Low	-	-	Low

^a Disease spike incidence in the blasted field (Number of spikes blasted)

^b Disease spike severity in the blasted field (Proportion of blasted in the spike)

^c Disease seed incidence in the seed sample (Number of infected seeds)

^d Disease seed severity in the seed sample (Amount of fungal biomass per infected seed)

+ High probabilities for detection

- Low probabilities for detection

+/- Inconsistent detection

Chapter 4 - Conclusions

Seedborne pathogens are one of the most common ways for pathogen introduction to new areas. The recent wheat blast outbreak in Bangladesh, the pathogen most probably introduced by MoT infected seeds, highlighting the importance to adopt more efficient detection systems.

The study in chapter 2 established the MoT detection threshold was 0.1% of MoT infected seeds in a seed sample by previous established PCR assay (MoT-3 specific primer). However, in the same study, the detection threshold varied as a function of the MoT severity (fungal biomass concentration) of the infected seeds in a seed sample. The results from this study suggest the infection timing in the field is crucial for detection sensitivity, as earlier the infection occurs in the field, the produced infected seeds might contain more fungal biomass increasing the likelihood for MoT detection. In addition, the sensitivity and specificity for MoT detection using the MoT-3 specific primer were verified. The MoT DNA in the presence of possible interfering DNA (host and *M. oryzae* Lolium and Oryza pathotype DNAs) did not inhibit nor affect the MoT detection sensitivity and specificity. This study strengthens, once more, the specificity of the MoT-3 specific primer against the controversy of MoT-3 primer limitations to discriminate the Triticum pathotype from the Oryza pathotype DNA.

The qualitative risk assessment tool designed in this study indicates that relying solely on a detection tool is not enough for MoT dissemination. Hypothetical case scenarios were established based on agronomical management (planting date x cultivar susceptibility) to study the likelihood of field disease, MoT seed infection and MoT detection. Given the case scenario of planting a susceptible cultivar under the non-favorable condition for MoT disease, it was determined the high likelihood of false negatives from the detection tool, representing a high risk of infected seeds being undetectable. Therefore, it is essential to complement the detection tool with field

assessments. In addition, given the availability of MoT disease forecasting, weather conditions could be used to determine the extent of epidemic from fields which seeds are produced. This study suggests the establishment of a detection system with an MoT detection tool complemented with field assessment and weather conditions to minimize the likelihood of false positive, consequently decrease the likelihood of MoT dissemination to non-MoT established areas. The next step might be critical to study the transmission rate from infected seeds to the plant stage established in the field, based on gradient incidence and severity MoT infected seeds ratios.