EXPLORING THE EFFECTS OF ENVIRONMENTAL FACTORS ON RICE BLAST DISEASE

DISSERTATION

A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy in the
Martin-Gatton College of Agriculture, Food, and Environment
at the University of Kentucky

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2023

ABSTRACT OF DISSERTATION

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Rice blast disease, caused by the ascomycete fungus *Magnaporthe oryzae*, occurs in over 85 countries and results in an annual crop loss of 10-30%, a corresponding nutrient value of meals for 60 million people. As a result, it is listed as a critical plant disease by the United Nations. Understanding factors affecting disease severity is of critical concern for food security. *M. oryzae* has been used as a model system for studying effector-triggered immunity (ETI) by understanding that ETI is primarily a plant response. *M. oryzae* has been used as a model to study fungal pathogenicity, host specificity, genome evolution, and population biology. This dissertation explores the environmental impacts on the disease progression of rice blast with a primary goal of understanding unexpected variation in infection phenotypes from one experiment to another.

Fungal strain 2539 was studied to characterize possible AVR genes. Using growth rate studies, artificial inoculation assays, and southern blots, 2539 was shown to have undergone massive, seemingly spontaneous, genome rearrangement, resulting in an unpredicted infection phenotype. This genomic rearrangement resulted in a newly observed virulence of 2539 on rice cultivar 51583 and higher disease ratings on rice cultivar Yt16. Resistance of CO39 to 2539 indicated that *AVR1-CO39* remained intact after the genomic rearrangement. Additional resistance of M2O2 to 2539 indicated a possible unidentified AVR gene in 2539 or broad resistance conveyed through *AVR1-CO39*.

Based on the irreproducibility of inoculation assay results, factors such as plant age, light, and time of day at inoculation were studied as influencers of the infection phenotype of *M. oryzae* strains. Plant age at inoculation displayed a high susceptibility of plants between 14-18 d, which correlated to the early tillering stage of rice plants. The light inoculations displayed a cultivar and strain-specific influence where one strain/cultivar experienced increased disease severity while another experienced decreased severity. The time of day inoculations displayed a slight trend of increased disease severity for inoculations performed later in the day but were dependent on the

stock. Irreproducibility between inoculation was observed in the control groups of these experiments, indicating a confounding factor not yet identified.

Uncontrolled temperature exposures were also studied as a possible factor contributing to the irreproducibility between experiments. Temperature treatments were performed pre and post-inoculation at 4℃ and 37℃. The results of these experiments indicated that the influence of temperature exposures pre and post-inoculation were strain and cultivar-specific. There was not an overall trend with any of the temperature treatments for all of the strains. However, post-inoculation heat treatment of 37℃ for 24 h did present an increased degree of infection for two 2539 stocks. This indicated a possible suppression of host resistance early in infection. Due to this result, post-inoculation 37℃ exposure was further studied with different fungal strains, multiple rice cultivars, and varying exposure times. The results supported the influence of brief exposure to higher temperature post-inoculation acting at the level of recognition.

The last topic covered in this dissertation was a final exploration into the confounding factor introducing irreproducibility between experiments. Fungal paper stocks had been used during plant inoculations to activate fungal stocks in the creation of inoculum. Since these were the only differences between experimental replicates, it was proposed that the fungal stocks were introducing variability in inoculum. The results supported the presence of clonal variation in fungal paper stocks. Although rare, one stock out of multiple taken from a single plate could be phenotypically different from the other stocks. This could cause a stark difference in disease incidence and severity on rice plants.

KEYWORDS: *Magnaporthe*, Rice, Host-Microbe Interaction, Variability, Climate

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EXPLORING THE EFFECTS OF ENVIRONMENTAL FACTORS ON RICE BLAST DISEASE

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To my sister, Kelsey Lamb. You are one of the most brilliant people I have ever met, but you never let that stop you from being kind. Keep being the person who helps, loves, and holds so much wisdom. Thank you because I never could have gotten to this point without you.

ACKNOWLEDGMENTS

First, I would like to thank my advisor, Dr. Mark Farman, for providing the opportunity and resources to complete this dissertation. I would also like to thank my advisory committee members for their time and expertise. A special thanks to Dr. Melissa Morgan for allowing me to broaden my teaching skills in her class and for forever solidifying my title as Dr. Baby Lamb. I am grateful to the DGS, Dr. Nicole Gauthier, for her dedication and support during this degree process. I would also like to express my deepest gratitude to my laboratory technician, Rebekah Ellsworth. Her training and support throughout my time in the laboratory was exceptional. She provided the expertise I needed to complete this degree in the face of many challenges, including an unexpected global pandemic.

I want to thank the plant pathology department's past and present student members for their expertise, support, encouragement, and friendship. A special thank you to Renata Belisario, Erica Fealko, Simran Goyal, Caleb Mathias, Rachel Sneed, and Gabdiel Yulifo-Soto for being brilliant, amazing lab mates and friends. Another thank you to Dr. Lou Hirsch, who guided me to this path during my undergraduate years and continues to be a bastion of encouragement whenever I drop by. Finally, I would like to express my deepest love for my original and found family, who have supported, encouraged, and helped me stay the course. Bethany, Emma, Lindsay, Rachel, and Stefani, I could not have asked for better sisters to walk with in life. Mom, Dad, Kelsey, and Tanner, thank you for pushing me to be better every day and loving me, degree or not. Thank you for reminding me that the heart of man plans his way, but the Lord establishes his steps.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1: LITERATURE REVIEW

1.1 Types of Plant-Microbe Interactions

Interactions between plants and microbes can be broadly divided into three categories: mutualistic, commensalistic, and parasitic (Newton et al., 2010). Mutualists are defined as an interaction that benefits without harming both the host and microbe. A common example of a plant mutualistic interaction is the arbuscular mycorrhizal fungi in the *Glomeromycota* phyla that extend the root absorption capabilities of over 80% of higher plants (Lagunas et al., 2015). Commensalists are defined as interactions that benefit the host or the microbe without affecting the nonbeneficiary party (Mathis & Bronstein, 2020). The reality and validity of this type of interaction are strongly debated as the pervading literature asserts that no biological interaction can be neutral (Iliev & Underhill, 2013; Luiselli, 2023). Nevertheless, examples of plant commensalism have been proposed in specific *Colletotrichum spp.* and *Epichloë spp* (Redman et al., 2001; Scott et al., 2018). Parasites are defined by an interaction that benefits the microbe and harms the host, in most cases creating a disease state (Kemen et al., 2015). The disease state involves complex behaviors and responses from both parties in the interaction, causing continual coadaptation.

Plant parasitism can be categorized by the behavior of the microbe, or pathogen, as it infects the host. Biotrophic pathogens generally form niche environments in the host to gain nutrients without killing the host or host tissues (Spoel et al., 2007). The tumor formation in host tissues by *Agrobacterium tumefaciens* allows the pathogen to exploit host resources while avoiding recognition, thereby extending the period of pathogen growth (Gonzalez-Mula et al., 2019). Necrotrophic pathogens destroy host tissues to

access resources and previously unavailable nutrients immediately, killing the host (Rajarammohan, 2021). The bacterial pathogen *Pectobacteria carotovorum* secrete large amounts of cell-well-degrading enzymes, liquifying host barriers and allowing for infection (Davidsson et al., 2013). Hemi-biotrophs are pathogens that begin infecting a host with a biotrophic phase and then switch to a necrotrophic stage (Zuluaga et al., 2016). The period for each stage is dependent upon the pathogen, host, and environment (Huang et al., 2020). The oomycete pathogen *Phytophthora infestans* transitions to a full necrotrophic phase when host recognition factors begin to destroy tissues (Lee & Rose, 2010). The bacterial pathogen *Pseudomonas syringae* pv. tomato transitions into the necrotrophic phase when there is a certain amount of bacterial population determined by quorum sensing (Chatterjee et al., 2007). Biotrophic, hemi-biotrophic, and necrotic pathogens have unique ways of entering hosts and avoiding detection.

1.2 The Plant Immune Response

The plant immune response is currently defined in the literature as a two-fold system made up of a passive and active response. The passive response or immunity specifies any general host barrier that impedes parasitism. This immunity would include the cell wall, the cuticle layer of leaves, and the Casparian strip in roots (Chassot et al., 2008; von Wangenheim et al., 2017). The pH and pressure within many plant organs inhibit pathogen egress, such as the xylem, which only highly specialized pathogens can invade (Venturas et al., 2017). The apoplast in some tissues of *Sorghum bicolor* contains an antimicrobial compound that is toxic to, among other pathogens, *Colletotrichum spp.* (Hueckelhoven, 2007). This type of nonspecific generalized immunity is the first barrier most pathogens must adapt to overcome.

The active immune response can be further separated into the basal and specific immune responses. Basal resistance response refers to the recognition of pathogen-associated molecular patterns (PAMPs) or microbe-associated molecular patterns (MAMPs) by pattern recognition receptors (PRRs) (Jones & Dangl, 2006). A MAMP is a specific pattern conserved across pathogen types (Bittel & Robatzek, 2007). Bacterial MAMPs include flagellin, lipopolysaccharides, peptidoglycan, and lipoteichoic acid (Erbs & Newman, 2012; Vijayan et al., 2018). Fungal MAMPS include beta-glucan and ergosterol (Fesel & Zuccaro, 2016; Klemptner et al., 2014). This pattern is recognized by PRRs, which are on the intramembrane proteins. Recognition of the MAMP is thought to induce pathogen-triggered immunity (PTI) in the apoplast, the region outside the cell membrane (Newman et al., 2013). The basal response can also be defined as innate and race-nonspecific immunity (Shafikova & Omelichkina, 2015).

The specific or race-specific immune response relies on the interaction between host resistance (R) genes and pathogen effectors. Effectors are small, secreted proteins that a pathogen releases into the apoplast or cytoplasm to varying degrees of concentration. These secreted proteins are thought to suppress PTI and aid pathogen proliferation through the host, also known as effector-triggered susceptibility (ETS) (Gong et al., 2015). If a host R gene recognizes an effector, it is then called an avirulence (AVR) gene because it initiates effector-triggered immunity (ETI) (Koeck et al., 2011). ETI causes a hypersensitive response (HR) reaction, which kills host cells that a pathogen has infiltrated and is thought to block pathogen movement through the host. The interaction between R genes and AVR genes is of interest for disease management because R genes can be bred into crops to promote specific pathogen resistance (Ashkani

et al., 2015; Wang et al., 2017). The difficulty with this breeding strategy is that many pathogens can quickly overcome one or two R genes within a plant population in a short time (Castagnone-Sereno, 2002; Ridout et al., 2006).

Effector-triggered immunity has been described as a gene-for-gene response (Flor, 1971). Under this premise, the plant response is categorized as either susceptible or resistant. It is generally accepted that if an AVR gene is present with the corresponding R gene, an incompatible reaction will occur, categorized as a resistant response. If the AVR gene is present without the corresponding R gene, the R gene is present without the corresponding AVR gene, or neither genes are present, a compatible reaction will occur, categorized as a susceptible response (Van Der Biezen & Jones, 1998). Flor first described this concept in 1955 with Melamspora lini, the causal agent of flax rust. Over the years, the gene-for-gene hypothesis has been utilized to describe the interaction of *Colletotrichum fructicola,* one of the causal agents of anthracnose in fruit, *Plasmopara viticola,* the causal agent of downy mildew in grapes, *Claviceps purpurea* the causal agent of ergot in cereals, *Magnaporthe oryzae* the causal agent of blast in rice, and many other pathosystems (Kaur et al., 2021). When a direct interaction or corresponding R gene could not be found within a system, the hypothesis was expanded to include guards and decoys, changing the hypothesis to be more quantitative (R. A. L. van der Hoorn & S. Kamoun, 2008). Still, the gene-for-gene hypothesis does not accept infection phenotypes that do not fit within the two categories of susceptible or resistant and could prejudice reported results.

The phenotypes of plant infection cover a spectrum of symptoms that are generally not contained within strict categories. Disease ratings in the field are

quantitative rather than qualitative, considering a wide range of partial infections in plants (Atoum et al., 2016; Thomas et al., 2018). The study of plant resistance genes and their identification led to combining disease phenotypes into groups (Mahlein et al., 2019; Reid et al., 2009; Santiago et al., 2010). For example, a rating system of six ratings would be used to record symptoms, and those ratings would be split into two groups: the first three as avirulent and the second as virulent. This grouping would allow for the segregation of progeny into two categories, thus allowing Mendelian genetics application for gene identification (Ellis et al., 2007; Omoigui et al., 2019). This breeding method has allowed for the discovery of R genes in crop plants such as rice, wheat, soybean, and corn (Busungu et al., 2016; Osorno et al., 2007; Qiu et al., 2020; Wang et al., 2020). However, this method may have also precluded the discovery of R/AVR genes that do not behave in an all or none fashion ascribed to the gene-for-gene hypothesis (Lawrence et al., 1995). Segregation analysis also creates a bias against the variability within biological systems by categorizing phenotypes in strict, reproducible definitions instead of quantitative, inconsistent spectrums (Mutka et al., 2016).

1.3 The Magnaporthe oryzae pathosystem

Magnaporthe spp. are heterothallic ascomycete fungi reported to cause disease in over 50 Poacea hosts, including rice, finger millet, and weeping lovegrass (Leung et al., 1988). Rice Blast caused by *M. oryzae* was first reported in China in 1637 and has since become a critical disease in over 85 countries (Couch et al., 2005; Eseola et al., 2021). Over 50% of the world's population relies on rice as a staple food crop, with over 90% of the world's rice production and consumption located in developing countries (Smith & Leong, 1994). Blast can cause 10-30% annual crop losses, which is enough food to feed

more than 60 million people. Disease impacts can devastate communities that rely on rice for critical nutritional value (Talbot, 2003). Epidemics are common in all countries where blast has occurred, with notable occurrences such as France in 1960, Butan in 1995, Japan in 2003, and the United States in 2010 (Bernaux, 1967; Hataya et al., 2004; "Rice blast disease epidemic - international conference," 2010; Thinlay et al., 2000). Severe epidemics are predicted to increase in Northern India, South East China, Japan, and Korea due to disease presence and changing weather patterns (Savary et al., 2012). The disease has not been eradicated from any country, and management issues persist (Szulczyk, 2022).

The fungus has a predicable infection cycle that begins at dawn or dusk when the relative humidity is high (Talbot, 2003). Following conidial adherence to the rice leaf, the conidium will germinate within approximately 2-4 h given the proper amount of leaf wetness (Cruz-Mireles et al., 2021). The germ tube will hook and form an appressorium 6-8 h post adherence (Eseola et al., 2021; Foster et al., 2017). Penetration of the leaf will occur 10-18 h post adherence with the formation of the primary invasive hypha and the secretion of initial effectors (Shipman et al., 2017). Invasive growth will begin 18-72 h post adherence, with the fungus colonizing the primary infected cell and moving to adjacent cells. Within approximately four days, lesions will be visible on plant surfaces, and within 6-15 d, the fungus can sporulate from plant surfaces (Giraldo et al., 2013; Khang et al., 2010).

M. oryzae is a hemi-biotroph with a sequential progress of infection. The biotrophic stage begins when the penetration peg enters the host cell wall, differentiating into a filamentous hypha (Yan & Talbot, 2016). The filamentous hypha is surrounded by

a host-derived extra-invasive hyphal membrane (EIHM) that keeps the hypha contained (Kankanala et al., 2007). While enclosed in the EIHM, the hypha does not penetrate the host cell membrane, keeping the host cell alive (Jones et al., 2021). A specialized body is then formed on the tip of the filamentous hypha named the biotrophic interfacial complex (BIC). The BIC is not contained in the EIHM and is the proposed location of the cytoplasmic effector section into host cells (Giraldo et al., 2013; Shipman et al., 2017). When the bulbous hyphae begin to differentiate from the filamentous hypha, the BIC is moved to the side of the invasive hypha (Eseola et al., 2021). Once the bulbous hyphae fill the primary infected host cell, the infection spreads into secondary cells (Cruz-Mireles et al., 2021). The initial entrance into secondary cells is identical to the primary infection site, with a filamentous hypha surrounded by an EIMH and tip BIC (Yoshida et al., 2016). The movement of the hyphae into secondary cells generally triggers the transition of *M. oryzae* to the necrotrophic phase in the primary infected cell (Wang et al., 2019). This behavior continues until the host is entirely infected or the fungus is recognized.

Effectors produced by *M. oryzae* can be cytoplasmic or apoplastic (Zhang & Xu, 2014). The cytoplasmic effectors have been shown to sequester in the BIC before secretion into host cells; those effectors will only be present in the host cell after the BIC has formed (Giraldo et al., 2013). Effectors shown in the literature to sequester in the BIC include AVR-Pita, AVR-Pizt, AVRPi9, Pwl1, Pwl2, and Bas1-3 (Gong et al., 2015; Jia & Martin, 2008; Palanna et al., 2023; Tanaka et al., 2010; Zhu et al., 2021). Pwl2 and Bas1 have been shown to continuously secrete into the host cytoplasm from the BIC long after the filamentous hypha differentiates into bulbous hyphae (Khang et al., 2010). Apoplastic

effectors such as Slp1, Bas4, and Bas113 accumulate between the fungal cell wall and the EIHM (Mentlak et al., 2012; Wu et al., 2015). Bas4 and MoCDIP1-5 are suspected to trigger the transition between the biotrophic and necrotrophic phases of fungal infection, indicating a late secretion into the host cell (Chen et al., 2013). The timing of effector release is critical to fungal infection, and at times, plant recognition as effectors can act as AVR genes with host or cultivar specificity.

Host-specific AVR genes in *Magnaporthe spp.* restrict the fungus to only a few susceptible plant species. Pwl1 and Pwl2 are AVR genes that prevent the fungus from infecting weeping lovegrass (Laugé & De Wit, 1998). As stated, Pwl2 is continuously secreted from the BIC, offering no chance for the fungus to escape recognition on weeping lovegrass (Khang et al., 2010). Pwt3 and Pwt4 are AVR genes that confer resistance to wheat (J. Li et al., 2020). Cultivar specific AVR genes are *Magnaporthe spp.* specific and confer resistance to groups within a plant species (Hu et al., 2022; McDonald & Stukenbrock, 2016). Although predominantly asexual in reproduction, *M. oryzae* is highly adaptive, and new strains regularly emerge in the field to overcome resistance (Valent et al., 1991). While host resistance is extremely stable for *Magnaporthe spp.,* cultivar resistance is highly unreliable, only conferring one or two seasons of resistance before the fungus overcomes the plants (Mbinda & Masaki, 2021; Zhu et al., 2016). Cultivar-specific resistance and the genes that confer it are far more common in the *Magnaporthe spp.* genome than host resistance genes (Lopez et al., 2019).

1.4 Oryza sativa

Rice is most susceptible to *M. oryzae* infection when the plant has reached the tillering stage (14-25 d) and the heading stage (55-70 d). The tillering stage will have the

onset of leaf blast, while the heading stage will have the onset of panicle and neck blast (Savary et al., 2012). In addition, environmental factors such as high nitrogen, high humidity, and warming temperatures can cause an increase in disease severity (Cheng et al., 2013; Sester et al., 2019). The season and timing of fungal infection are critical for disease development. This crucial timing is due not only to the climate but also to the development of the rice plant, which confers both passive and active immune responses.

Passive immune responses in rice include physiological changes to leaf structures and cell structures. The activation of the OsMYB30 gene has been associated with the lignification of sclerenchyma cells, thickening the cells and inhibiting the penetration peg of many pathogenic fungi, including *M. oryzae* (W. Li et al., 2020). Plant-mediated changes or suppression of leaf volatile expression allows the host to avoid pathogen detection (F. Li et al., 2022). As many pathogens participate in seeking behavior on host surfaces before germination or hooking, changes to those surfaces cause the pathogen to act as though it is on a nonhost (Oh et al., 2008). Rice uptake of silicon causes the sequestration of silicon in rice cells, inhibiting pathogen penetration and movement within cells (Van Bockhaven et al., 2013). Recent research has even proposed mutualistic relationships between rice and nonpathogenic bacterial species as a means of plant defense (Kumar et al., 2021; Patel et al., 2022). These types of defenses are strongest in mature rice plants, explaining in part why blast is most common at the tillering and heading stages of rice when new growth is present (Nandy et al., 2010).

Race nonspecific immunity of rice to *M. oryzae* involves recognizing fungal-specific proteins. Recognition of MAMPs for *M. oryzae* is rare compared to race-specific resistance (Kanda et al., 2019). Chitin, a component of fungal cell walls, is recognized by

multiple PRRs of rice and brings about cell death (Kishimoto et al., 2010). Recent research has suggested that chitin-induced cell death in rice due to water modulation in host cells (Attia et al., 2020). Chitin is a nonspecific MAMP of most fungi; thus, receptors that recognize the protein components of chitin are highly conserved in the rice genome (Zhang et al., 2021).

Race-specific rice immunity to *M. oryzae* requires the recognition of fungal effectors secreted into the host by R proteins. The most common types of R proteins/genes in rice are nucleotide-binding domains and leucine-rich repeat domains (NLRs) (Yu et al., 2018). NLRs are intracellular receptors that experience a confirmational change upon recognition, inducing a MAP kinase cascade involving salicylic acid that results in the release of reactive oxygen species (ROS) and cell death characterized by HR (Liu et al., 2017; Mizuno et al., 2020). The interaction between R proteins and AVR genes is still greatly debated and weakly understood. Two cultivar-specific AVR genes have been characterized as directly interacting with an R protein, AVR1-CO39, and AVR-Pia, with RGA4 and RGA5, respectively (Cesari et al., 2013). Other AVR genes are suspected of indirectly interacting with R proteins through a guard or decoy.

The guard model was proposed to rectify a rising issue with the gene-for-gene hypothesis of direct interaction due to the discovery of a large amount of AVR genes and comparably few R genes in different pathosystems, including *M. oryzae* (Renier A. L. van der Hoorn & Sophien Kamoun, 2008). The model describes an R protein "guarding" multiple molecules and signaling when one of those molecules interacts with an AVR protein (Holt et al., 2003). The guard model asserts that there could be a reduced

population of R genes linked to many effector targets (Van der Hoorn et al., 2002). An additional model dubbed the decoy model, was proposed to answer the polymorphic nature of R genes. This model maintained that the R proteins still "guarded" target proteins; however, the target proteins would have no fitness advantage acting as a decoy to the pathogen (Kroj et al., 2016). The target molecule would have a high binding affinity for the effector, and, in the absence of an R gene, the effector would not gain a benefit or virulence advantage through the binding (Cesari et al., 2014). The interaction of the AVR-Pizt gene in *M. oryzae* with Piz-t in rice has been suggested to adhere to the decoy model (Park et al., 2016; Tang et al., 2017).

1.5 Environmental factors in the plant-microbe interaction

The Food and Agricultural Organization of the United Nations (FAO-UN) defined Climate Change as an increase in combined surface-air and sea-surface temperatures, averaged over the globe, over 30 years ((IPCC), 2023). Data collected from the NASA Earth Observations (NEO) has shown an overall global mean temperature increase of 1.0℃ since 1880 (Hoegh-Guldberg et al., 2019). While a general increase in global air temperature is concerning in the long term, rising temperature anomalies in focused locations are of immediate importance. The National Center for Atmospheric Research (NCAR) predicted an increase in the intensity and frequency of heat waves during the 21st century (Meehl & Tebaldi, 2004). The National Oceanic and Atmospheric Administration (NOAA) confirmed that the frequency of heat waves has risen from an average of two per year in the 1960s to an average of six per year in the 2010s and 2020s. The temperature of heat waves has also increased to an average of 2.3℃ above the local mean during the 2020s ((NOAA), 2022). Shifting temperatures due to climate change

creates a vulnerability in agricultural systems by introducing variability to the growing season (Bede-Fazekas & Somodi, 2023). Staple crops such as wheat, maize, rice, and soybeans are threatened globally due to unpredictable changes in hardness zones (Velásquez et al., 2018). All agricultural crops are also vulnerable to the spread of new pathogens or pathogen adaptations under a changing climate (Garrett et al., 2014).

Increased temperatures and humidity can cause the persistence of pathogens and pathogen vectors in areas where they have not been present or had extended periods of viable infection (Bebber, 2015; Nnadi & Carter, 2021). Pathogen spread can be due to a myriad of factors, including the increase of biofilm formation and quorum sensing in bacteria (Hasegawa et al., 2005), altered mating behavior in vectors (Curnutte et al., 2014), or movement dynamics in nematodes (Dusenbery, 1988). The molecular aspects of the plant-microbe interaction are also susceptible to rising temperatures. The systems of PTI and ETI have been heavily researched through the lens of climate change (Cheng et al., 2013). The study of protein kinetics has established the optimal temperature for protein interactions and gene expression. Since PTI and ETI are, at the primary level, an interaction of proteins, studies have shown optimal temperatures for those interactions.

At 33°C, the hypersensitive response activated by the *Cf4* and *Cf9* R genes in tomatoes against *Cladosporium fulvum* is suppressed, and the *SNC1* R gene in *Arabidopsis sp.* is directly inhibited at temperatures 28°C and above (Zhu et al., 2010). The resistance of rice to *Xanthomonas oryzae* mediated by the *Xa7* R gene was increased at a temperature combination of 35°C day and 31°C night (Webb et al., 2010). MacQueen et al. found that R genes were better adaptable to the environments of their host plants (i.e., R genes in drought-tolerant, desert plants are inherently better able to function under

increased temperatures than those in a tundra plant) (MacQueen & Bergelson, 2016). The effects of temperature on the plant-microbe interaction can be extremely specific and distinct to the individuals involved. The pathosystem of *Magnaporthe oryzae* displays discrete temperature effects depending on the fungal strain and rice cultivar utilized.

1.6 Infection phenotypes and ratings

A rating scale is prevalently utilized in plant pathology to record disease severity or incidence effectively (Chiang et al., 2016; Chiang et al., 2017). A disease incidence rating provides data on the occurrence of disease in a host (Bock, Chiang, et al., 2022; Chiang & Bock, 2022). A disease severity rating provides data on the progress or acuteness of symptoms of a disease on a host (Abbas et al., 2021; Luvisi et al., 2017; Peña et al., 2013). Disease severity ratings can be focused on a particular plant part, and there can be separate scales for certain symptom types (Abdelraheem et al., 2021; Schneider et al., 2013). The development of artificial intelligence (AI) has allowed for ratings to be performed utilizing sensor-based measurements or fully automated programs (Bock et al., 2020; Bock et al., 2010). Challenges to utilizing any level of AI for disease ratings occur when machine learning fails to differentiate disease symptoms from host responses (Pandian et al., 2019; Prabha, 2021).

Categorization of infection phenotype for *M. oryzae* has been performed in the literature commonly by using a leaf and lesion rating system. Lesions are categorized based on current thinking of how *M. oryzae* progresses through the host. Pinpoint brown or HR flecks are categorized as incompatible reactions since they denote host recognition (Hayashi et al., 2016). Brown lesions have a more divisive categorization in the literature (Hayashi et al., 2016; Yaegashi, 1978). For this work, brown lesions are categorized as

virulent since viable conidiospores were recovered from plant tissues with brown lesions. White lesions are categorized as a susceptible reaction and can be split into two groups (Das et al., 2021). Spreading white lesions are the most susceptible reaction as they display no host recognition and uninhibited fungus growth (Valent et al., 1991). Brown ridged white lesions are a more intermediate susceptible reaction as the fungus grew uninhibited for a time before host recognition halted the spread (Heath et al., 1990). Rating systems have allowed for the isolation of AVR genes through progeny segregation (Ellingboe, 1992; Leung et al., 1988; Silue et al., 1992; Valent et al., 1986; Yaegashi & Asaga, 1981).

1.7 Dissertation outline

This dissertation explored different influences on the host-microbe interaction of *M. oryzae* on rice. Fungal strain 2539 was first selected as a viable source of undiscovered AVR genes. Validation of strain identity in 2539 laboratory stocks through culture assays, plant inoculations, and southern blots led to identifying a genomic rearrangement potentially affecting AVR genes. Plant inoculation assays were used to characterize differences in infection phenotype of the mutant 2539 stocks on different rice cultivars. During these inoculations, infection phenotypes were found to be irreproducible between experiments. The cause of this irreproducibility was explored under different environmental treatments, including plant age at inoculation, light exposure, and time of day at inoculation. Variable temperature exposures pre and post-inoculation at 4℃ and 37℃ were also studied as factors contributing to irreproducibility. Specific treatment of 37℃ post-inoculation for 24 h resulted in stable differences in infection phenotype that were further explored using multiple rice cultivars and fungal

strains. Using fungal paper stocks within plant inoculation assays was explored as a final factor in experimental irreproducibility. Fungal paper stocks had been used during plant inoculations to activate fungal stocks in the creation of inoculum. Since these were the only differences between experimental replicates, it was proposed that the fungal stocks were introducing variability in inoculum.

CHAPTER 2: STRAIN VALIDATION OF 2539 STOCKS

2.1 Background

M. oryzae strain 2539 was bred by the Ellingboe lab in 1988 using fungal strains pathogenic to rice, finger millet, weeping lovegrass, and goosegrass. Strain 2539 is hermaphroditic, with the *MAT1-1* utilized as the primary mating type in crossing experiments (Leung et al., 1988). 2539 infects very few rice cultivars and is a generally poor rice pathogen (Smith & Leong, 1994). 2539 is useful because it is highly fertile and avirulent on most rice cultivars. Therefore, it is amenable to genetic analysis (map-based cloning) and potentially a good source of avirulence genes. In 1998, the Leong lab identified the presence of *AVR1-CO39* on the first chromosome of 2539, and no other AVR genes have been identified in 2539 since then (Farman & Leong, 1998). In fact, in 2010, the Zheng lab proposed that *AVR1-CO39* is the predominant locus governing broad resistance in 2539 and posited that no other AVR genes were present in the strain (Zheng et al., 2011).

Previous work in the Farman lab resulted in several single-spore cultures from 2539 that were found to be fully virulent on previously resistant rice cultivar 51583, which implied that a stable mutation to virulence had occurred. In order to further study the source of this mutation in the 2539 single-spore cultures, it was necessary to validate the strain identity of the mutant stocks compared to known 2539 wild-type stocks. Validation was accomplished using artificial inoculation assays and culture growth comparisons. The results of these experiments predicated the need to analyze the genomic arrangement of the 2539 stocks utilizing Southern blots. The initial use of the telomeric probe did not clarify strain identity for the mutant stocks, necessitating using

the Pot2-MGL probe. Telomeric fingerprinting allows for the specific identification of *M. oryzae* strains based on the banding pattern in a Southern blot with an expected 14 bands for 2539 strains. Pot2_MGL fingerprinting also allows for the specific identification of *M. oryzae* strains utilizing internal transposons, which provide more stable banding patterns than the telomeric regions.

2.2 Material and Methods

2.2.1 Rice cultivation

Rice seeds were soaked in deionized water (DI) for approximately 12 h, surface-sterilized by automated shaking for 10 min in a 50% bleach solution, and rinsed in DI water. Plastic plant pots (5.97 cm x 5.46 cm x 5.92 cm) were filled with moistened, coarse-ground vermiculite, and 12-15 seeds were sown in each pot. The pots were labeled with tags, placed in plastic trays (27.94 cm x 54.28 cm x 6.2 cm), flooded with DI water, and covered with a transparent plastic lid. The seeds were incubated in a growth chamber using a 27°C for 16 h light and 21°C for 8 h dark cycle (COVIRON® PGC Flex-2 Tier growth chamber, 500 µmol/m^2/sec) while maintaining the humidity at less than 80%. After seedling emergence (~7 d), the plastic cover was removed, and the trays were watered twice weekly with Hoagland's solution. Hoagland's solution was made using N 210 ppm, P 31 ppm, S 64 ppm, Cl 0.65 ppm, B 0.5 ppm, Na 0.023 ppm, Mg 48.6 ppm, K 235 ppm, Ca 160 ppm, Mn 0.5 ppm, Zn 0.05 ppm, Cu 0.02 ppm, Mo 0.048 ppm, and Fe 2.9 ppm in DI water. Plants were inoculated at 14 d post-planting unless otherwise noted in specific experimental procedures.

2.2.2 Plant inoculations

The inoculum preparation and the inoculation procedure were performed at the same time of day for every experiment unless otherwise noted. On the same day the seeds were sown, the fungal cultures were activated from frozen stocks by placing fungal stocks grown on filter paper disks on oatmeal agar. Oatmeal agar was made by straining 25 g of rolled oats per liter of DI water and mixing 15 g of agar per liter of DI water. Oatmeal plates were supplemented with 100 µg/ml of ampicillin (Goldbiotechnology®) to inhibit bacterial contamination. Cultures were then grown at 25℃ under continuous illumination. After 14 d, the plates were flooded with 2 ml of a 0.25% gelatin suspension in water, and the fungal colony's surface was massaged with a sterilized bacterial cell spreader to liberate the conidia. The solution was filtered through 0.2 µM Miracloth (Cal BioChem™), the spores were quantified using a hemocytometer, and concentrations were adjusted to 10^5/ml of spores using 0.25% gelatin.

Once the spores were harvested and in suspension, plants were placed into a single Myco-bag (Fisherbrand™) unless otherwise noted, and approximately 200 ml of water was added to increase the humidity. Aerosol inoculation was conducted using a glass sprayer at 20 psi. The bags were sealed with tape and incubated in the dark for 20 h at 22℃. The glass sprayer was cleaned with 70% ethanol and DI water between each fungal strain. After 20 h, the bagged pots were moved to the growth chamber, and the bags were left slightly open for ~1 h to allow the humidity levels to equilibrate. The pots were removed from the bags, and the plants were maintained in the growth chamber under the same environmental conditions described above.

2.2.3 Imaging and quantification

Disease observations and imaging were performed 7 d post-inoculation. All secondary leaves from an individual pot or from multiple pots of the same cultivar inoculated with the same strain were cut and taped to a black binder to provide a clean background. Leaves were scanned in full color on an EPSON GT-1500 scanner. Three samples of the first, second, and third leaves were collected and stored on labeled notecards in case of labeling issues or loss of digital files. The sample leaves were chosen based on how representative they were from the entire pot. Leaves with physical damage due to transport, abnormal growth, or other problems were avoided for collection; however, this information was recorded. Ratings of the secondary leaves were performed using the rating scales shown in subsequent chapters specific to the experimental design and fungal strains.

2.2.4 Statistical analysis

Separate groups of plants were analyzed using an unpaired, unequal variance t-test. Inoculations comparing the entire inoculation were analyzed using an ANOVA (Analysis of variance). Average means and standard deviations were determined using Excel (Microsoft 365© Office 2021). Statistical analysis was performed in R (version 2023.06.2 + 561) and Excel.

2.2.5 DNA extraction

Test tubes containing 10 ml Complete Medium were inoculated with a single mycelial agar plug from actively growing cultures and 10 µl ampicillin. Complete Medium was made with 1% sucrose, 0.6% yeast extract, and 0.6% casamino acids in 300 ml nanopore water. The test tubes were incubated on a slant at room temperature, shaking at 150 rpm for 5-7 d until the white mycelial mass had encompassed greater than 1/3 of

the 10 ml culture volume. The mycelial mass was harvested by filtration through Miracloth. The mycelium was dried on paper towels and then transferred to a 15 ml conical tube (Falcon®). The mycelial plugs were frozen at -20 ℃ and then lyophilized for 24 hrs. The lyophilizer apparatus was filled with paper towels to avoid losing samples. The lysis buffer was warmed in a water bath at 65°C. Lysis buffer was made with 0.5% SDS and 0.05 M Tris-Cl adjusted to pH 8 and 1 mM dithiothreitol. Mycelial pellets were broken up against the sides of the 15 ml conical tube using a glass rod, avoiding the bottom of the tube. Then, 1.5 ml of lysis buffer was added to the falcon tube, and a glass rod was used to disperse mycelial clumps until a smooth slurry was formed. The tube was capped and left at room temperature for 30 min, inverting the tube by hand once at 15 min. Then, 1 ml of phenol/chloroform/isoamyl alcohol solution (25:24:1) was added, the cap was replaced, and the tube was carefully and briefly vortexed, and left at room temperature for 30 min, inverting the tube by hand once at 15 min. The slurry was split into two Phasemaker™ Tubes (Invitrogen™) for a volume of 1.25 ml in each tube. This was centrifuged (Eppendorf™) for 10 min at 15,000 rpm. Then, 1 ml of supernatant was removed and transferred to a 2 ml microfuge tube with 600 µl of isopropanol and mixed by inverting the tube by hand at least ten times. The tubes were immediately centrifuged for 10 min at full speed in a microfuge (15,000 rpm). After discarding the supernatant, the pellet was rinsed with 1 ml of 70% ethanol and centrifuged for 5 min at 14,000 rpm). The pellets dried on the benchtop overnight. The pellets were then resuspended in 100 µl of TE + RNAse A (1 µg/ml, 10 µl/ml TE). DNA was quantified using a Qubit. In order to

visualize the samples, the DNA was loaded onto a 0.7% Agarose gel in 0.5 x TBE (2 µl dye, 2 µl sample, and 4 µl ladder) and run for 80 minutes at 80V. The gel was stained for 10 min with EtBr and washed for 10 min with nanopore water. The gel was imaged with a UV gel imager (Bio-Rad Laboratories ©). Stocks utilized in experiments are listed in Table 2-1.

2.2.6 DNA digestion

DNA was extracted and quantified using Qubit. Concentrations were adjusted so that each stock had the same concentration. Each sample had 1 µg of genomic DNA digested. The total volume of DNA had to be less than 20% of the total restriction reaction. After digestion, a mini gel was run overnight at 20 V for 9 h with 5 µl aliquot of digested DNA and 2 µl of loading dye. This step checks for complete digestion and confirms equal DNA loading. The mini gel was stained with EtBr for 10 min and washed for 10 min in nanopore water. This was imaged with a UV gel imager.

2.2.7 Big gel

A 0.7% agarose gel was made using 0.5X TBE in the medium gel apparatus with tape on the edges to prevent leakages. The agarose was cooled to 55 ℃ before pouring into the gel tray. The gel was allowed to solidify for 1 h. Once the agarose gel solidified, the gel was placed in a 4 ℃ fridge overnight. The gel was placed in the running tank, and the samples were loaded with 1 µl of 5X loading dye for every 3 µl of a sample. The loaded samples sat in the wells for 15 min before activating the electrophoresis unit. The DNA samples were electrophoresed overnight (maximum 35 V) until the smallest fragments were about to run off the gel. This was approximately 18 - 24 h running time

for the gel size and fragments). The gel was stained with EtBr and imaged. The gel was exposed to as little UV light as possible when photographed.

2.2.8 Electroblotting

A Whatman® nitrocellulose membrane, and two pieces of Whatman ® ashless filter paper, were cut according to the gel cast size. The membrane, mesh, pads, and Whatman paper were soaked in fresh TBE buffer. The electroblotting apparatus (Bio-Rad Laboratories ©) was layered: electrode, mesh, pad, Whatman paper, gel upside down (bottom of wells nearest to the membrane), membrane, Whatman paper, pad, mesh, electrode. After adding each layer to expel bubbles, a test tube was rolled over the gel, membrane, and Whatman paper sandwich. The electroblotter was set up and attached to the power supply. The electroblotter was run for 2h at 12V. The blot was removed, and the wells on the membrane were marked with a pencil. The gel and Whatman papers were disposed of in the trash. The membrane was denatured in 0.4 N NaOH (1.6 g/100 ml) for 10 min. The blot was neutralized with 2X SSC for 10 min. The DNA on the membrane was crosslinked using a Spectrolinker™. The blot was put into a glass hybridization cylinder using forceps. The cylinder was sealed, and 5 ml of hybridization solution was added. The hybridization solution was made with 6X SSC, 5X Denhardt's solution (100X = 2% BSA, 2% Ficoll™, 2% polyvinylpyrrolidone), 50% formamide, and 5% SDS. The membrane was incubated at 65 ℃ in a hybridization oven (Cole-Parmer® HI-200) for 30 min.

2.2.9 Probing

In order to make the probe, DNA (25 ng) and nanopore water were mixed to a final volume of 17.5 µl in an Eppendorf tube and boiled in water for 2 min to denature the probe. This was immediately moved to ice, and the following reagents were added: 5 µl Buffer, 1 µl BSA, 0.5 µl Klenow and 1 µl 32P. The reaction mixture was incubated in a 37 ℃ water bath for 1h. The reaction was halted by adding 50 µl of stop buffer. A Sephadex G-50 column™ (Cytiva©) was assembled (Column and 800 µl of Sephadex G-50 suspended in a TE collection tube). The Sephadex column was spun down for 15 s, and the TE flow-through was discarded. The probe was cleaned by adding the reaction mixture to the top of the Sephadex slant and spun down for 30 s. The flow-through was put into a new labeled tube, as this was the cleaned probe, and the Sephadex column was discarded in a hot waste container. The probe was denatured by adding 12 µl of 2 N NaOH to the reaction mix. This was incubated at room temperature for 8 min. Finally, 12 µl of 1 M Tris-HCl, pH 7.4, was added. The prehybridization solution was discarded down the drain, and 10 ml of fresh hybridization solution was added to the membrane. The probe was then added to the cylinder. This was hybridized overnight at 65 ℃.

2.2.10 Washing and Imaging

Once the probe had hybridized overnight, 20 ml of 2X SSC was added into the cylinder to dilute the probe, which was dumped into the hot waste. Then, 60 ml of 2X SSC was added to the cylinder, which was incubated at 65 ℃ for 20 min. This wash was discarded into the hot waste. Another 60 ml of 2X SSC was added and incubated at 65 ℃ for 20 min. Following the second wash, a Phosphor screen was placed on a lightbox to clear just before the final wash. The second wash was discarded in the hot waste. For the

final wash, 60 ml of 0.1X SSC/0.1% SDS was added and incubated at 65 ℃ for 20 min. This wash was disposed of down the drain with running water. The blot was removed from the cylinder using forceps and placed on white paper towels to dry. A piece of Saran wrap was cut, and the membrane was wrapped face down. The membrane was exposed face up on the Phosphor Imager screen and imaged on a Typhoon™, according to program parameters.

2.3 Results

2.3.1 Inoculation of 2539 MH and 2539 ss4* on 51583

This experiment aimed to validate the wild-type and mutant 2539 stock phenotypes on 51583. The wild type 2539 MH was expected to be avirulent on 51583, and the mutant type 2539 ss4* was expected to be virulent on 51583, according to inoculations performed by a previous student in the Farman lab (Heist unpublished). The inoculation of 2539 MH on 51583 resulted in an unexpected virulent response. Plants developed HR flecks, brown lesions, and multiple expanding white lesions. Lesions were not relegated to areas of damage on the leaves or leaves edges. This would suggest that the fungus could infect most of the leaf, not highly susceptible areas. The plants inoculated with 2539 ss4* displayed high levels of infection severity (Figure 2-1). Unlike 2539 MH, the virulent reaction of ss4* was expected as the previous student found similar results after isolating the rare white lesion on 51583. The results of this experiment indicated that the 2539 MH stock utilized for inoculations was a possible contaminant due to the unexpected virulence on 51583 of the stock. Both morphological and DNA tests were performed to validate the authenticity of 2539 MH.

It is necessary to mention that during this initial artificial inoculation assay, the growth chamber utilized for plant growth and holding, malfunctioned, leading to a temperature increase of 25℃ to 37℃ for 24 h before the error was detected. This malfunction occurred directly after the 51583 plants inoculated with 2539 MH and 2539 ss4* were returned to the chamber following the 20 h dark period at 21℃. The 51583 plants were grown in the chamber under normal 25℃ conditions and inoculated when they reached 14 d. After inoculation, the plants were placed in a dark room at 22℃ for 20 h. The plants were then placed back in the chamber, which malfunctioned, going into alarm for 24 h before the plants were removed and placed in a working chamber. As plants were only exposed to a brief period of high temperatures and were not visibly damaged in the process, this malfunction was noted but not predicted to influence the infection phenotypes. It was later determined to be a vital confounding variable that influenced virulence and precipitated numerous heat experiments described in later chapters.

2.3.2 Plate morphology of 2539 MH and 2539 ss4*

Concurrent with the artificial plant inoculation assays, the wild type 2539 MH stock and mutant 2539 ss4* were grown in culture. This experiment aimed to validate the plate morphology of the two stocks. The stocks were expected to grow rapidly on oatmeal agar with dark grey coloration and high sporulation classic to the 2539 strain (Leung et al., 1988). The mutant 2539 ss4* stock displayed the expected rapid growth rate and high level of sporulation after 14 d of growth on the plate. In contrast, the wild-type 2539 MH stock grew noticeably slower, with a beige coloration and low sporulation (Figure 2-2). The plate morphology of 2539 MH was unexpected as it differed from the

accepted 2539 strain morphology in the culture. Based on the results of this experiment and the plant inoculation assays described previously, 2539 MH was predicted to be a contaminant. These phenotypic and morphological results indicated a need to genetically verify the strain identity of 2539 MH.

2.3.3 Telomere fingerprints of 2539 wild-type and mutant culture stocks to verify the strain identity

This experiment aimed to determine the strain identity of 2539 MH by comparing the telomere fingerprint of the stock to known 2539 strain fingerprints. *M. oryzae* strains have a predicated telomeric repeat, allowing strain identification in a blot. The results of the telomere fingerprint blot revealed that the wild type 2539 MH stock was not a contaminant, despite the anomalous growth and infection phenotype previously noted in experiments. Based on the telomere fingerprint blot, 2539 MH was 2539 strain identity. The wild-type stocks displayed matching bands in addition to the known MoTeR elements and rDNA. Surprisingly, the 2539 mutant stocks could not be confirmed to have 2539 identity due to the differences in band sizes compared to the wild-type stocks. All mutant stocks were missing ten of the fourteen expected telomeric fragments for strain 2539. The mutant stocks also contained nine novel fragments not present in strain 2539 (Figure 2-3A). These results indicated that the mutant strains could be contaminants and not 2539 strain identity, revealing the need to further verify the mutant stocks' strain identity.

2.3.4 Confirmation of strain identity using Pot2-MGL fingerprint

This experiment aimed to verify the stain identity of the mutant stocks and rule out the possibility that the mutant stocks were contaminating progeny from a concurrent inoculation when they were first isolated. The arrangement of Pot2 within the genome

differs for many *M. oryzae* strains, allowing the different strains to be distinguished when Pot2 is used as a marker. If the mutant stocks were contaminants, they would not have similar bands with the parental stocks after probing with Pot2. The fingerprints of each 2539 stock were almost identical after Pot2 probing (Figure 2-3B). This negated any alternative interpretations that the different growth patterns or infection phenotypes were due to the mutant stocks being a contaminant strain. This result clarified that although the mutant stocks have 2539 strain identities, they each contain massive genomic rearrangement in the telomeric and sub-telomeric regions of the chromosome.

2.4 Discussion

The results of this chapter support the occurrence of a spontaneous and severe genomic rearrangement of the telomeric regions of *M. oryzae* 2539 stocks during either plant inoculation or fungal storage. The genomic shift may have caused a gain of virulence on the 2539 mutant stocks on 51583, which had previously been resistant to the strain. Southern blotting analysis indicated changes in the telomeric and sub-telomeric regions of the chromosome. The chromosome tips are the most variable regions of the *M. oryzae* genome; therefore, probes for these regions are ideal for DNA fingerprinting. This is due to the ability to detect several sequences (at least 14 in *M. oryzae),* and the resulting hybridization profiles tend to be variable among different strains, but similar for sub-cultures (Farman & Leong, 1995). The results also indicated that internal regions of the chromosome were intact through the use of the Pot2_MGL probe. Pot2 and MGL are high-copy transposons that reside at internal locations of the M. oryzae genome and are less prone to rearrangement (Farman and Kim, 2005). Pot2 is 1,857 bp in length and has terminal inverted repeats, and there are approximately 100 copies of Pot2 per haploid

genome (Kachroo et al., 1994). These results present a concern for the long-term storage of fungal stocks.

Laboratory fungal stocks are commonly stored in freezer conditions as filter paper or glycerol stocks (Gupta et al., 2020; Hiruma & Saijo, 2016). Numerous subculturing of fungal stocks is supported to introduce mutations and loss of traits (Curtis et al., 2023; Trejo-Aguilar et al., 2013). The results discussed in this chapter indicate that activation and immediate usage of fungal stock in experiments may not be ideal. Stocks that have been subcultured numerous times or stored for long periods should be validated before any use in experiments. Evidence of mutation in other fungal genera in freezer storage indicates that some form of genetic validation should be performed regularly on stocks (Kretschmer et al., 2014; Newton & Johnson, 1939).

Approximately 50% of the AVR genes in *M. oryzae* map to the telomeric and sub-telomeric regions of the chromosomes (Rehmeyer et al., 2006). These areas are highly variable and can change rapidly from one generation of the fungus to the next (Rahnama et al., 2021). This adaptation is highly advantageous for the fungus, as *M. oryzae* reportedly only asexually reproduces in the field except for an isolated case in Yunnan, China (Couch et al., 2005; Hayashi et al., 1997; Saleh et al., 2012). Without sexual recombination, the fungus would need to evolve mechanisms of genomic variability in order to overcome host resistance. MoTeR elements are one such predicted mechanism resulting in highly unstable regions of the telomeres (Starnes et al., 2012). Changes to the telomeric and sub-telomeric areas of the chromosomes could lead to decreased or increased infection in host plants depending on the AVR genes involved (Huang et al., 2014; Orbach et al., 2000; Sone et al., 2013). This is one possible reason

for the lack of stable disease resistance in rice cultivars for over two seasons (Ballini et al., 2008; Goncharova et al., 2020). High levels of variability or massive changes to the genome, as discussed in this current research, may mirror the circumstances of host shifts leading to the emergence of wheat blast in 1985 or the more recent reports of *M. oryzae* infection of banana (Gladieux et al., 2018; J. Li et al., 2022; Murakami et al., 2000).

M. oryzae is not the only fungus reported to have an unstable genome, leading to gains or losses of infection on hosts. *Puccinia graminis,* the causal agent of wheat stem rust, was reported to have a gain of function mutation while being kept in refrigerator storage in the laboratory (Newton & Johnson, 1939). *Fusarium sp.,* including the economically critical *F. graminearum, F. verticilliodies,* and *F. oxysporum,* have been found to have large transposable elements in pathogenicity-related areas of the chromosome, which are variable during asexual reproduction (Ma et al., 2010). Given the speed and efficiency of asexual production compared to sexual reproduction, it is logical that many plant pathogenic fungi would evolve mechanisms of genetic variability or instability during mitosis (Lang et al., 2009; Sun & Heitman, 2011). Targeting areas of the genome related to pathogenicity would be highly advantageous for a fungus continually attempting to outpace host resistance. This also emphasizes a critical need for stacked genes in resistance breeding, as fungal adaptation could quickly overcome reliance upon one or two genes (Kumari et al., 2018; Li et al., 2023).

2.5 Conclusions

This chapter explored the validation of strain identity of 2539 laboratory fungal stocks. Initial plant inoculation assays resulted in unexpected virulent phenotypes of wild-type 2539 MH stock on 51583. Additionally, this stock differentiated itself during

culture growth, compared to the mutant stocks and other wild-type stocks. Southern blots were performed to validate the strain identity of 2539 MH. The strain was found to have 2539 identity through a telomeric fingerprint. Unexpectedly, the mutant stocks could not be confirmed in identity with the telomeric fingerprint, necessitating the use of Pot2_MGL. The blot probing with Pot2_MGL confirmed the identity of the mutant stocks as 2539 strains. The main conclusion found in this chapter was that the mutant 2539 stocks underwent genomic rearrangement in the telomeric and sub-telomeric regions of the chromosome. Rearrangement in these chromosome regions could lead to changes in the infection ability of the mutant stocks. Further work should be performed to determine the potential effects of the genomic rearrangement on the ability of the mutant stocks to infect different rice cultivars.

Table 2-1: *Magnaporthe* Stocks Utilized for Experiments

Isolate Name	Host	Year	Type	Source
2539 MH	*Oryza sativa*	2014	Wild Type	2539 subculture
2539 FG	*Oryza sativa*	2007	Wild Type	2539 subculture
2539 5.5.01	*Oryza sativa*	2007	Wild Type	2539 subculture
2539 5.5.02	*Oryza sativa*	2007	Wild Type	2539 subculture
2539 ss1	*Oryza sativa*	2014	Mutant	2539 single spore from lesion of 51583
2539 ss2	*Oryza sativa*	2014	Mutant	2539 single spore from lesion of 51583
2539 ss3	*Oryza sativa*	2014	Mutant	2539 single spore from lesion of 51583
2539 ss4	*Oryza sativa*	2014	Mutant	2539 single spore from lesion of 51583
2539 ss4*	*Oryza sativa*	2015	Mutant	2539 ss4 subculture made
2539 ss5	*Oryza sativa*	2014	Mutant	2539 single spore from lesion of 51583

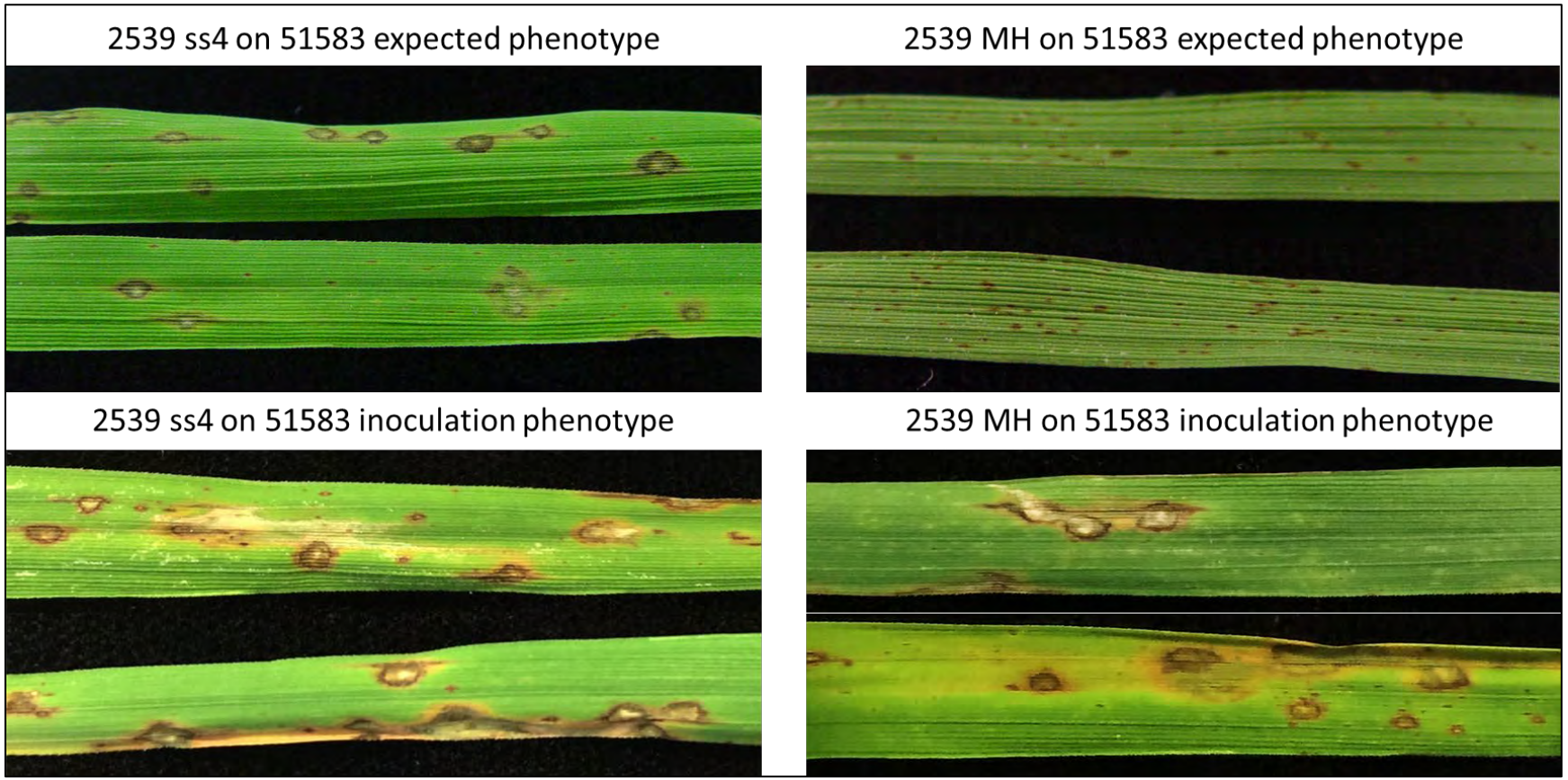


Figure 2-1 Unexpected Phenotype of 2539 MH on 51853 Rice cultivar 51583 was inoculated with 2539 MH and 2539 ss4. The figure displays the expected phenotypes for both 2539 MH and 2539 ss4 on 51583. While the 2539 ss4 phenotype matched the previous inoculations, the virulent 2539 MH phenotype was unexpected. These are the plants that were in the chamber during the malfunction.

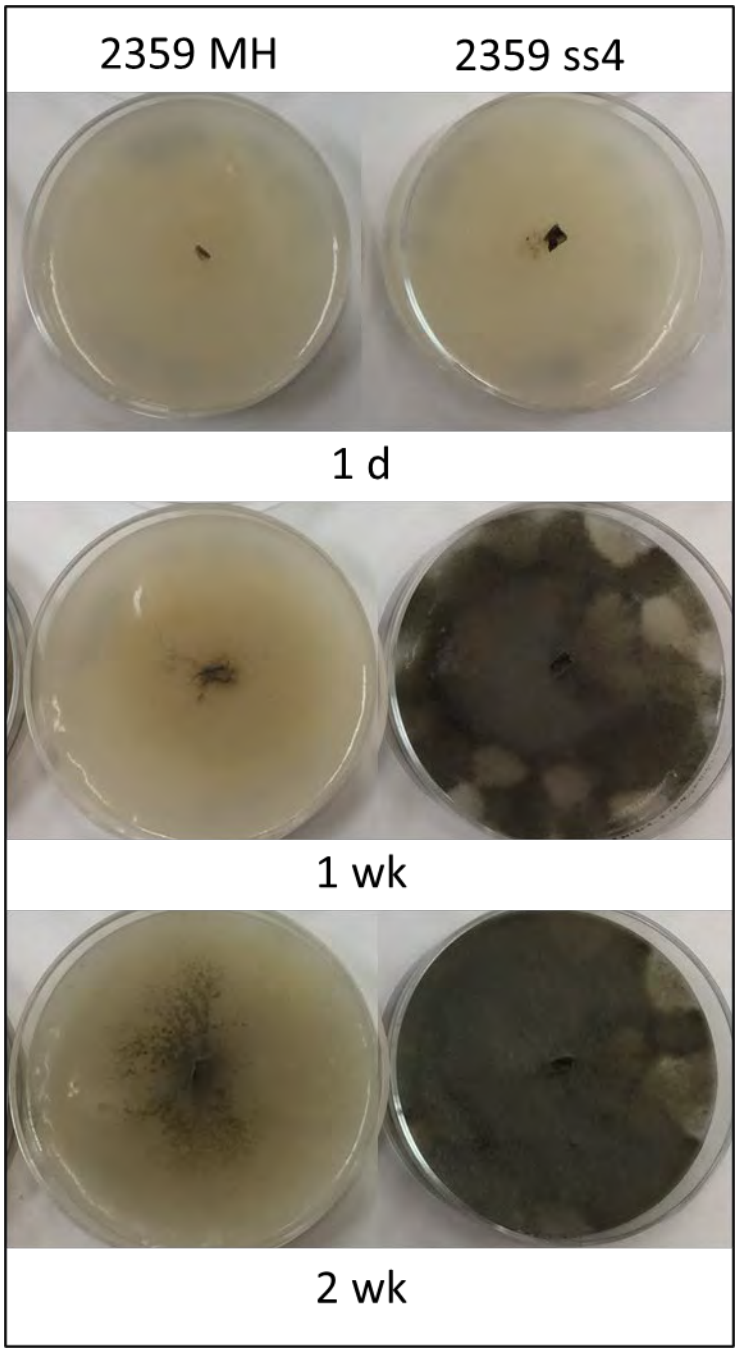


Figure 2-2 Delayed Growth and Low Sporulation of 2539 MH Plates of 2539 MH and 2539 ss4 are shown for comparison of growth rate over 2 wk. 2539 MH is the wild type parental stock and 2539 ss4 is the mutant single-spore stock.

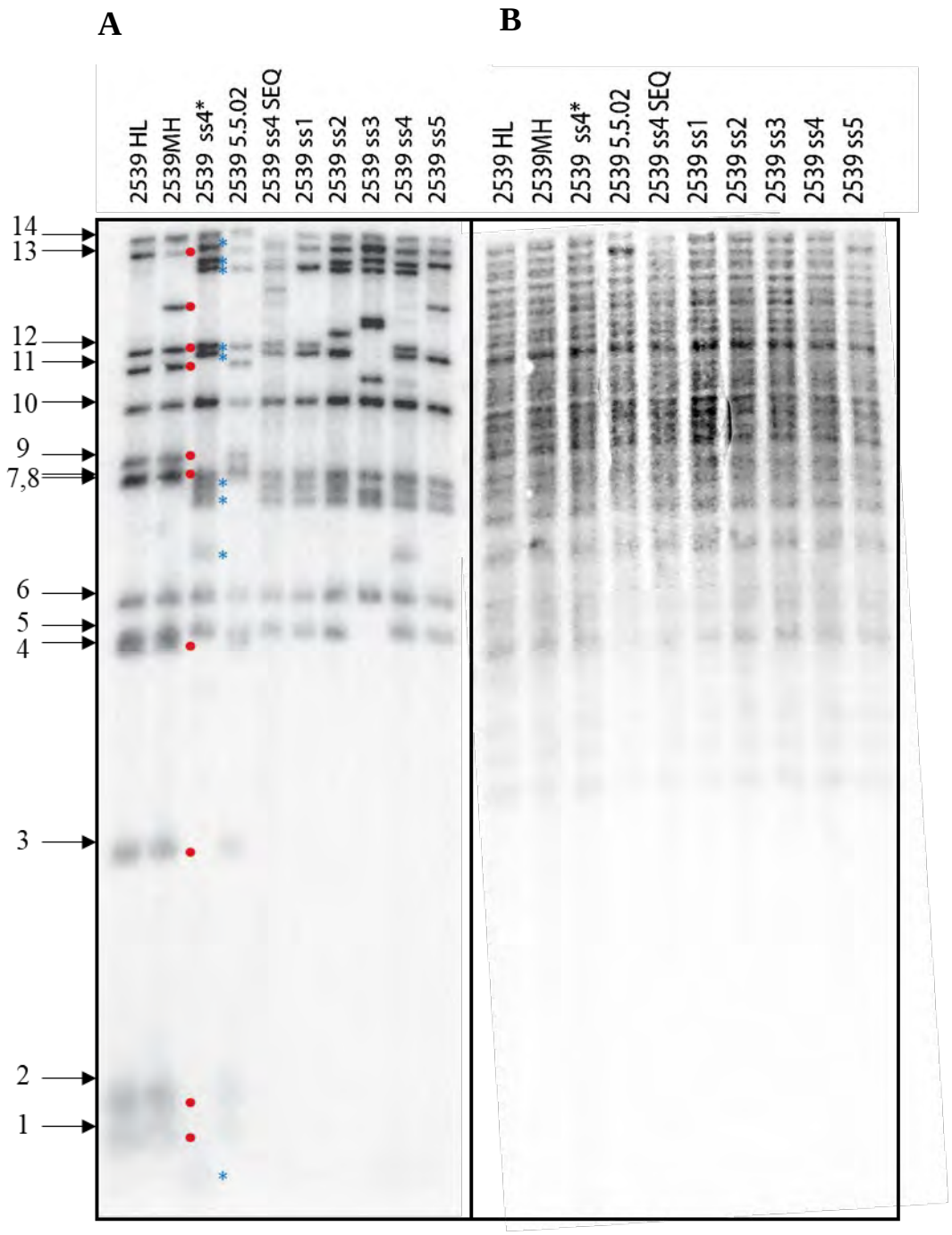


Figure 2-3: Southern Blot Analysis of 2539 Stocks (A) Telomere fingerprints of 2539 stocks with red dots marking the telomere fragments present in the wild type 2539 and absent in the single spore mutant. Blue asterisks mark novel telomere fragments present in the single spore mutants. (B) POT2_MGL fingerprints of 2539 stocks displaying tandem repeats throughout the genome

CHAPTER 3: PHENOTYPIC COMPARISON OF 2539 STOCKS IN VIVO

3.1 Background

Strain 2539 was bred in the Ellingboe lab in 1988, utilizing strains pathogenic on weeping lovegrass, finger millet, and rice (Leung et al., 1988). The only AVR gene to date identified in 2539 is *AVR1-CO39* (Farman & Leong, 1998). This led Zheng et al. to posit that *AVR1-CO39* was the only AVR gene in 2539 governing broad resistance (Zheng et al., 2011). Strain 2539 is a poor rice pathogen but can be bred with fertile *M. oryzae* strains (Silue & Notteghem, 1990; Talbot, 2003). Research was performed on this fungal strain to further explore the influences of the genomic rearrangement discussed in Chapter 2. The 2539 stocks were predicted to have greater virulence on different rice cultivars than wild-type strains, based on the genomic rearrangements in the telomeric regions.

Rice is considered to have been domesticated approximately 6,000 years ago in Japan (Couch et al., 2005). Rice has since been divided into two major subspecies: African and Asian rice (Badro et al., 2020; Chauhan et al., 2017). The Asian rice subspecies can be divided into *Indica* and *Japonica* (Khush et al., 2003). Asian rice types are popularly utilized for blast research (Wang & Valent, 2009; Zhang et al., 2015). Asian rice cultivars 51583, Yt16, CO39, and M2O2 are utilized for experimentation with *M. oryzae* and are not generally used in commercial rice production (Laborte et al., 2017). Rice cultivar CO39 is known to carry the reciprocal R gene *Pi-CO39* for *AVR1-CO39* in 2539 (Cesari et al., 2013; Farman & Leong, 1998). Rice cultivar 51583, Yt16, and M2O2 do not possess R gene *Pi-CO39* (Jia & Martin, 2008; Jia et al., 2000; Leung et al., 1988). Yt16, M2O2, and CO39 are resistant to 2539 infection, while 51583 is susceptible

(Chauhan et al., 2002). This indicates that there may be more AVR genes in the genome of 2539 other than *AVR1-CO39*. The genomic rearrangement of the 2539 stocks may have disrupted the other unidentified AVR genes and changed the ability of 2539 to infect Yt16 and M2O2. These experiments aimed to compare the infection phenotype of the wild-type and mutant 2539 stocks on rice cultivars 51583, Yt16, M2O2, and CO39.

3.2 Materials and Methods

Plant inoculations matched the protocols described in Chapter 2. Inoculations were replicated on five separate days. The stocks utilized in the experiments are listed in Table 3-1. Cultivars utilized in the experiments are listed in Table 3-2.

Disease ratings for secondary leaves were made using the scale shown in Figure 3-1. Ratings were used to determine the severity of the disease. Ratings 0-3 represent resistance levels on the host (avirulent), while ratings 4-8 represent levels of susceptibility on the host (virulent). Ratings of 4 and above were categorized as virulent, while ratings below 4 were categorized as avirulent. Statistical analysis was performed using an unpaired, unequal variance t-test. Analysis of variance was performed for the replications of each stock to ensure that data could be combined.

3.3 Results

3.3.1 Inoculation of 51583 with 2539 stocks

The goal of this experiment was twofold. First, the infection phenotype of the wild-type 2539 stocks on 51583 was compared to the phenotype of 2539 MH on 51583. It was necessary to determine if the virulence of 2539 MH on 51583 was an anomaly or if the other wild-type 2539 stocks would also display virulence on 51583. Second, the infection phenotypes of six mutant stocks on 51583 were compared to those of five wild-

type stocks on 51583. Based on the genomic rearrangement of the mutant stocks described in Chapter 2, it was necessary to determine if the mutant stock would be more virulent on 51583 compared to the wild-type stocks.

Overall, the results of this inoculation displayed a lack of virulence on 51583 after inoculation with both the mutant stocks and the wild-type stocks, with some variability across experimental replicates. The virulence of 2539 MH on 51583 discussed in Chapter 2 was confirmed to be an anomaly. All the wild-type stocks displayed an average rating below the level of virulence across the five replications. The mutant stocks resulted in higher average disease ratings than wild-type stocks.

The average secondary leaf rating across the five experimental replicates was less than 4, categorized as avirulent for wild-type stocks. Stock 5.5.01 had an average rating of 2.51±0.91 across the five inoculations, with no replicate displaying virulence. Stock 5.5.02 had an average rating of 3.2±0.97 across the five inoculations, with only the second replicate displaying virulence. Stock FG had an average rating of 3.22±0.43 across the five inoculations, with no replicate displaying virulence. Stock HL had an average rating of 3.12±0.32 across the four inoculations, with no replicate displaying virulence. The second replicate of stock HL was dropped due to plate contamination. Stock MH had an average rating of 1.64±0.68 across the four inoculations, with no replicate displaying virulence. The second replicate of stock MH was dropped due to plate contamination. All of these results are displayed in Figure 3-2. These results indicated the inability of the wild-type stocks to cause virulent reactions reliably on 51583. These results also indicated that the virulence of 2539 MH on 51583, described in

Chapter 2, was anomalous compared to these experimental replicates and the other wild-type stocks.

Stock ss1 had an average rating of 4.27±0.5 across the five inoculations, with the first, second, and third replicates displaying virulence. Stock ss2 had an average rating of 3.94±0.65 across the five inoculations, with the first and second replicates displaying virulence. Stock ss3 had an average rating of 3.8±1.01 across the five inoculations, with the first and third replicates displaying virulence. Stock ss4 had an average rating of 1.16±0.45 across the five inoculations, with no replicate displaying virulence. Stock ss4* had an average rating of 3.94±0.8 across the five inoculations, with the third and fifth replicates displaying virulence. Stock ss5 had an average rating of 3.16±0.92 across the five inoculations, with only the third replicate displaying virulence. All of these results are displayed in Figure 3-2. While some mutant stocks displayed virulent reactions in some replications, the overall combined average for each stock displayed avirulence.

Although the majority of the two stock types, mutant or wild-type, did not result in a virulent reaction on 51583, the mutant stocks produced a higher average rating than the wild-type stocks. The average rating was higher for the mutant stocks than the wild-type stocks for the first replicate with a $p<0.05$, the third with a $p<0.01$, and the fourth replicate with a $p<0.01$. The comparison for the second replicate was not functional due to the lack of results for 2539 MH and 2539 HL. All of these results are displayed in Figure 3-3. These results indicate a behavioral difference between the mutant and wild-type stocks supported by the genomic rearrangement discussed in the previous chapter, despite their inability to reliably cross the standard virulence threshold.

3.3.2 Inoculation of Yt16 with 2539 stocks

This experiment aimed to determine whether the increased disease severity of the mutant stocks on 51583 could be mirrored on another cultivar with different resistance genes. Generally, the 2539 strain is unable to infect rice cultivar Yt16. The expected results of this experiment were that the wild-type stocks would be avirulent on Yt16 due to previous avirulence observed in inoculations. The mutant stocks were predicted to have higher disease ratings than the wild-type stocks while remaining under the level of virulence similar to what was observed in the 51583 inoculations. Overall, the results of this inoculation displayed a lack of virulence on Yt16 after inoculation with both the mutant stocks and the wild-type stocks, with some variability across experimental replicates. The mutant stocks resulted in higher disease ratings compared to the wild-type stocks.

The average secondary leaf rating across the five experimental replicates was less than 4, categorized as avirulent for the wild-type and mutant stocks. Stock 5.5.01 had an average rating of 0.32±0.41 across the five inoculations, with no replicate displaying virulence. Stock 5.5.02 had an average rating of 0.47±0.52 across the five inoculations, with no replicate displaying virulence. Stock FG had an average rating of 0.32±0.37 across the five inoculations, with no replicate displaying virulence. Stock HL had an average rating of 0.65±0.28 across the four inoculations, with no replicate displaying virulence. The second replicate of stock HL was dropped due to plate contamination. Stock MH had an average rating of 0.13±0.13 across the four inoculations, with no replicate displaying virulence. The second replicate of stock MH was dropped due to plate contamination. All of these results are displayed in Figure 3-4. These results indicated the inability of the wild-type stocks to cause any virulent reactions on Yt16.

Stock ss1 had an average rating of 0.81±0.35 across the five inoculations, with no replicates displaying virulence. Stock ss2 had an average rating of 1.31±0.55 across the five inoculations, with no replicates displaying virulence. Stock ss3 had an average rating of 0.95±0.33 across the five inoculations, with no replicates displaying virulence. Stock ss4 had an average rating of 0.05±0.07 across the five inoculations, with no replicate displaying virulence. Stock ss4* had an average rating of 0.8±0.3 across the five inoculations, with no replicate displaying virulence. Stock ss5 had an average rating of 0.88±0.32 across the five inoculations, with no replicate displaying virulence. All of these results are displayed in Figure 3-4. These results indicated the mutant stocks' inability to cause any virulent reactions on Yt16, although the mutant stocks did have higher disease ratings compared to the wild-type stocks.

Although a majority of the inoculations on Yt16, with either the mutant stocks or wild-type stocks, did not result in a virulent reaction, the mutant stocks produced a higher combined average rating than the wild-type stocks. The average rating was higher for the mutant stocks than the wild-type stocks for the first and fourth replicates with a $p<0.01$. The comparison for the second replicate was not functional due to the lack of results for 2539 MH and 2539 HL. All of these results are displayed in Figure 3-5. These results indicate a behavioral difference between the mutant and wild-type stocks supported by the genomic rearrangement discussed in the previous chapter.

3.3.3 Inoculation on M2O2 with 2539 stocks

This experiment aimed to determine whether the increased disease severity of the mutant stocks on 51583 and Yt16 could be mirrored on another cultivar with different resistance genes. Generally, the 2539 strain is unable to infect rice cultivar M2O2. The

expected results of this experiment were that the wild-type stocks would be avirulent on M2O2. The mutant stocks were predicted to have higher disease ratings than the wild-type stocks but still lacked virulence, similar to the observation in the 51583 and Yt16 inoculations. Overall, the results of this inoculation displayed complete avirulence on M2O2 after inoculation with both the mutant and wild-type stocks. The mutant stocks displayed no difference in disease ratings compared to the wild-type stocks. Leaf scans of the M2O2 inoculations are included in the Appendices of this dissertation for reference.

3.3.4 Inoculation on CO39 with 2539 stocks

This experiment aimed to determine whether the increased disease severity of the mutant stocks on 51583 and Yt16 could be mirrored on another cultivar with different resistance genes. Generally, the 2539 strain cannot infect rice cultivar CO39 due to the presence of *AVR1-CO39*. It was possible that the genomic rearrangement had altered of deleted *AVR1-CO39* in the mutant stocks. The expected results of this experiment were that the wild-type stocks would be avirulent on CO39. The mutant stocks were predicted to have higher disease ratings than the wild-type stocks without reaching the level of virulence, as similarly observed in the 51583, Yt16, and M2O2 inoculations. Overall, the results of this inoculation displayed complete avirulence on CO39 after inoculation with both the mutant and wild-type stocks. The mutant stocks displayed no difference in disease ratings compared to the wild-type stocks. Leaf scans of the CO39 inoculations are included in the Appendices of this dissertation for reference.

3.4 Discussion

The experiments described in this chapter were performed to elucidate the differences between in vivo infection of the wild-type 2539 stocks and the mutant 2539

stocks. The mutants stocks displayed higher disease ratings on 51583 compared to the wild-type stocks. The mutant stocks displayed higher disease ratings on Yt16 compared to the wild-type stocks. All 2539 stocks were completely avirulent on M2O2. All 2539 stocks were completely avirulent on CO39. The loss of the second replication of 2539 HL and 2539 MH may have caused the combined average rating of the wild-type stocks to appear higher for that replicate. This, in turn, gave the appearance that the wild-type stocks displayed higher virulence ratings compared to the mutant stocks in the second replication on 51583 in Figure 3-3.

Based on the genomic rearrangement described in Chapter 2, the mutant stocks were hypothesized to have increased virulence on rice compared to the wild-type stocks. The inoculations on 51583 and Yt16 supported this prediction as the mutant stocks displayed higher disease ratings than the wild-type stocks despite their inability to reach the level of virulence. The inoculations on CO39 and M2O2 indicated that, although the mutant stocks did have telomeric rearrangement, some avirulence factors were still expressed in the mutant stocks, leading to host resistance. This would most likely include *AVR1-CO39*, resulting in an avirulent reaction upon interaction with *Pi-CO39*, which is known to be in rice cultivar CO39.

The results described above indicate that telomeric rearrangement of the mutant 2539 stocks, noted in Chapter 2, influenced the infection phenotypes on 51583 and Yt16. Zheng et al. posited that *AVR1-CO39* is the predominant locus 2539 that governs broad resistance (Zheng et al., 2011). *Pi-CO39* has not been reported in the genome of either 51583 or Yt16 (Jia et al., 2000). AVR1-CO39 may interact indirectly or directly with other R proteins in 51583 and Yt16, leading to host resistance. The direct interaction of

AVR1-CO39 with *RGA4* and *RGA5* was recently reported (Cesari et al., 2013). Whether 51583 or Yt16 possess these R genes has yet to be determined. *AVR1-CO39* expression is reportedly low in 2539 and does not accumulate in large quantities even when present (Ribot et al., 2013). It could be proposed that the genomic rearrangement of the mutant stocks influenced the expression of *AVR1-CO39,* causing a greater reduction in expression; however, this seems unlikely due to the continued complete resistance of rice cultivar CO39 against the mutant stocks (Chauhan et al., 2002). It seems more likely that 2539 has at least one other AVR gene that is recognized by 51583 and at least one other AVR gene that is recognized by Yt16, leading to host resistance against the pathogen. If only one single AVR gene were conveying resistance on both 51583 and Yt16, then the mutant stocks would have been virulent on both cultivars, which was not supported by the avirulent results.

The simplest explanation for the higher disease ratings of the mutant stocks on 51583 and Yt16 could be the deletion of AVR genes recognized by the two cultivars, as has been reported for many AVR/R interactions (Fujisaki et al., 2015; Kanzaki et al., 2012; Longya et al., 2019). However, the interaction of AVR genes and R genes can be extremely complex, resulting in infection phenotypes displaying partial resistance like the results described in this chapter. *Leptosphaeria maculans*, the causal agent of phoma stem canker on oilseed rape, was reported to have a complex interaction between multiple AVR and R genes during infection (Ghanbarnia et al., 2018). In *L. maculans, AVRLm4-7* suppresses recognition of *AVRLm3* by acting directly on the resistance gene *Rlm3*. If *AVRLm4-7* were deleted, the host would recognize infection by interacting with *AVRLm3* and *Rlm3* (Plissonneau et al., 2016). In this case, a deleted AVR gene leads to resistance

due to the role of the gene in immune suppression (Lazar et al., 2022). If the genomic rearrangement of the mutant stocks was not a simple deletion of an AVR gene(s) that interacted directly with R gene(s) for recognition but rather a deletion that influenced suppression or expression of multiple genes, this could explain the unstable virulence of the mutant stocks on 51583 and the unstable resistance on Yt16.

3.5 Conclusions

The results of these experiments indicate that the genomic rearrangement in the telomeric and sub-telomeric regions of the 2539 mutant stocks potentially influenced a newly observed virulence on 51583. The mutant stocks were also able to cause a slight increase in avirulent host reaction factors, such as HR flecks on Yt16. M2O2 and CO39 maintained resistance against the mutant 2539 stocks, indicating the stability of *AVR1-CO39* and another possible unidentified AVR gene. Future work could be performed to characterize the specific mutations in the mutant stocks genetically. Once identified, a gene predictor algorithm could be utilized to isolate possible new AVR genes in 2539. Alternatively, if no other AVR genes can be identified, specific interactions of Yt16 and M2O2 with *AVR1-CO39* could be studied.

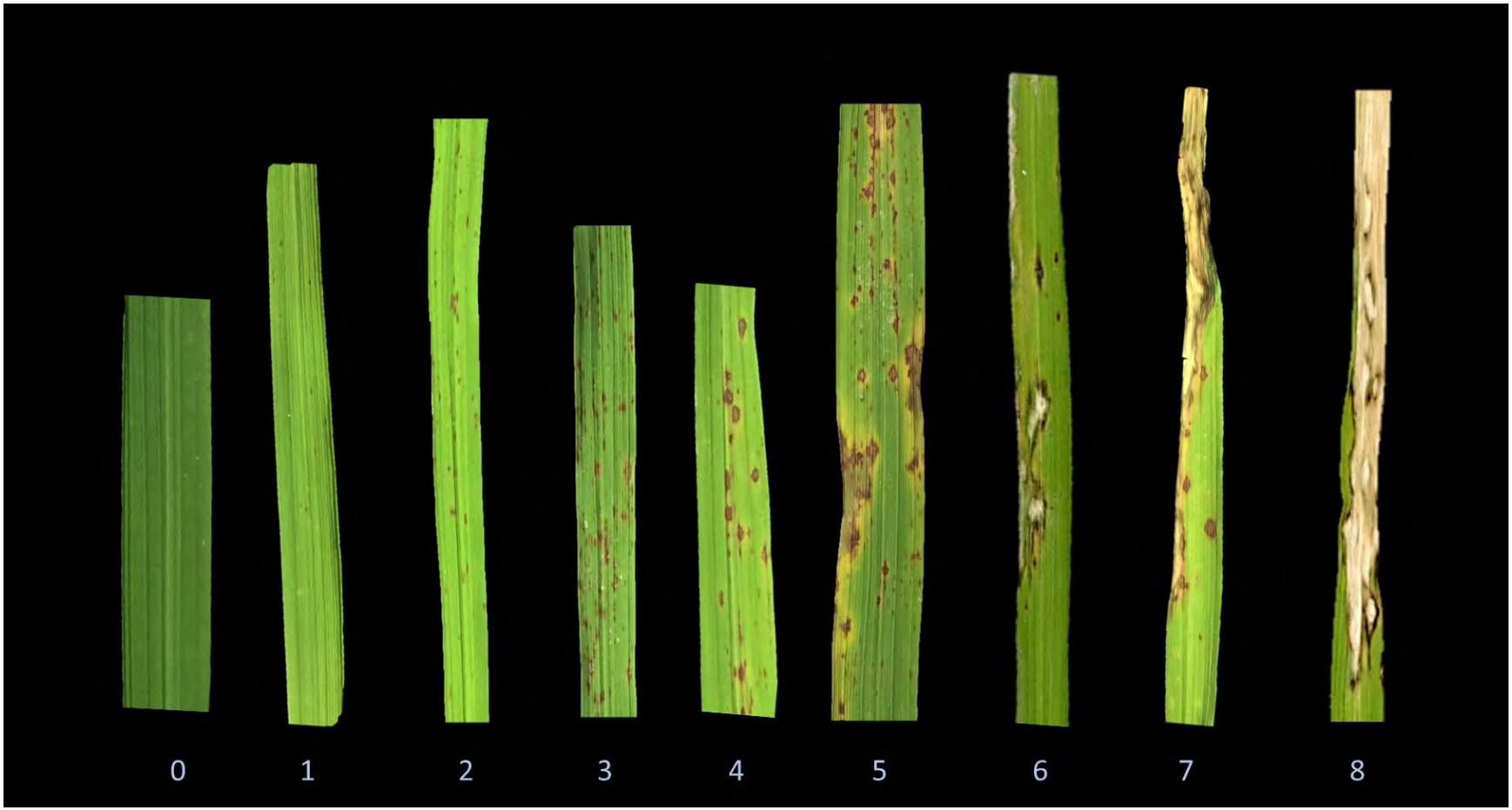


Figure 3-1 Disease Severity Ratings Severity ratings are on a scale from 0-8. Ratings of 0-3 are categorized as avirulent and show levels of resistance. Ratings of 4-8 are categorized as virulent and shows levels of susceptibility.

Table 3-1: Magnaporthe stocks utilized for experiments

Isolate Name	Host	Year	Type	Place of Isolation	Source
2539 HL	Oryza sativa	1988	Wild-Type	Laboratory Strain	H. Leung, bred strain
2539 MH	Oryza sativa	2014	Wild-Type	Laboratory Strain	M. Heist, subculture of 2539
2539 FG	Oryza sativa	2007	Wild-Type	Laboratory Strain	Subculture of 2539
2539 5.5.01	Oryza sativa	2007	Wild-Type	Laboratory Strain	Subculture of 2539
2539 5.5.02	Oryza sativa	2007	Wild-Type	Laboratory Strain	Subculture of 2539
2539 ss1	Oryza sativa	2014	Mutant	Laboratory Strain	M. Heist, 2539 single spore from lesion of 51583
2539 ss2	Oryza sativa	2014	Mutant	Laboratory Strain	M. Heist, 2539 single spore from lesion of 51583
2539 ss3	Oryza sativa	2014	Mutant	Laboratory Strain	M. Heist, 2539 single spore from lesion of 51583
2539 ss4	Oryza sativa	2014	Mutant	Laboratory Strain	M. Heist, 2539 single spore from lesion of 51583
2539 ss4*	Oryza sativa	2015	Mutant	Laboratory Strain	M. Heist, subculture of 2539 ss4
2539 ss5	Oryza sativa	2014	Mutant	Laboratory Strain	M. Heist, 2539 single spore from lesion of 51583

Table 3-2: Rice Cultivars Utilized for Experiments

Cultivar Name	Type	Year	Origin	Known R genes	Reference Source
51583	Indica		USSR		(Leung, et. al, 1988)
CO39	Indica		India	Pi-CO39, Pi-a	(Miah, et al., 2013), (Bryan, et al., 2000), (Tsunematsu, et al., 2000)
M202	Japonica	1985	United States		(Costanzo and Jia, 2010), (Wang, et al., 2007)
Yt16				Pt-r	(Bryan, et al., 2000), (Zhao, et al., 2018)

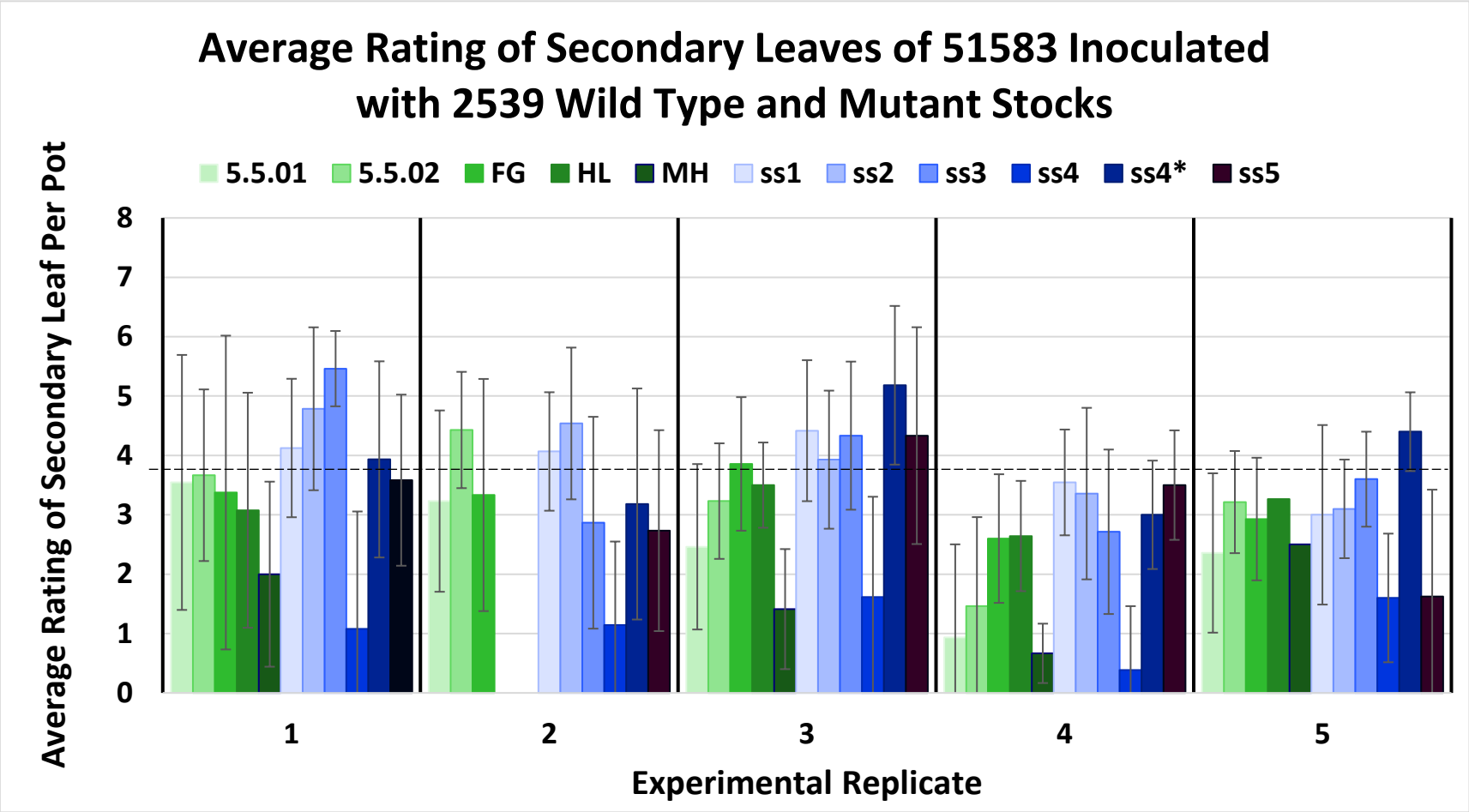


Figure 3-2 Average Rating of Secondary Leaves of 51583 Inoculated with 2539 Wild-Type and Mutant Stocks Severity ratings are on a scale from 0-8. Ratings of 0-3 are categorized as avirulent and show levels of resistance. Ratings of 4-8 are categorized as virulent and shows levels of susceptibility. The green stocks in the legend are the wild-type stocks and the blue stocks in the legend are the mutant stocks. 2539 HL and MH were dropped in rep 2 due to plate contamination. Each bar represents an approximate n=15.

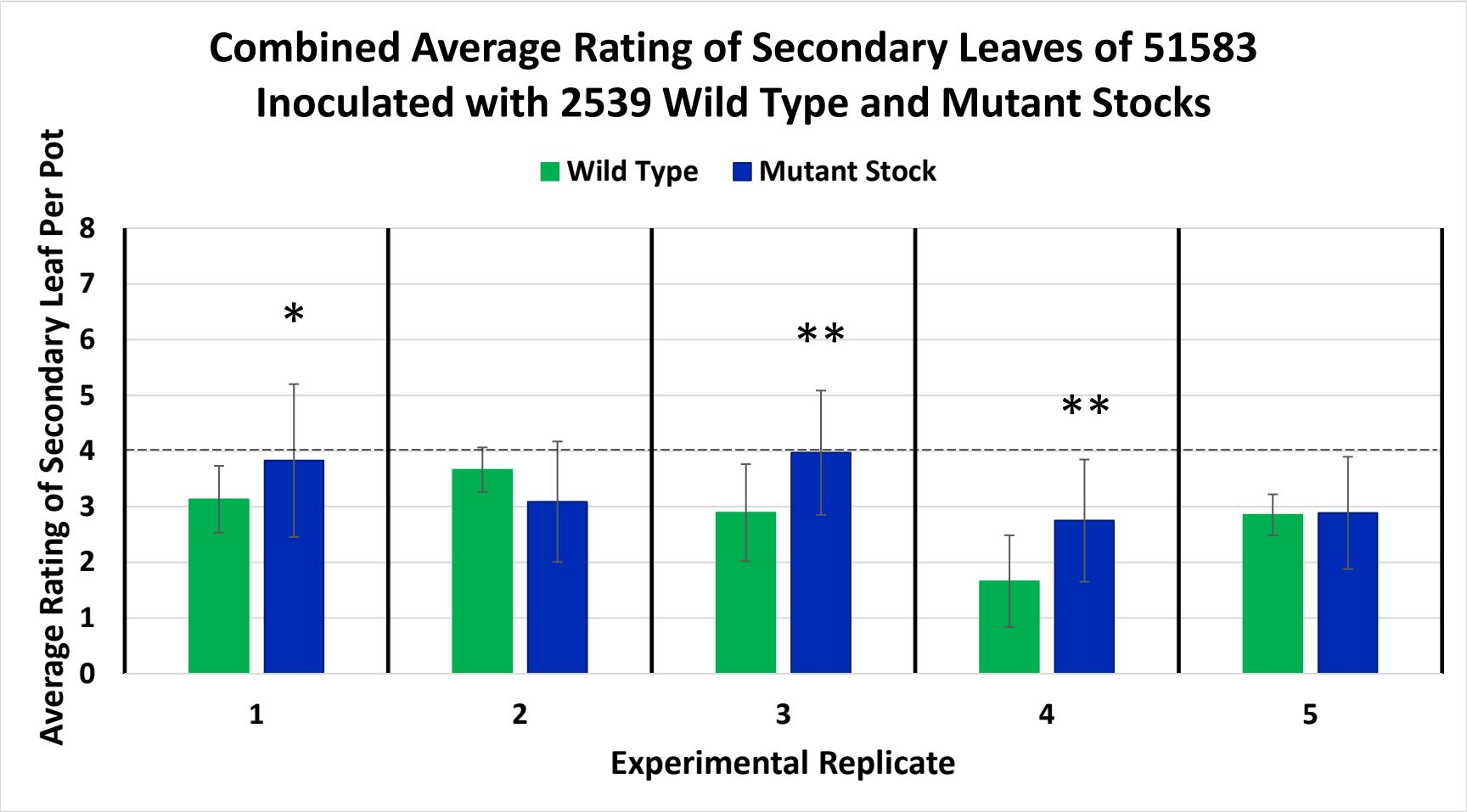


Figure 3-3 Combined Average Rating of Secondary Leaves of 51583 Inoculated with 2539 Wild-Type and Mutant Stocks Severity ratings are on a scale from 0-8. Ratings of 0-3 are categorized as avirulent and show levels of resistance. Ratings of 4-8 are categorized as virulent and shows levels of susceptibility. The green stocks in the legend are the wild-type stocks and the blue stocks in the legend are the mutant stocks. 2539 HL and MH were dropped in rep 2 due to plate contamination.

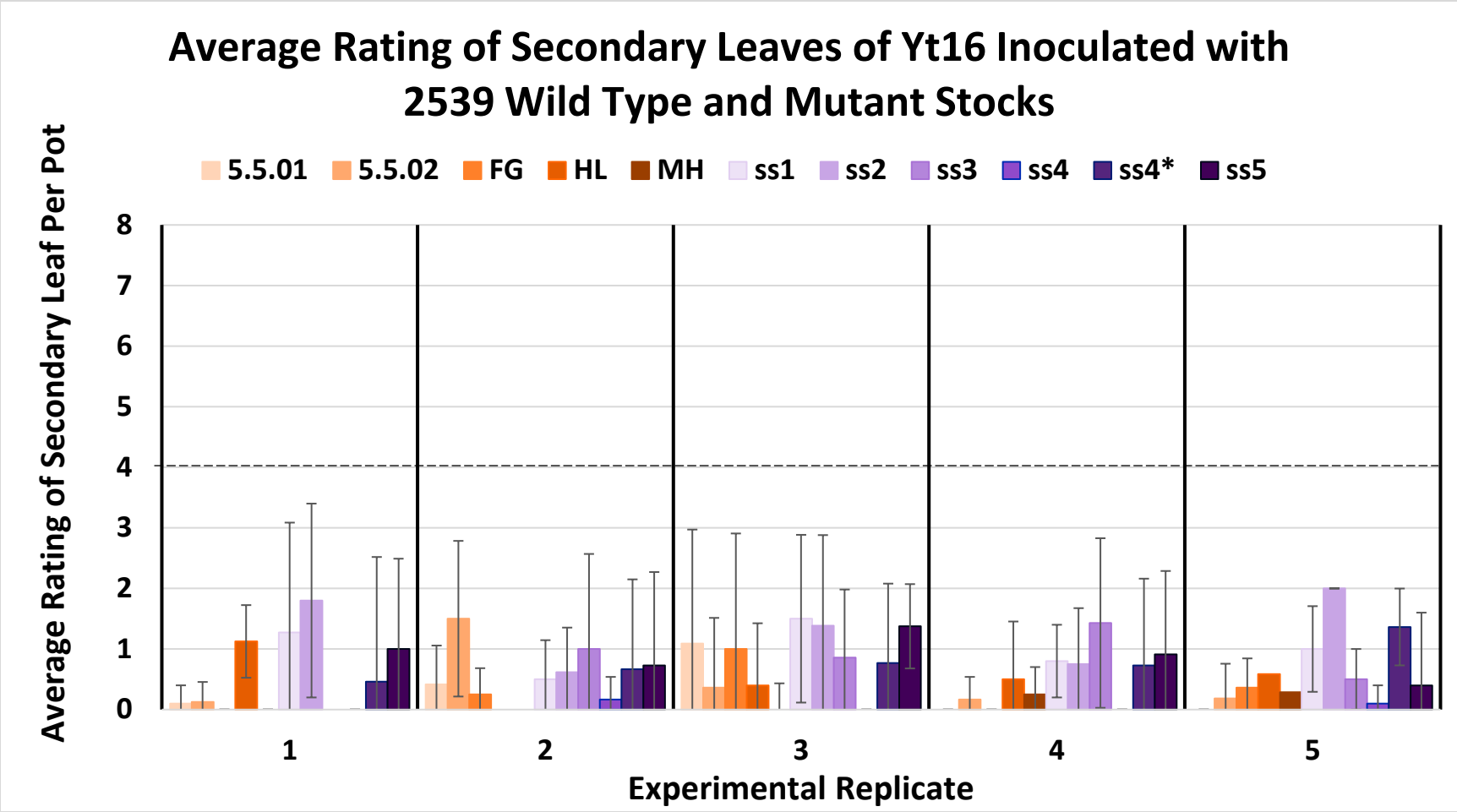


Figure 3-4 Average Rating of Secondary Leaves of Yt16 Inoculated with 2539 Wild-Type and Mutant Stocks Severity ratings are on a scale from 0-8. Ratings of 0-3 are categorized as avirulent and show levels of resistance. Ratings of 4-8 are categorized as virulent and shows levels of susceptibility. The orange stocks in the legend are the wild-type stocks and the purple stocks in the legend are the mutant stocks. 2539 HL and MH were dropped in rep 2 due to plate contamination. Each bar represents an approximate n=15.

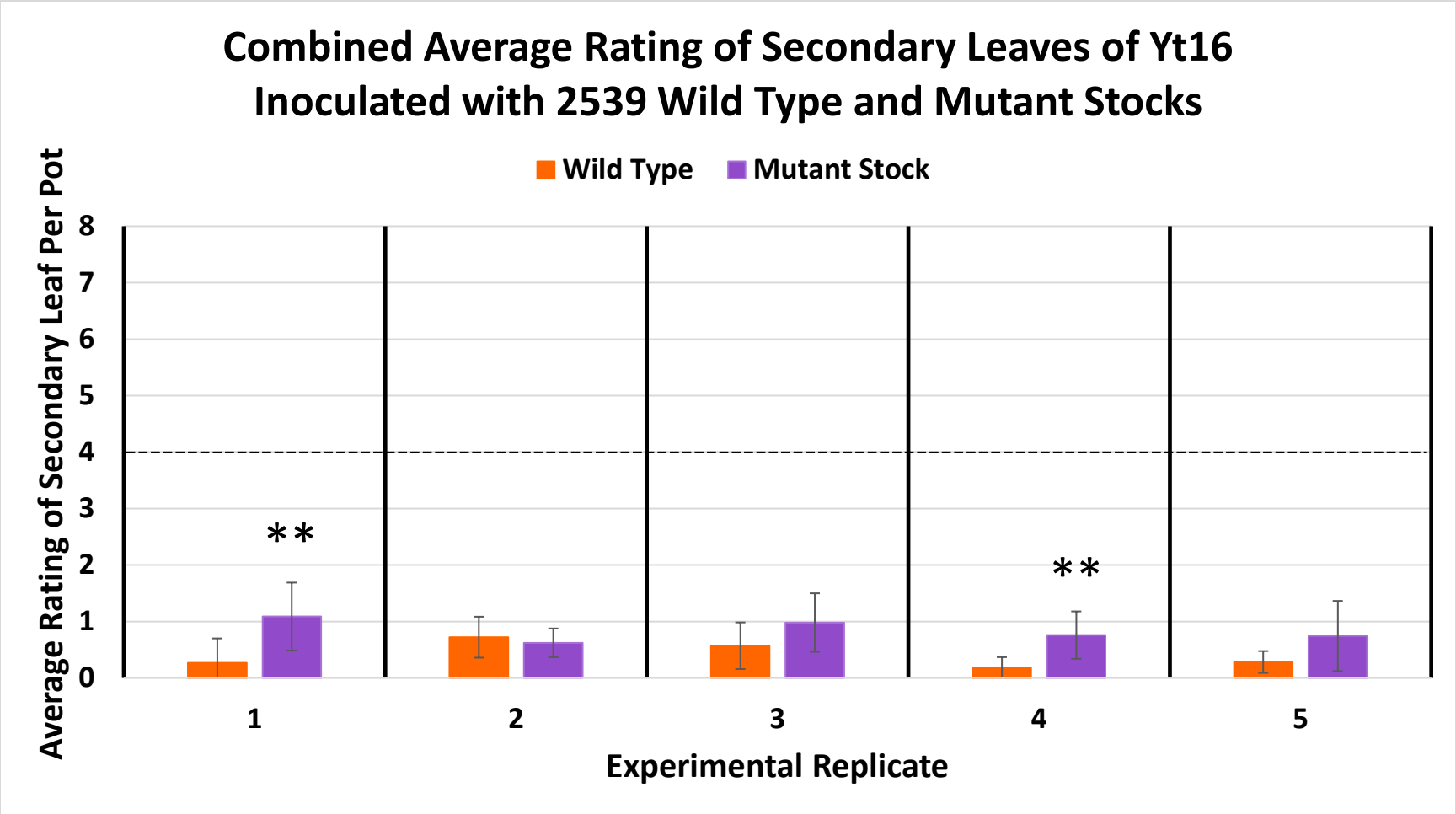


Figure 3-5 Combined Average Rating of Secondary Leaves of Yt16 Inoculated with 2539 Wild-Type and Mutant Stocks Severity ratings are on a scale from 0-8. Ratings of 0-3 are categorized as avirulent and show levels of resistance. Ratings of 4-8 are categorized as virulent and shows levels of susceptibility. The orange stocks in the legend are the wild-type stocks and the purple stocks in the legend are the mutant stocks. 2539 HL and MH were dropped in rep 2 due to plate contamination.

CHAPTER 4: EXPLORATION OF EXTERNAL FACTORS INFLUENCING PHENOTYPIC REPRODUCIBILITY

4.1 Background

It is well documented that plant age influences the physiology of plant tissues. Rice has two documented stages when it is most susceptible to rice blast: tillering and heading (Otofuji, 1987; Yamamoto et al., 1995). Studies have proposed that this susceptibility is due to the salicylic acid concentration, nitrogen uptake, and density of leaves in rice (Hasegawa & Horie, 1996; Iwai et al., 2007). Rice development can be hindered by environmental factors such as plant submergence and light diffusion through the leaf canopy (Hu et al., 2015; Sasaki & Zhao, 2000). Light is necessary for plant survival and is used as energy in photosynthesis (Ballaré et al., 2012; Carvalho & Castillo, 2018; Poorter et al., 2019). However, high-intensity light can cause sun scorch and stress plants (Nigam, 1934; Quigley & Mulhall, 2002). The circadian rhythm of plants is based on the type of light exposure during various times of day (Creux & Harmer, 2019; Más & Yanovsky, 2009). Since pathogens have specific times during the day conducive to infection, it is possible in an artificial setting to use light queues to raise resistance in plants (Smith et al., 2022). This would indicate that in a natural setting, plants are more susceptible to pathogens at a given time of day (Roden & Ingle, 2009).

The experiments in this chapter sought to address potential influences of irreproducibility between experiments. During routine plant inoculations, disease severity would unexpectedly change from one replicate to another. Fungal strains inoculated onto a rice cultivar displaying full susceptibility in one replication would show partial resistance in another. Due to the restrictions and facilities used for inoculations, the age of

the plant, light exposure, and time of day at inoculation were chosen as potential confounding variables. All of these factors were tested in isolated experiments to determine the type of influence each had on the occurrence of disease severity.

4.2 Materials and Methods

4.2.1 Plant age inoculations

The procedure matched the plant inoculation protocol listed in Chapter 2. The fungal stocks were grouped on different inoculation days so that the number of plants to be inoculated would exceed the time point for the inoculation and introduce more variability. The planting was staggered, so the fungal plates grew for 14 d by the day of inoculation. Seeds were started at 21 d, 18 d, 16 d, 14 d, and 7 d before the day of inoculation. These times were chosen based on major developmental changes that occur in rice at different ages as shown in Figure 4-1. There were two replications of 14 d plants referred to as 14-1 and 14-2.

4.2.2 Light treatment inoculation

The procedure matched the plant inoculation protocol listed in Chapter 2. Following the 20 h dark period, plants were separated into two treatment groups. The normal/control groups were placed in normal growth conditions. The treatment groups were placed in a growth chamber under 24 h light. The CONVIRON® PGC Flex – 2 Tier growth chamber emits 500 µmol/m^2/sec of light intensity with a base height of 635 mm from the shelf to the light source.

4.2.3 Time of day inoculations

The procedure matched the plant inoculation protocol listed in Chapter 2. Fungal stocks used in this inoculation were all torn from the same fungal paper stock. Four

culture plates were activated using the same single paper stock. Plants were inoculated at 7 am, 10 am, 1 pm, and 4 pm on the same day, Eastern Standard Time. Stocks utilized in the experiments are listed in Table 4-1. Cultivars utilized in the experiments are listed in Table 4-2.

4.2.4 Ratings and statistical analysis

Disease ratings for secondary leaves were made using the scale shown in Figure 3-1. Ratings were used to determine the severity of the disease. Ratings 0-3 represent resistance levels on the host (avirulent), while ratings 4-8 represent levels of susceptibility on the host (virulent). Ratings of 4 and above were categorized as virulent, while ratings below 4 were categorized as avirulent. Statistical analysis was performed using an unpaired, unequal variance t-test. The R^2 values were also calculated for inoculations to determine the adherence of inoculation to a line of best fit.

4.3 Results

4.3.1 Plant age inoculation

This experiment aimed to determine whether the updated growth chambers utilized for experiments influenced the physiology of the plants and caused differences in infection phenotypes compared to previously established results in the Farman lab. Plants were inoculated at 14 d according to protocol. This age was chosen because it is the early tillering stage of rice and has been shown to be highly susceptible to blast infection (Rodrigues et al., 2003). The inoculations were staggered several days apart to determine if the plant age would change the infection phenotype, thus correlating to a possible difference in plant development time. This would imply differences in efficiency between the new growth chamber and the old, nonfunctional growth chamber.

The results of this experiment did not support a difference in growth chambers as a reason for the lack of infection phenotype reproducibility. Infection phenotypes would shift between avirulent and virulent for the same stock inoculated on plants of the same age on different days. The results did display that the rice plants were most susceptible between 14 – 18 d, which aligns with the known susceptibility of the tiller stage in the literature.

Stock 2539 MH was avirulent on 51583 plants at every age. 2539 MH had an R^2 of 0.17 with 14-1 and 0.35 with 14-2 using a second-order polynomial trendline, indicating a lack of susceptibility during the peak tillering stage. All other stocks fluctuated in phenotypic expression based on the inoculation day. Stock 2539 HL was virulent on the 14-1 plants and not virulent on the 14-2 plants. This showed a shift between infection phenotypes on a given inoculation day. 2539 HL had an R^2 of 0.77 with 14-1 and 0.95 with 14-2 using a second-order polynomial trendline indicating susceptibility during the peak tillering stage. 2539 FG had an R^2 of 0.99 for 14-1 and 14-2 using a second-order polynomial trendline indicating susceptibility during the peak tillering stage. 2539 5.5.01 had an R^2 of 0.99 for 14-1 and 14-2 using a second-order polynomial trendline indicating susceptibility during the peak tillering stage. 2539 5.5.02 had an R^2 of 0.95 for 14-1 and 0.99 for 14-2 using a second-order polynomial trendline indicating susceptibility during the peak tillering stage. The stocks 2539 FG, 5.5.01, and 5.5.02 were not inoculated onto the sixteen-day-old plants. All of these results are displayed in Figure 4-2.

2539 ss1 had an R^2 of 0.73 for 14-1 and 14-2 using a second-order polynomial trendline indicating susceptibility during the peak tillering stage. 2539 ss2 had an R^2 of

0.89 for 14-1 and 0.71 for 14-2 using a second-order polynomial trendline indicating susceptibility during the peak tillering stage. 2539 ss3 had an R^2 of 0.90 for 14-1 and 0.73 for 14-2 using a second-order polynomial trendline indicating susceptibility during the peak tillering stage. 2539 ss4 had an R^2 of 0.80 for 14-1 and 0.67 for 14-2 using a second-order polynomial trendline indicating susceptibility during the peak tillering stage. 2539 ss4* had an R^2 of 0.62 with 14-1 and 0.99 with 14-2 using a second-order polynomial trendline indicating susceptibility during the peak tillering stage. 2539 ss5 had an R^2 of 0.99 for 14-1 and 14-2 using a second-order polynomial trendline indicating susceptibility during the peak tillering stage. All of these results are displayed in Figure 4-3.

FR13 had an R^2 of 0.55 for 14-1 and 0.58 for 14-2 using a second-order polynomial trendline, indicating a weak susceptibility during the peak tillering stage. Guy11 had an R^2 of 0.34 for 14-1 and 0.25 for 14-2 using a second-order polynomial trendline indicating general susceptibility during multiple plant ages. ML33 had an R^2 of 0.73 for 14-1 and 0.52 for 14-2 using a second-order polynomial trendline indicating susceptibility during the peak tillering stage. O254 had an R^2 of 0.87 for 14-1 and 0.66 for 14-2 using a second-order polynomial trendline indicating susceptibility during the peak tillering stage. SSID116 was avirulent on the 14-1 plants and virulent on the 14-2 plants, switching between phenotypes on a given inoculation day. SSID116 had an R^2 of 0.16 for 14-1 and 0.04 for 14-2 using a second-order polynomial trendline, indicating a lack of susceptibility during the peak tillering stage. All of these results are displayed in Figure 4-4.

4.3.2 Light treatment inoculation

This experiment aimed to determine whether uncontrollable light fluctuations during plant transport were causing the differences in infection phenotypes from previously established results in the Farman lab. Due to biological safety restrictions and facility constraints, plants were moved for inoculation between buildings. Although the plants were bagged inside secondary containers, potential light exposure was unavoidable when removing plants from growth chambers or containers. If the issues of reproducibility in the infection phenotypes were due to light fluctuations, the expected results of this experiment would display a generalized increase or decrease in infection for all the fungal strains after light exposure. The results of this experiment did not support additional light exposure as a reason for a lack of infection phenotype reproducibility. Interestingly, the results indicated that light exposure influenced interactions specific to the strain and cultivar, as each pair displayed a unique reaction to light.

2539 HL on 51583 exposed to light had an average rating of 1.94±0.91 compared to the average rating of 2.75±0.66 in the control, leading to a statistically significant decrease in infection with a $p<0.05$. 2539 HL on LTH exposed to light had an average rating of 1.82±0.39 compared to the average rating of 2±0.77 in the control. 2539 HL on Yt16 exposed to light had an average rating of 1±0.00 compared to the average rating of 2.4±0.8 in the control, leading to a statistically significant decrease in infection with a $p<0.05$. Guy11 on 51583 exposed to light had an average rating of 5±0.41 compared to the average rating of 6.23±0.89 in the control, leading to a statistically significant decrease in infection with a $p<0.01$. Guy11 on LTH exposed to light had an average rating of 4.5±0.5 compared to the average rating of 2.64±1.54 in the control, leading to a

statistically significant increase in infection with a $p<0.01$. Guy11 on Yt16 exposed to light had an average rating of 3.2±1.47 compared to the average rating of 3.2±1.47 in the control, indicating no light exposure influence. ML33 on 51583 exposed to light had an average rating of 6.6±0.49 compared to the average rating of 7.54±0.5 in the control, leading to a statistically significant decrease in infection with a $p<0.01$. ML33 on LTH exposed to light had an average rating of 5.1±0.54 compared to the average rating of 7.64±0.48 in the control, leading to a statistically significant decrease in infection with a $p<0.01$. ML33 on Yt16 exposed to light had an average rating of 5.29±0.7 compared to the average rating of 6.08±1.86 in the control. These results indicate that exposure to light can cause an increase, decrease, or no change in infection phenotype depending on the strain and cultivar paired for inoculation. All of these results are displayed in Figure 4-5.

4.3.4 Time of day inoculation

This experiment aimed to determine whether the time of day during plant inoculations was causing the differences in infection phenotypes compared with previously established results in the Farman lab. The timing of plant inoculations was not previously controlled according to the Farman lab plant inoculation protocol; therefore, plants were inoculated at any time of the day that was convenient for the completion of the experiments. If the issues of reproducibility in the infection phenotypes were due to the time of day at inoculation, the expected results of this experiment would display a generalized increase or decrease in infection for all the fungal strains depending on the time of day at inoculation. The results of this experiment supported the hypothesis that infection phenotypes were influenced by the time of day at inoculation. This was displayed through disease ratings that became more severe with inoculations performed

later in the day. However, some stocks did not follow any trend of increased or decreased infection given the time of day, indicating that the reproducibility issues of inoculations were not solely due to the time of day at inoculation.

The average disease rating for 2539 HL at 7 am was 2.33±0.47, categorized as an avirulent reaction. The average disease rating for 2539 HL at 10 am was 3.33±1.25, categorized as an avirulent reaction. The average disease rating for 2539 HL at 1 pm was 5±0.00, categorized as a virulent reaction. The average disease rating for 2539 HL at 4 pm was 5.67±0.47, categorized as a virulent reaction. The average ratings across the four time points resulted in an R^2 value of 0.98, indicating a strong linear increase as inoculations were performed later in the day. The average disease rating for 2539 MH at 7 am was 3.33±0.47, categorized as an avirulent reaction. The average disease rating for 2539 MH at 10 am was 3±0.47, categorized as an avirulent reaction. The average disease rating for 2539 MH at 1 pm was 4±1.41, categorized as a virulent reaction. The average disease rating for 2539 MH at 4 pm was 4.67±0.47, categorized as a virulent reaction. The average ratings across the four time points resulted in an R^2 value of 0.76, indicating a moderate linear increase as inoculations were performed later in the day. All of these results are displayed in Figures 4-6 and 4-7.

4.4 Discussion

The experiments described in this chapter were performed to ascertain the potential cause of the irreproducibility seen between experiments and the differences between current inoculation phenotypes compared to previously observed results in the Farman lab. The inoculations focused on plant age supported the already established susceptibility of rice during the tillering stage but did not clarify the cause of

irreproducibility between experiments. Plants of the same age could switch between avirulent and virulent reactions. The inoculations focused on light exposure displayed a cultivar and strain-specific interaction that again did not clarify the cause of irreproducibility in the experiments. The inoculations focused on the time of day did indicate that the inoculation time influenced the infection phenotype; however, there was still irreproducibility between seemingly identical fungal stocks. All of these results did help to constrain the protocol for artificial plant inoculation assays to further control for external variables.

One challenge of studying a plant disease is the number of variables involved in the host-microbe interaction. Plant age is already well described in the blast pathosystem to greatly influence infection success and progression (Roumen, 1992; Yang et al., 1998). Light has also been extensively documented in influencing fungal and plant germination, fungal sporulation, and plant resistance in the blast pathosystem (Lee et al., 2006; Liang et al., 2022; Shirasawa et al., 2012). *M. oryzae* is frequently utilized as a model pathogen for understanding the host-pathogen interaction, yet this model has shown itself to be unpredictable in several ways (Ebbole, 2007). The Ellingboe lab noted Guy11 and 2539 progeny switching between avirulent and virulent infection phenotypes on two different rice cultivars during the initial studies of the newly bred 2539 strain (Leung et al., 1988). The rapid mutation characteristic of *M. oryzae* has led to difficulties maintaining stocks in vitro for extended periods or subculturing the stocks as mutations accumulate rapidly, leading to phenotypic changes (Jeon et al., 2013). As supported by the results described in this chapter, researchers not only have to contend with the complexities involved in the

host-pathogen interaction but may encounter unexpected irreproducibility in the *M. oryzae* interaction.

The complexities of defining the host-pathogen interaction while avoiding the inclusion of background variability in each specific fungal stock-cultivar interaction emphasize the need to determine how each interaction should be analyzed carefully. Diagnostic sensitivity determines how many positive results an analysis can provide, while diagnostic specificity determines how many true negative results an analysis can provide (Saah & Hoover, 1997). Sensitivity and specificity are necessary to diagnose a disease state or identify differences in treatments; however, they are inversely related (Bartol, 2015). A sensitive diagnosis will provide positive results at the expense of negative results, and a specific diagnosis will provide negative results at the expense of positive results (Woodruff & Baron, 1989). The challenge in plant disease diagnostics is identifying sensitive assays to determine disease state/severity while not identifying every minuscule aspect of the interaction that will contain variability (Cardwell et al., 2018; Fletcher et al., 2006). This is the reason why disease severity ratings, or incidence ratings, are predominantly used in plant pathological studies to determine the disease state on a host rather than counting individual lesions (Atoum et al., 2016; Bock, Pethybridge, et al., 2022; Siddiqua et al., 2022). Despite using a simplified threshold system rating virulent versus avirulent reactions, the results still displayed irreproducibility between experiments due to phenotypic variability between replicates. This poses a problem since *M. oryzae* is a model organism, yet irreducibility arises even in strictly controlled experiments. Results concerning a specific treatment or gene effect could be clouded by

spontaneous phenotypic changes that are still evident during an insensitive diagnostic analysis.

4.5 Conclusions

The results of the experiments in this chapter indicated that the influence of the plant age, light exposure, and time of day at inoculation were strain and cultivar-specific. The age of plant inoculations displayed high susceptibility during the tillering stage of rice (14-25 d), which was already well documented in rice literature. The light inoculations displayed a cultivar and strain-specific influence where one strain/cultivar experienced increased disease severity while another experienced decreased severity. The time of day inoculations displayed a slight trend of increased disease severity for inoculations performed later in the day but were dependent on the stock.

Although each factor influenced the occurrence of disease severity, none of the factors displayed the large differences in disease severity previously observed between replicates. Notably, large differences in disease severity were seen during these inoculations within control groups and plants treated identically. The cause of irreproducibility between experiments was not one of the factors being tested and was a confounding variable in these inoculations. These inoculations did provide information that was used to improve the plant inoculation protocol in the Farman lab. Further research should be performed to clarify the source of irreproducibility between experiments. Exposure of plants to brief amounts of variable temperatures was of particular interest following these experiments.

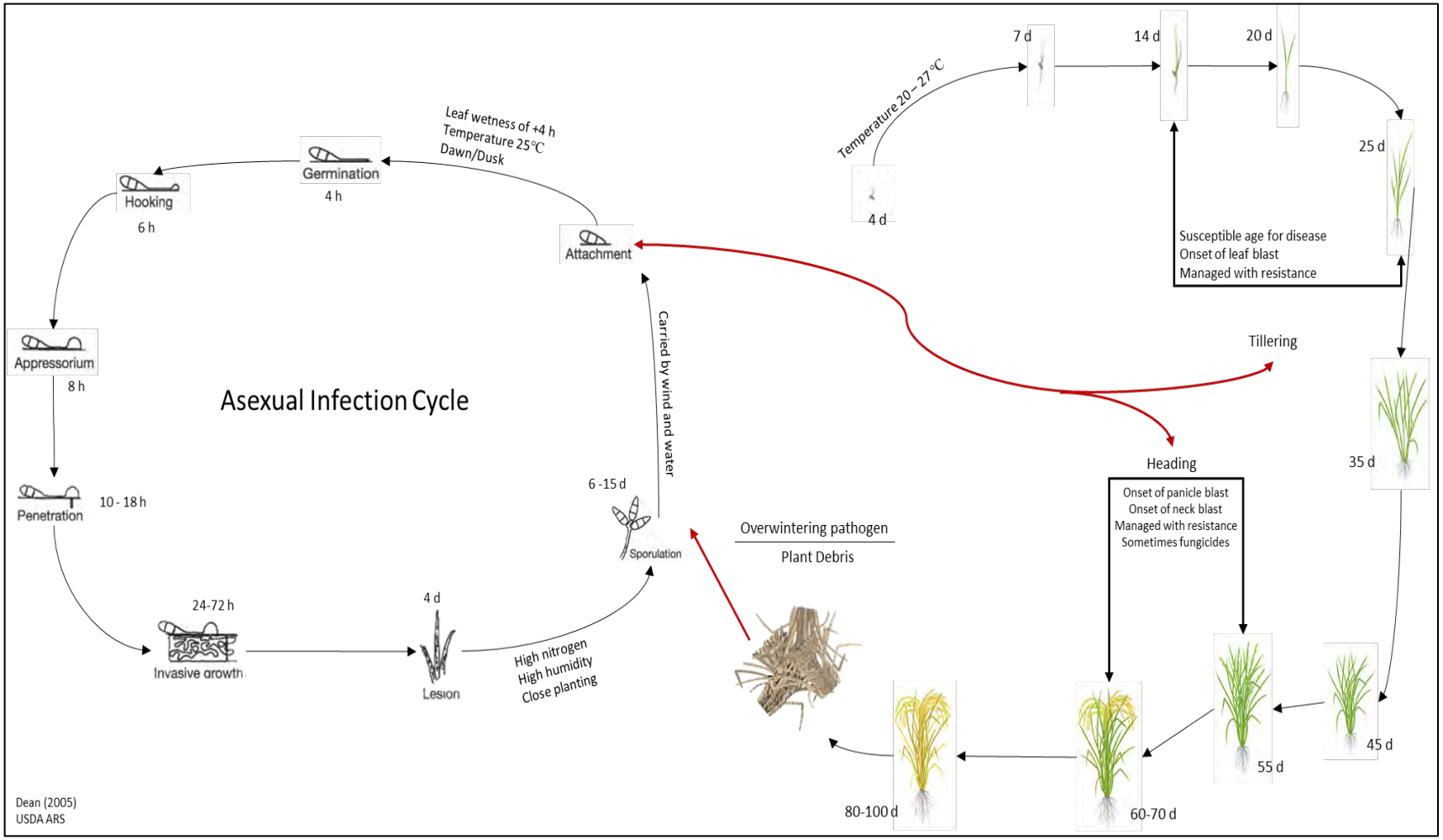


Figure 4-1 Disease cycle of M. oryzae on rice The asexual infection of the rice blast fungus is displayed in the diagram. Rice development is also shown with relevant timings for fungal susceptibility.

Table 4-1: *Magnaporthe* stocks and strains utilized for experiments

Isolate Name	Host	Year	Type	Place of Isolation	Source
2539 HL	*Oryza sativa*	1988	Wild-Type	Laboratory Strain	H. Leung, bred strain
2539 MH	*Oryza sativa*	2014	Wild-Type	Laboratory Strain	M. Heist, subculture of 2539
2539 FG	*Oryza sativa*	2007	Wild-Type	Laboratory Strain	Subculture of 2539
2539 5.5.01	*Oryza sativa*	2007	Wild-Type	Laboratory Strain	Subculture of 2539
2539 5.5.02	*Oryza sativa*	2007	Wild-Type	Laboratory Strain	Subculture of 2539
2539 ss1	*Oryza sativa*	2014	Mutant	Laboratory Strain	M. Heist, 2539 single spore from lesion of 51583
2539 ss2	*Oryza sativa*	2014	Mutant	Laboratory Strain	M. Heist, 2539 single spore from lesion of 51583
2539 ss3	*Oryza sativa*	2014	Mutant	Laboratory Strain	M. Heist, 2539 single spore from lesion of 51583
2539 ss4	*Oryza sativa*	2014	Mutant	Laboratory Strain	M. Heist, 2539 single spore from lesion of 51583
2539 ss4*	*Oryza sativa*	2015	Mutant	Laboratory Strain	M. Heist, subculture of 2539 ss4
2539 ss5	*Oryza sativa*	2014	Mutant	Laboratory Strain	M. Heist, 2539 single spore from lesion of 51583
ML33	*Oryza sativa*	1986	Wild-Type	Mali	D. Tharreau, field isolation
Guy11	*Oryza sativa*	1979	Wild-Type	French Guyana	J. Notteghem, field isolation
SSID116	*Oryza sativa*	2013	Wild-Type	United States	Y. Jia, field isolation
O-254	*Oryza sativa*	-	Wild-Type	Japan	B. Valent, field isolation
FR13	*Oryza sativa*	1988	Wild-Type	France	D. Tharreau, field isolation

Table 4-2: Rice Cultivars Utilized for Experiments

Cultivar Name	Type	Year	Origin	Known R genes	Reference Source
51583	*Indica*		USSR		(Leung, et. al, 1988)
Lijiangxintuanheigu (LTH)	*Japonica*	2001	China		(Tsunematsu, et al., 2000)
Yt16				*Pt-r*	(Bryan, et al., 2000), (Zhao, et al., 2018)

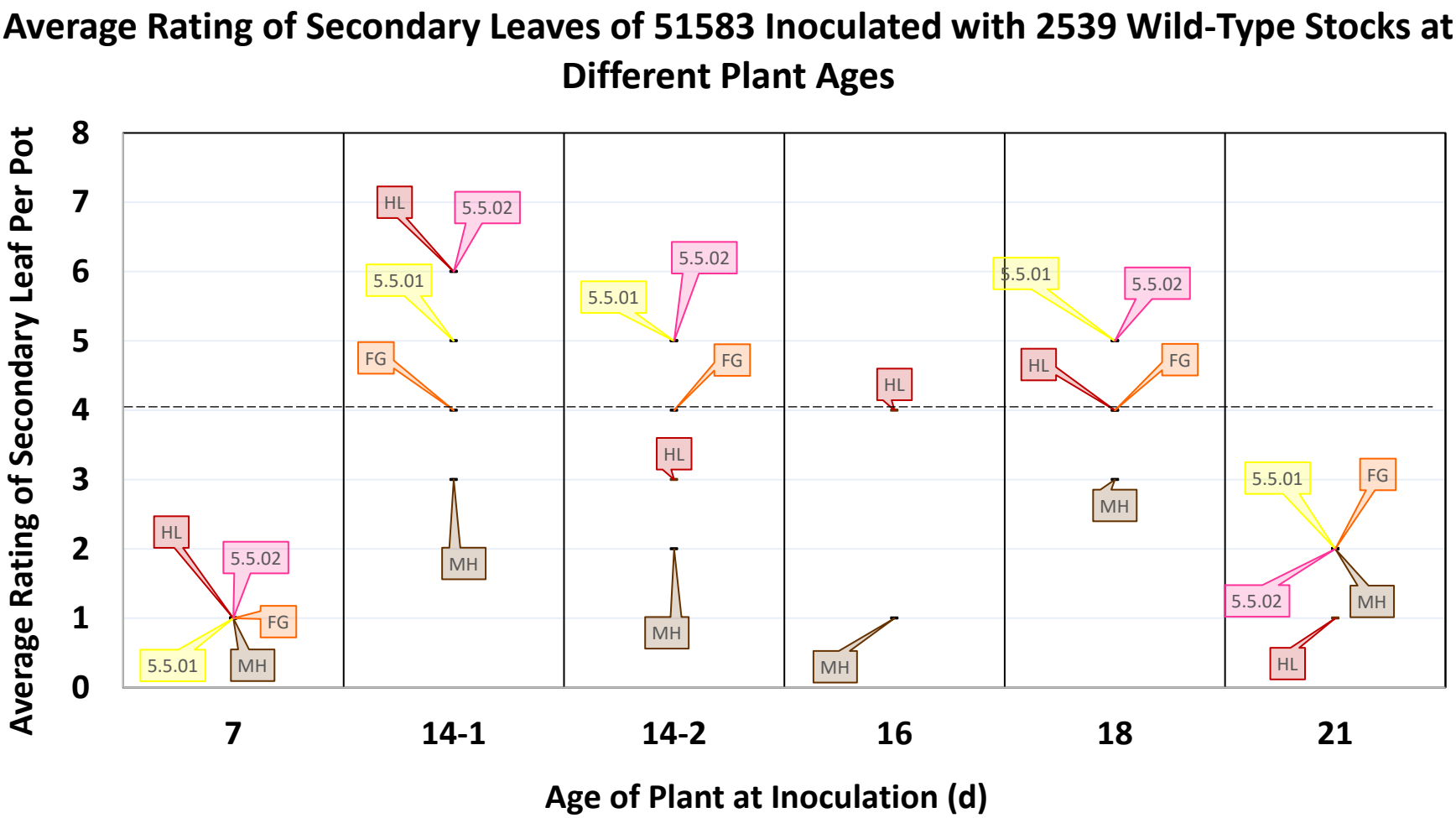


Figure 4-2 Average rating of secondary leaves of 51583 inoculated with 2539 wild-type stocks at different plant ages The x axis displays the age of the plant in days at the time of inoculation and the y axis displays the virulence rating of each of the stocks within each day column. Ratings of 4 and above are categorized as virulent while ratings below 4 are categorized as avirulent. Each point represents an approximate n=3.

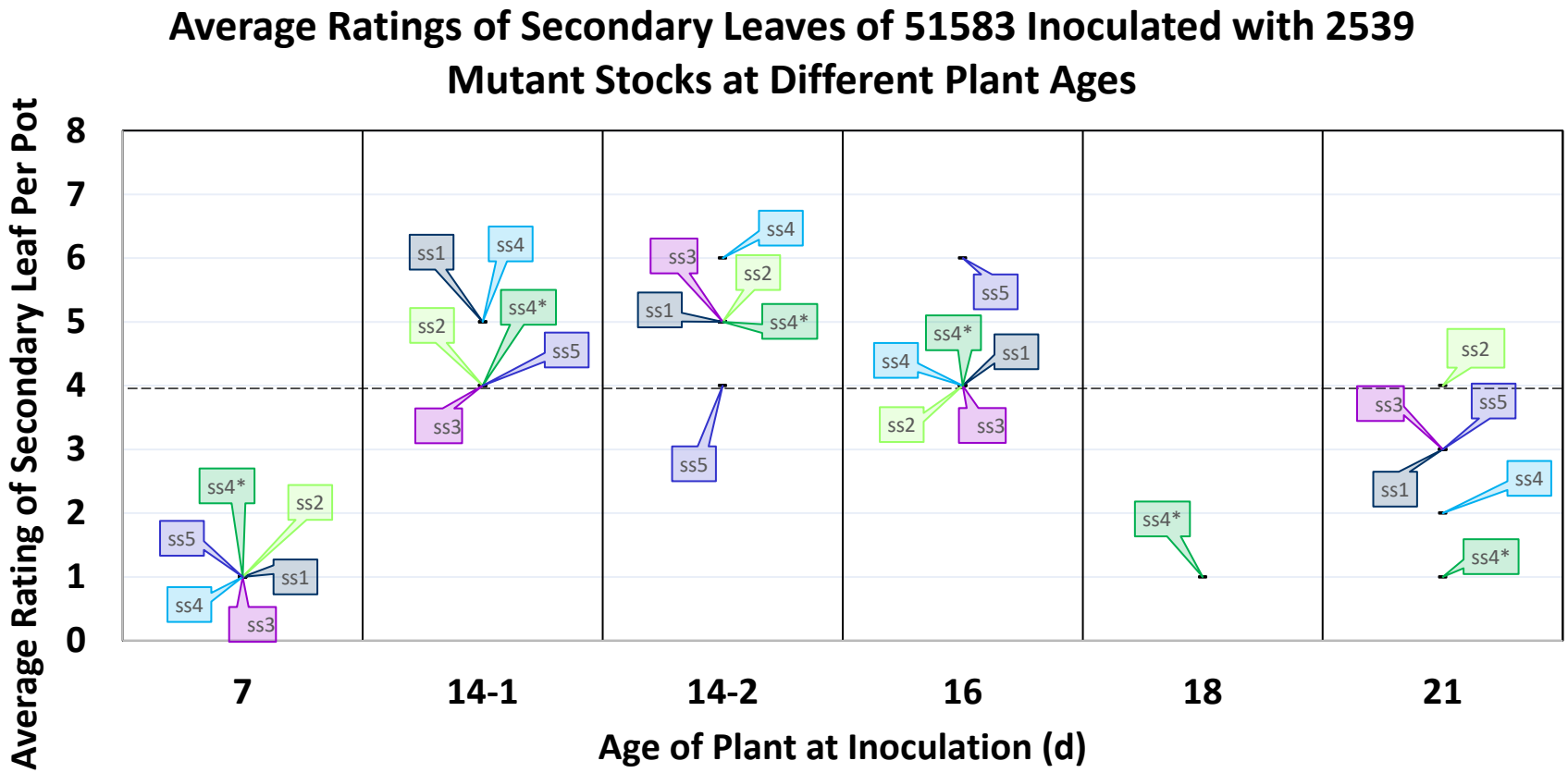


Figure 4-3 Average rating of secondary leaves of 51583 inoculated with 2539 mutant stocks at different plant ages The x axis displays the age of the plant in days at the time of inoculation and the y axis displays the virulence rating of each of the stocks within each day column. Ratings of 4 and above are categorized as virulent while ratings below 4 are categorized as avirulent. Each point represents an approximate n=3.

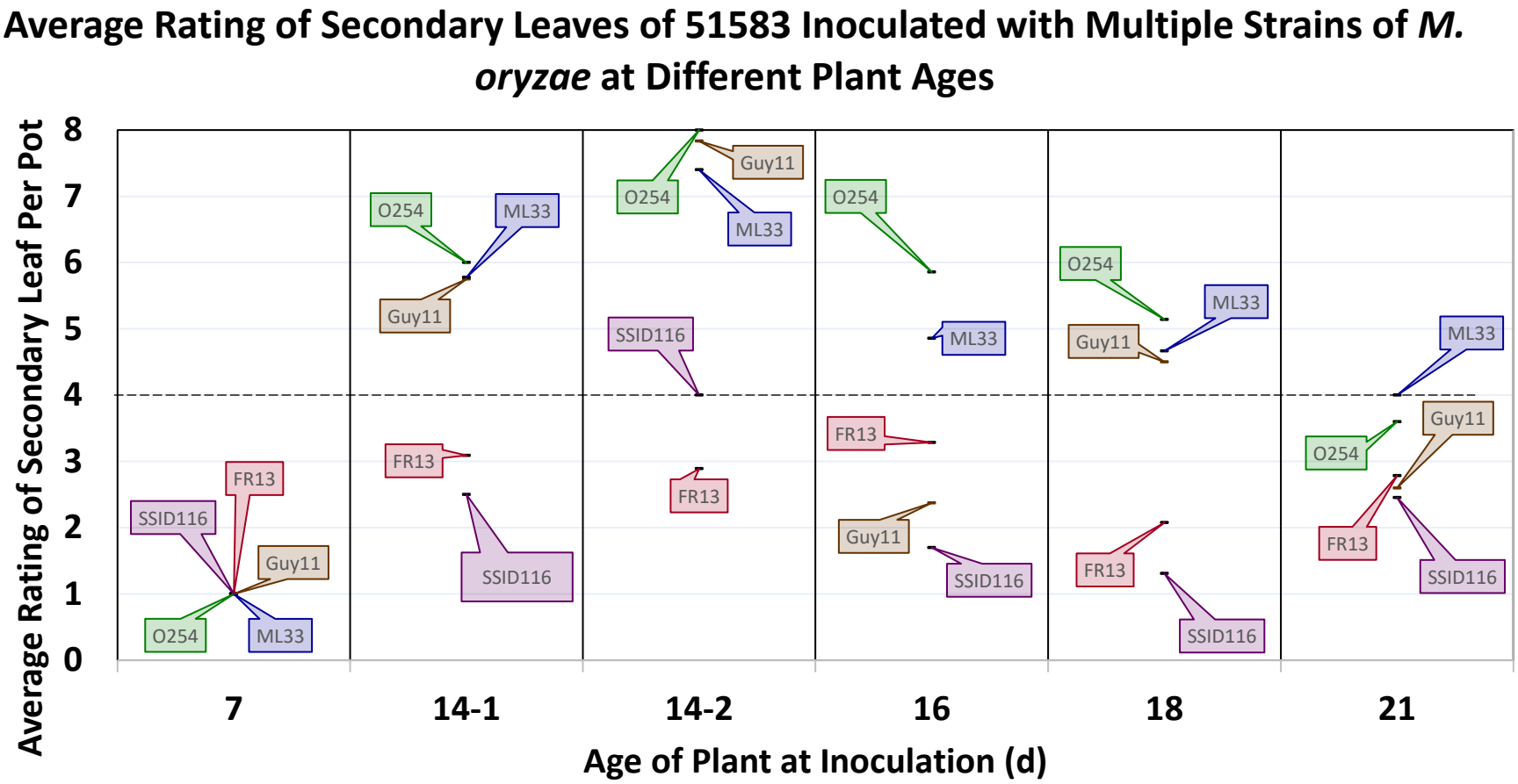


Figure 4-4 Average rating of secondary leaves of 51583 inoculated with multiple strains of M. oryzae at different plant ages x axis displays the age of the plant in days at the time of inoculation and the y axis displays the virulence rating of each of the stocks within each day column. Ratings of 4 and above are categorized as virulent while ratings below 4 are categorized as avirulent. Each point represents an approximate n=3.

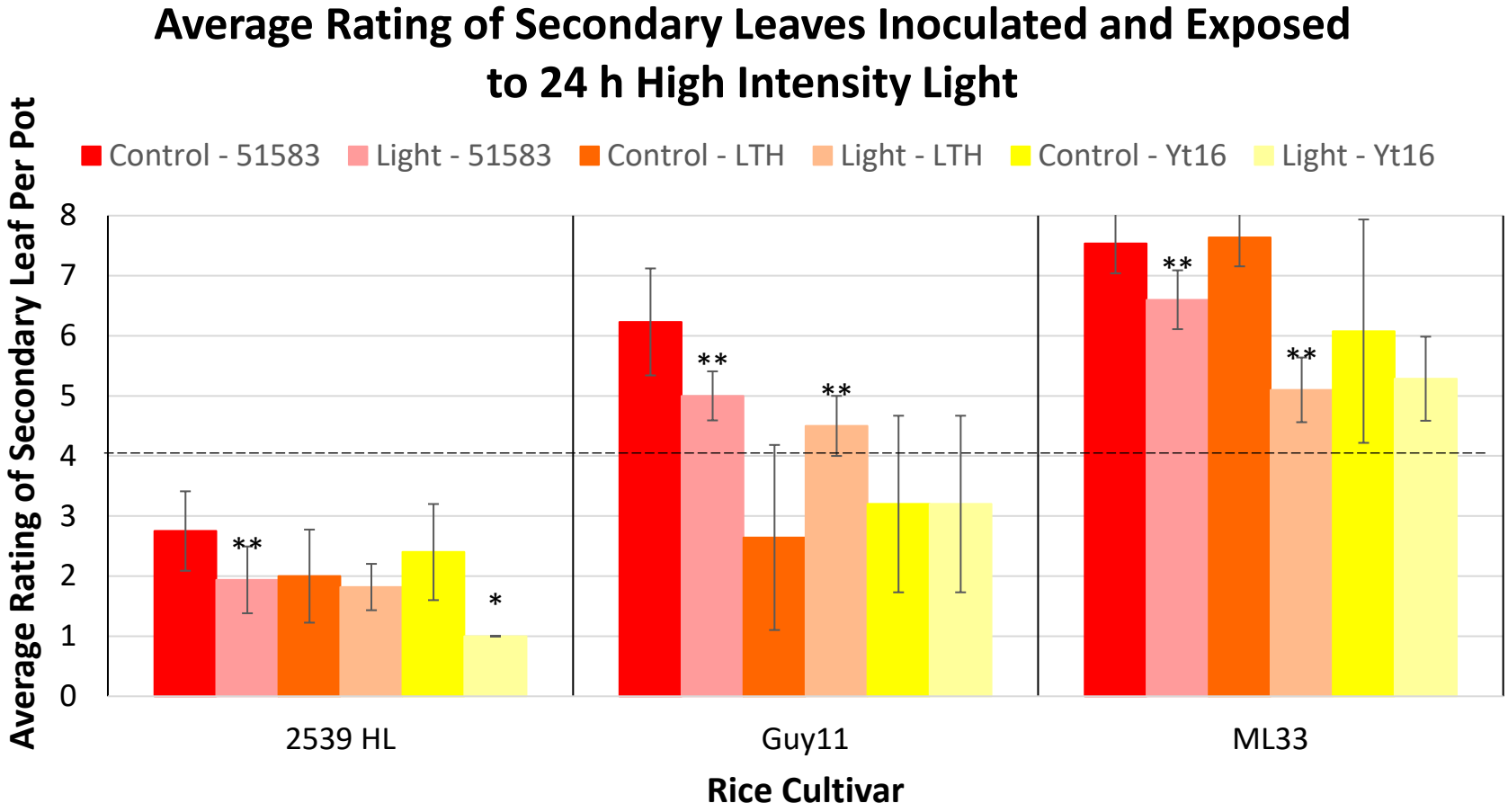


Figure 4-5 Average rating of secondary leaves inoculated and exposed to 24 h high intensity light Red colors denote inoculation on rice cultivar 51583, orange colors denote inoculation on rice cultivar LTH, and yellow colors denote inoculation on rice cultivar Yt16. Ratings of 4 and above are categorized as virulent while ratings below 4 are categorized as avirulent. One * denote $p<0.05$ and two ** denote $p<0.01$. Each bar represents an approximate n=15.

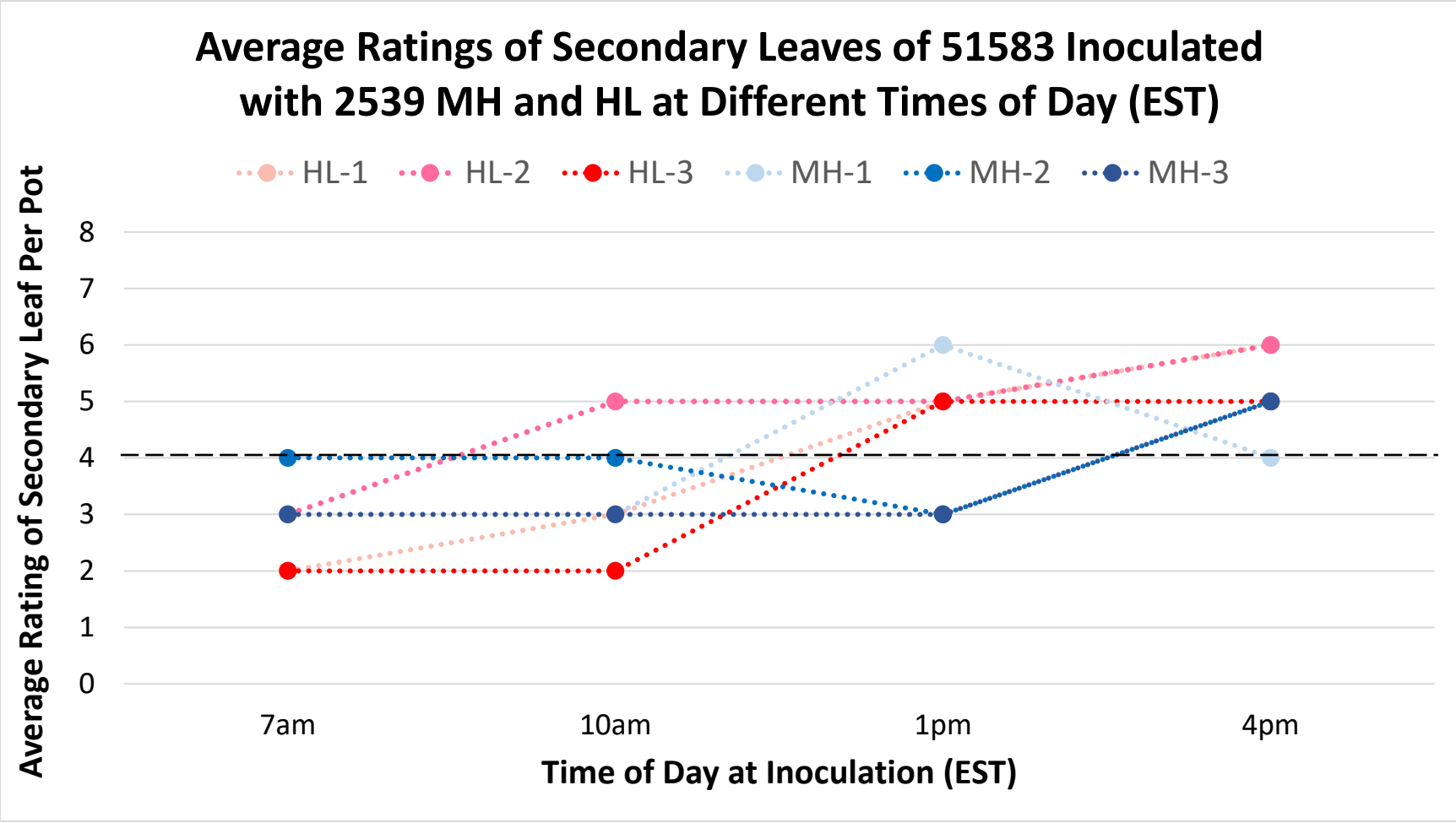


Figure 4-6 Average ratings of secondary leaves of 51583 inoculated with 2539 MH and HL at different times of day (EST) Red colors denote 2539 HL stocks and blue colors denote 2539 MH stocks. Ratings of 4 and above are categorized as virulent while ratings below 4 are categorized as avirulent. Each point represents an approximate n=3.

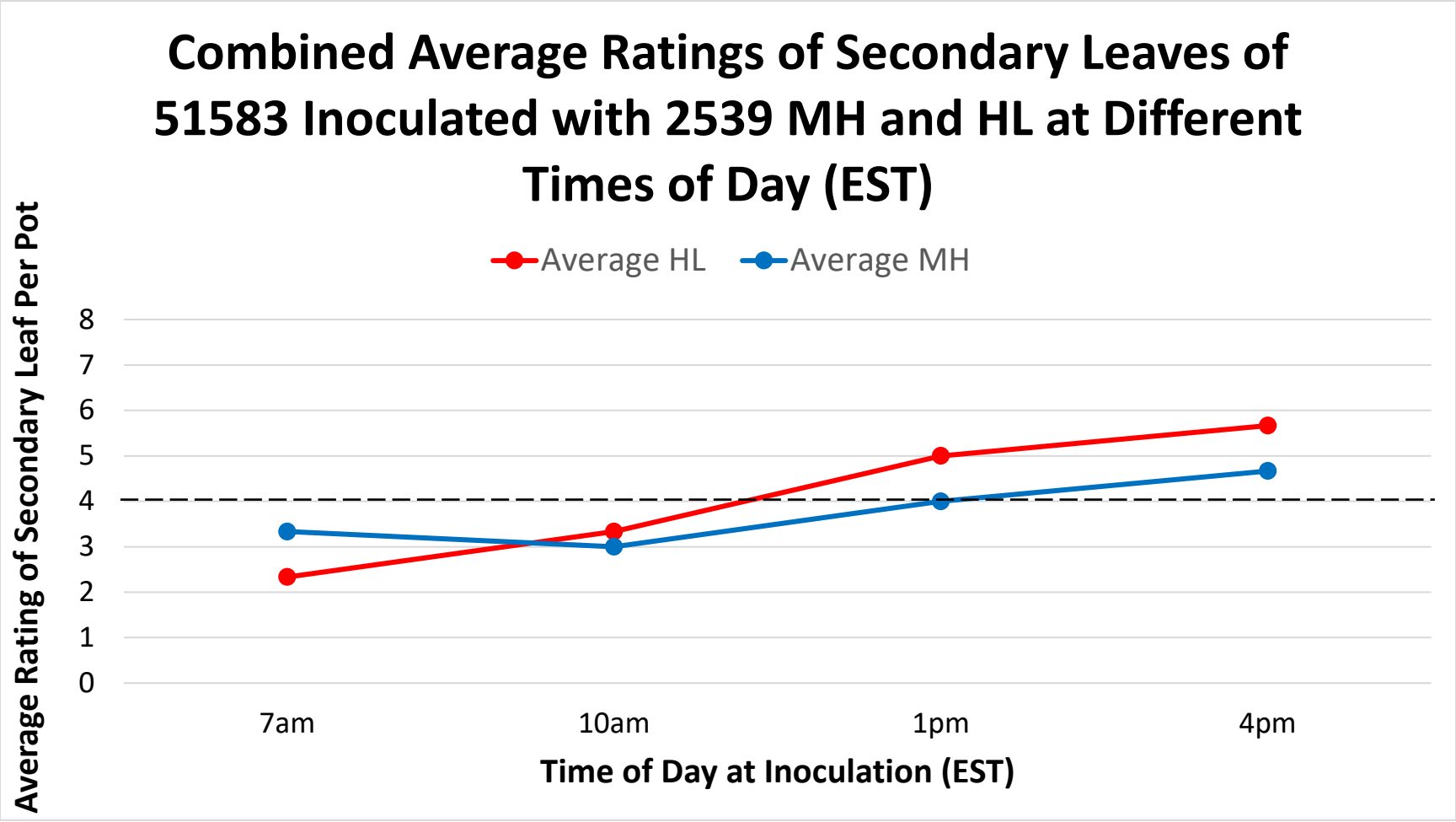


Figure 4-7 Combined average ratings of secondary leaves of 51583 inoculated with 2539 MH and HL at different times of day (EST) Red color denote 2539 HL stocks and blue color denote 2539 MH stocks. Ratings of 4 and above are categorized as virulent while ratings below 4 are categorized as avirulent.

CHAPTER 5: THE INFLUENCE OF TEMPERATURE ON INFECTION PRE AND POST-INOCULATION

5.1 Background

Temperature is widely accepted as a major influence on pathogen survival and proliferation (Desaint et al., 2021; Laine, 2007; Sabburg et al., 2015). *Erwinia amylovora,* the causal agent of Fireblight, has an optimal temperature of 28°C. Temperatures below this can cause the bacteria to enter stasis, and temperatures above this will kill the bacteria (Santander & Biosca, 2017). The proliferation of *Sclerotinia* in lettuce depends heavily on temperature and humidity for fungal sporulation (Lohmeier et al., 2013). Low temperatures can slow the germination of rice seedlings, and high temperatures in rice paddies can inhibit rice heading (Rau et al., 2021; Shim et al., 2020). This implies that temperature can not only stress the host, making it more susceptible to pathogen infection, but also provide a conducive environment for pathogen proliferation. Exposures to brief temperature variations could have been the confounding variable causing the irreproducibility between experiments discussed in Chapter 4.

The experiments in this chapter sought to explore the effects of brief exposure to cold and hot temperatures on the occurrence of disease severity in rice pre and post-inoculation with *M. oryzae*. The malfunction of the growth chamber discussed in Chapter 2 precipitated exploring this confounding variable. Due to the restriction of facilities and equipment, plants were transported outside during the summer and winter. Although plants were stored in secondary containers, brief exposures to different temperatures were unavoidable. Purposeful manipulation of temperature during pre and post plant inoculation sought to replicate the uncontrollable temperature variation during plant

transport to determine if this was the confounding variable contributing to irreproducibility.

5.2 Materials and Methods

5.2.1 Temperature treatment inoculations

The procedure matched the plant inoculation protocol listed in Chapter 2. The pre-treatments of 4℃ and 37℃ included a control, 10 min, 30 min, 1 h, and 24 h exposure before inoculation and the 20 h dark room period at 22℃. The delayed pre-treatment was exposed to the change in temperature and then set back in the growth chamber for 1 h before inoculation. The immediate pre-treatment was exposed to the temperature and inoculated immediately after the allotted time. The post-treatment of 4℃ and 37℃ included a control, 10 min, 30 min, 1 h, and 24 h exposure after inoculation and the 20 h dark room period at 22℃. There were also treatments of non-continuous exposure 1 d, 2 d, 3 d, and 4 d after inoculation. The delayed pre-treatment, immediate pre-treatment, short exposure post-treatment, and non-continuous post-treatment were all inoculated on different days. The groups exposed to 4℃ and 37℃ were also inoculated on different days according to their treatments since only one chamber was available for temperature treatments. Therefore, there were eight separate inoculation days for this experiment. Only one replicate was performed for each temperature exposure experiment. There were three plants per treatment type. Stocks utilized in the experiments are listed in Table 5-1. Cultivars utilized in the experiments are listed in Table 5-2.

5.2.2 Ratings and statistical analysis

Disease ratings for secondary leaves were made using the scale shown in Figure 3-1. Ratings were used to determine the severity of the disease. Ratings 0-3 represent

resistance levels on the host (avirulent), while ratings 4-8 represent levels of susceptibility on the host (virulent). Ratings of 4 and above were categorized as virulent, while ratings below 4 were categorized as avirulent. Statistical analysis was performed using an unpaired, unequal variance t-test.

5.3 Results

5.3.1 Cold temperature exposures

This experiment aimed to determine whether uncontrolled exposure to cold temperatures during plant transport was causing the differences observed in infection phenotypes compared to previously established results in the Farman lab. Due to biological safety restrictions and facility constraints, plants were moved between buildings for inoculation. Although the plants were bagged inside secondary containers, exposure to cold temperatures was unavoidable when removing plants from growth chambers or containers. If exposure to cold temperatures pre or post-inoculation were the cause of the irreproducibility between experiments, the expected results would be a generalized increase or decrease in all infection phenotypes for the specific treatment regardless of strain or cultivar. The results of this experiment did not support exposure to cold temperatures as a reason for a lack of infection phenotype reproducibility. Interestingly, the results indicated that exposure to cold temperatures at specific intervals and times influenced interactions specific to the strain and cultivar, as each pair displayed a unique reaction to the 4℃ exposures.

5.3.1.1 Pre-inoculation

2539 HL on 51583 exposed to 4℃ pre-inoculation had an average rating of 4.33±0.47 for the control, 4±0 for the 10 min exposure, 4±0 for the 30 min exposure,

3.33±0.47 for the 1 h exposure, and 4±0.82 for the 24 h exposure. 2539 ss1 on 51583 exposed to 4℃ pre-inoculation had an average rating of 7±0 for the control, 6±0 for the 10 min exposure, 3.67±0.47 for the 30 min exposure, 4.67±0.47 for the 1 h exposure, and 5±0 for the 24 h exposure. The 30 min and 1 h exposure plants resulted in a statistically significant decrease in infection with a $p<0.01$. Guy11 on 51583 exposed to 4℃ pre-inoculation had an average rating of 5±0 for the control, 5.33±0.47 for the 10 min exposure, 5.67±0.47 for the 30 min exposure, 6±0.82 for the 1 h exposure, and 7.33±0.47 for the 24 h exposure. The 24 h exposure plants resulted in a statistically significant increase in infection with a $p<0.05$. These results indicate that pre-inoculation exposure to 4℃ for shorter periods does not generalize the infection phenotype. Specific influences on ss1 and Guy11 under specific times were evident; however, these results did not clarify the experiment's main goal, determining the factor(s) influencing the overall reproducibility between experiments. All of these results are displayed in Figure 5-1.

5.3.1.2 Pre-inoculation with delay

2539 HL on 51583 exposed to 4℃ pre-inoculation and delayed in inoculation for 1 h had an average rating of 4.33±0.47 for the control, 4±0.82 for the 10 min exposure, 3.33±0.47 for the 30 min exposure, 1.33±0.47 for the 1 h exposure, and 4.33±0.47 for the 24 h exposure. The 1 h exposure plants resulted in a statistically significant decrease in infection with a $p<0.01$. 2539 ss1 on 51583 exposed to 4℃ pre-inoculation and delayed in inoculation had an average rating of 4.33±0.47 for the control, 4±0 for the 10 min exposure, 3.67±0.47 for the 30 min exposure, 4±0.82 for the 1 h exposure, and 4.67±0.47 for the 24 h exposure. Guy11 on 51583 exposed to 4℃ pre-inoculation and delayed in inoculation had an average rating of 8±0 for the control, 7.33±0.47 for the 10 min

exposure, 7.67±0.47 for the 30 min exposure, 6±0.82 for the 1 h exposure, and 6±0 for the 24 h exposure. The 1 h and 24 h exposure plants resulted in a statistically significant decrease in infection with a $p<0.05$. These results indicate that a pre-inoculation exposure to 4℃ for shorter periods with a 1 h delay before inoculation does not have a generalized influence on infection phenotype. Specific influences on HL and Guy11 under specific times were evident; however, these results did not clarify the experiment's main goal, determining the factor(s) influencing the overall reproducibility between experiments. All of these results are displayed in Figure 5-2.

5.3.1.3 Post-inoculation short term

2539 HL on 51583 exposed to 4℃ post-inoculation had an average rating of 4.67±0.47 for the control, 4.33±0.82 for the 10 min exposure, 2.33±0.47 for the 30 min exposure, 3.67±0.94 for the 1 h exposure, and 4.67±0.47 for the 24 h exposure. The 30 min exposure plants resulted in a statistically significant decrease in infection with a $p<0.01$. 2539 ss1 on 51583 exposed to 4℃ post-inoculation had an average rating of 2.67±0.47 for the control, 3.67±0.47 for the 10 min exposure, 2.67±0.47 for the 30 min exposure, 4±0 for the 1 h exposure, and 4±0 for the 24 h exposure. The 1 h and 24 h exposure plants resulted in a statistically significant increase in infection with a $p<0.05$. Guy11 on 51583 exposed to 4℃ post-inoculation had an average rating of 3.33±2.36 for the control, 6.67±0.47 for the 10 min exposure, 5.33±0.47 for the 30 min exposure, 5.33±0.47 for the 1 h exposure, and 5.67±0.47 for the 24 h exposure. These results indicate that post-inoculation exposure to 4℃ for shorter periods does not generalize the infection phenotype. Specific influences on HL, ss1, and Guy11 under specific times were evident; however, these results did not clarify the experiment's main goal,

determining the factor(s) influencing the overall reproducibility between experiments. All of these results are displayed in Figure 5-3.

5.3.1.4 Post-inoculation long term

2539 HL on 51583 exposed to 4℃ post-inoculation had an average rating of 0.33±0.47 for the control. All the treatment plants inoculated with 2539 HL, 2539 ss1, and Guy11 succumbed to the 24 h noncontinuous exposure to 4℃. 2539 ss1 on 51583 exposed to 4℃ post-inoculation had an average rating of 4.33±0.47 for the control, 0.67±0.47 for the 3 d exposure, and 0.33±0.47 for the 4 d exposure. The 1 d and 2 d plants succumbed to the 24 h noncontinuous exposure to 4℃. The 4 d exposure plants resulted in a statistically significant decrease in infection with a $p<0.01$. Guy11 on 51583 exposed to 4℃ post-inoculation had an average rating of 5±0 for the control and 2.33±2.05 for the 2 d exposure. These results indicate that post-inoculation exposure to 4℃ for longer periods does not generalize the infection phenotype. Specific influences on ss1 and Guy11 under specific times were evident; however, these results did not clarify the experiment's main goal, determining the factor(s) influencing the overall reproducibility between experiments. All of these results are displayed in Figure 5-4.

5.3.2 Hot temperature exposures

This experiment aimed to determine whether uncontrolled exposure to hot temperatures during plant transport was causing the differences observed in infection phenotypes compared to previously established results in the Farman lab. Due to biological safety restrictions and facility constraints, plants were moved for inoculation between buildings. Although the plants were bagged inside secondary containers, exposure to hot temperatures was unavoidable when removing plants from growth

chambers or containers. If exposure to hot temperatures pre or post-inoculation was the cause of the irreproducibility between experiments, the expected results would be a generalized increase or decrease in all infection phenotypes for the specific treatment regardless of strain or cultivar. The results of this experiment did not support exposure to hot temperatures as a reason for the lack of infection phenotype reproducibility. Interestingly, the results indicated that exposure to hot temperatures at specific intervals and times influenced interactions specific to the strain and cultivar, as each pair displayed a unique reaction to the 37°C exposures. Specifically, the results of the 24 h post-inoculation 37°C exposure displayed similarities to the results of the chamber malfunction described in Chapter 2.

5.3.2.1 Pre-inoculation

2539 MH on 51583 exposed to 37°C pre-inoculation had an average rating of 3±0.82 for the control, 3±0 for the 10 min exposure, 2.33±0.47 for the 30 min exposure, 1.33±0.47 for the 1 h exposure, and 2±1.41 for the 24 h exposure. 2539 HL on 51583 exposed to 37°C pre-inoculation had an average rating of 2±1.41 for the control, 3±0.82 for the 10 min exposure, 2.33±0.47 for the 30 min exposure, 1±0.82 for the 1 h exposure, and 2.33±2.05 for the 24 h exposure. 2539 ss1 on 51583 exposed to 37°C pre-inoculation had an average rating of 3.67±0.47 for the control, 2.33±0.47 for the 10 min exposure, 2.33±0.47 for the 30 min exposure, 3±0.82 for the 1 h exposure, and 1.33±0.47 for the 24 h exposure. The 24 h exposure plants resulted in a statistically significant decrease in infection with a $p<0.01$. Guy11 on 51583 exposed to 37°C pre-inoculation had an average rating of 7.67±0.47 for the control, 8±0 for the 10 min exposure, 8±0 for the 30 min exposure, 7.33±0.47 for the 1 h exposure, and 7±0.82 for the 24 h exposure. These results

indicate that pre-inoculation exposure to 37℃ for shorter periods does not generalize the infection phenotype. Specific influences on ss1 under specific times were evident; however, these results did not clarify the experiment's main goal, which was to determine the factor(s) influencing the overall reproducibility between experiments. All of these results are displayed in Figure 5-5.

5.3.2.2 Pre-inoculation with delay

2539 MH on 51583 exposed to 37℃ pre-inoculation and delayed in inoculation for 1 hr had an average rating of 3.33±1.25 for the control, 3±0.82 for the 10 min exposure, 4±0 for the 30 min exposure, 1.33±1.25 for the 1 h exposure, and 1.67±0.47 for the 24 h exposure. 2539 HL on 51583 exposed to 37℃ pre-inoculation and delayed in inoculation for 1 h had an average rating of 4±0 for the control, 3±0.82 for the 10 min exposure, 3.67±0.47 for the 30 min exposure, 3.67±0.94 for the 1 h exposure, and 3.33±0.47 for the 24 h exposure. 2539 ss1 on 51583 exposed to 37℃ pre-inoculation and delayed in inoculation had an average rating of 4.33±0.47 for the control, 4±0 for the 10 min exposure, 4±0 for the 30 min exposure, 3±0 for the 1 h exposure, and 4±0 for the 24 h exposure. The 1 h exposure plants resulted in a statistically significant decrease in infection with a $p<0.05$. Guy11 on 51583 exposed to 37℃ pre-inoculation and delayed in inoculation had an average rating of 6.67±0.47 for the control, 7.33±0.47 for the 10 min exposure, 6.67±0.47 for the 30 min exposure, 7±0.82 for the 1 h exposure, and 7±0 for the 24 h exposure. These results indicate that a pre-inoculation exposure to 37℃ for shorter periods with a 1 h delay before inoculation does not have a generalized influence on infection phenotype. Specific influences on ss1 under specific times were evident; however, these results did not clarify the experiment's main goal, which was to determine

the factor(s) influencing the overall reproducibility between experiments. All of these results are displayed in Figure 5-6.

5.3.2.3 Post-inoculation short term

2539 MH on 51583 exposed to 37℃ post-inoculation had an average rating of 4.67±0.47 for the control, 4.33±0.47 for the 10 min exposure, 4±0 for the 30 min exposure, 3.67±0.47 for the 1 h exposure, and 5.33±0.47 for the 24 h exposure. 2539 HL on 51583 exposed to 37℃ post-inoculation had an average rating of 4.67±0.47 for the control, 3.67±0.47 for the 10 min exposure, 3.67±0.47 for the 30 min exposure, 4.67±0.47 for the 1 h exposure, and 5.67±0.47 for the 24 h exposure. 2539 ss1 on 51583 exposed to 37℃ post-inoculation had an average rating of 5±0.82 for the control, 4.67±0.47 for the 10 min exposure, 4.33±0.47 for the 30 min exposure, 5±0.82 for the 1 h exposure, and 4.33±0.47 for the 24 h exposure. Guy11 on 51583 exposed to 37℃ post-inoculation had an average rating of 8±0 for the control, 8±0 for the 10 min exposure, 7±0.82 for the 30 min exposure, 4±2.94 for the 1 h exposure, and 6.67±0.47 for the 24 h exposure. The 24 h exposure plants resulted in a statistically significant decrease in infection with a $p<0.05$. These results indicate that post-inoculation exposure to 37℃ for shorter periods does not generalize the infection phenotype. Specific influences on Guy11 under specific times were evident; however, these results did not clarify the experiment's main goal, which was to determine the factor(s) influencing the overall reproducibility between experiments. All of these results are displayed in Figure 5-7.

5.3.2.4 Post-inoculation long-term

2539 MH on 51583 exposed to 37℃ post-inoculation had an average rating of 4.33±0.47 for the control, 4±0 for the 1 d exposure, 3.33±0.94 for the 2 d exposure,

4.33±0.47 for the 3 d exposure, and 4.33±0.47 for the 4 d exposure. 2539 HL on 51583 exposed to 37℃ post-inoculation had an average rating of 4.33±0.47 for the control, 5±0 for the 1 d exposure, 5±0 for the 2 d exposure, 3±1.41 for the 3 d exposure, and 2±1.41 for the 4 d exposure. 2539 ss1 on 51583 exposed to 37℃ post-inoculation had an average rating of 4±0 for the control, 5±0 for the 1 d exposure, 5±0 for the 2 d exposure, 4.33±0.47 for the 3 d exposure, and 4±0.47 for the 4 d exposure. Guy11 on 51583 exposed to 37℃ post-inoculation had an average rating of 5.67±0.47 for the control, 5.67±0.47 for the 1 d exposure, 5.67±0.47 for the 2 d exposure, 5.33±0.47 for the 3 d exposure, 5.67±0.47 for the 4 d exposure, and 6.67±0.47 for the 24 h exposure. These results indicate that post-inoculation exposure to 37℃ for longer periods does not generalize the infection phenotype. These results did not clarify the experiment's main goal, determining the factor(s) influencing the overall reproducibility between experiments. All of these results are displayed in Figure 5-8.

5.3.3 2539 stocks 37℃ 24 h post-inoculation exposure

This experiment aimed to explore the stability of the increased disease severity on plants after exposure to 24 h of 37℃ post-inoculation observed in the hot temperature inoculations. Based on the results described in the previous experiments, it seemed possible that exposure to 37℃ at the critical time of fungal proliferation in the host allowed the fungus to go unrecognized. If this longer period of 37℃ exposure did suppress host resistance, the expected results would be increased disease severity in the hosts after exposure to heat. These experimental results supported the influence of heat on host resistance suppression as all plants exposed to 37℃ for 24 h post-inoculation displayed higher disease severity ratings than the controls.

2539 5.5.01 on 51583 exposed to 37°C for 24 h post-inoculation had an average rating of 4.23±0.42, compared to the average rating of 3.93±0.7 in the control. 2539 5.5.02 on 51583 exposed to 37°C for 24 h post-inoculation had an average rating of 5.43±0.82, compared to the average rating of 4.11±1.2 in the control, leading to a statistically significant increase in infection with a $p<0.05$. 2539 FG on 51583 exposed to 37°C for 24 h post-inoculation had an average rating of 5.08±0.47, compared to the average rating of 3.4±0.88 in the control leading to a statistically significant increase in infection with a $p<0.01$. 2539 HL on 51583 exposed to 37°C for 24 h post-inoculation had an average rating of 4.8±0.75, compared to the average rating of 3.27±0.44 in the control, leading to a statistically significant increase in infection with a $p<0.01$. 2539 MH on 51583 exposed to 37°C for 24 h post-inoculation had an average rating of 3.93±0.44, compared to the average rating of 3.38±0.92 in the control. The wild-type stocks displayed a trend of increased infection after exposure to heat, although both MH and 5.5.01 failed to reach a significant difference. All of these results are displayed in Figure 5-9.

2539 ss1 on 51583 exposed to 37°C for 24 h post-inoculation had an average rating of 6.69±0.46, compared to the average rating of 4.6±0.49 in the control, leading to a statistically significant increase in infection with a $p<0.01$. 2539 ss2 on 51583 exposed to 37°C for 24 h post-inoculation had an average rating of 6.67±0.47, compared to the average rating of 4.2±0.65 in the control, leading to a statistically significant increase in infection with a $p<0.01$. 2539 ss3 on 51583 exposed to 37°C for 24 h post-inoculation had an average rating of 6.73±0.86, compared to the average rating of 4.36±0.61 in the control, leading to a statistically significant increase in infection with a $p<0.01$. 2539 ss4

on 51583 exposed to 37℃ for 24 h post-inoculation had an average rating of 6.91±0.29, compared to the average rating of 4.07±0.46 in the control, leading to a statistically significant increase in infection with a $p<0.01$. 2539 ss4* on 51583 exposed to 37℃ for 24 h post-inoculation had an average rating of 6.58±0.76, compared to the average rating of 3.92±1 in the control leading to a statistically significant increase in infection with a $p<0.01$. 2539 ss5 on 51583 exposed to 37℃ for 24 h post-inoculation had an average rating of 7.44±0.5, compared to the average rating of 4.14±0.52 in the control, leading to a statistically significant increase in infection with a $p<0.01$. All the mutant stocks displayed significant increases in disease severity after exposure to 24 h of 37℃ post-inoculation, consistent with the prediction that the heat was suppressing host resistance. All of these results are displayed in Figure 5-9.

5.4 Discussion

The experiments described in this chapter were performed to elucidate the potential cause of the irreproducibility seen between experiments and the differences between current inoculation phenotypes compared to previously observed results in the Farman lab. The results of the experiments involving pre and post exposures to 4℃ and 37℃ did not support the hypothesis that uncontrolled temperature exposures were the cause of the irreproducibility between experiments. If temperature exposures were the cause of irreproducibility, the expected results would have been the same trend for the specific time exposure regardless of the strain or cultivar involved in the inoculation. Instead, the results supported a strain and cultivar interaction under the influence of a specific temperature and time. This could indicate an influence on the R genes, AVR genes, or an interaction between the two at critical times during infection.

For example, exposure of 51583 plants to 4℃ pre-inoculations displayed a significant decrease in infection for 2539 ss1 at 30 min and 1 h, indicating possible fungal suppression or host resistance inducement. Alternatively, exposure of 51583 plants to 4℃ pre-inoculations displayed a significant increase in infection for Guy11 at 24 h, indicating possible fungal inducement or host resistance suppression. Exposure to 37℃ for 24 h post-inoculation was particularly interesting as multiple stocks displayed stable, significantly increased infection, indicating potential suppression of host resistance. Studies have supported general increases or decreases in resistance for whole plants during *M. oryzae* infection under specific temperatures. Exposure to colder temperatures suppresses whole plant-specific resistance to *Magnaporthe* infection (Koga et al., 2004; Xiong & Yang, 2003). Exposure to warmer temperatures suppresses whole plant-specific and non-specific (partial) resistance to *Magnaporthe* infection (Du et al., 2021; Otofuji, 1987). The results described in this chapter indicate a more nuanced influence of temperature, perhaps at the level of recognition for specific AVR/R interactions.

Short-term temperature exposures are well documented in other pathosystems to influence host resistance. Resistance in barley leaves was induced with an oxidative burst in planta against *Blumeria graminis* f. sp. *hordei* after exposure to 50℃ for one minute (Vallélian-Bindschedler et al., 1998). Resistance in Swiss oats was suppressed, allowing for increased disease severity and mycotoxin production in *Fusarium Langsethiae* after exposure to 10℃ for 12 h (Schöneberg et al., 2019). The literature has also documented specific influences of temperature on R genes. The R gene $Ry_{chc,}$ which confers resistance to potato virus Y in potatoes, is disabled at temperatures of 28℃ for 5 d (Ohki et al., 2018). The R gene *Sr15,* which confers resistance to *Puccinia gramninis* f. sp. *tritici* in

wheat is disabled at temperatures of 22-25℃ (Gao et al., 2019). R gene *Pi54*, which confers resistance to *M. oryzae* in rice, is induced by pre-exposure to 35℃ for 7 d (Onaga et al., 2017). Temperature effects on R genes or general host resistance in rice have been described with long-term exposures over multiple days (Qiu et al., 2022; Yokotani et al., 2013). This research has shown phenotypic expression changes under short-term temperature exposures. It is possible that some R genes in rice can be suppressed with brief temperature exposures. Further work exploring the effects of post-inoculation 37℃ could be performed to determine if the effect is generalized to all strains on cultivars or if the interaction is specific and acts at the recognition level.

5.5 Conclusions

The results of the experiments in this chapter indicated that the influence of temperature exposures pre and post-inoculation were strain and cultivar-specific. The results from the 4℃ treatments did not present a generalized trend, and post-inoculation long-term treatment killed the plants. Additionally, the results from the 37℃ treatments did not present a generalized trend. Interestingly, a post-inoculation treatment of 37℃ for 24 h displayed stable disease severity increase for some strains on some cultivars. Although each temperature and timing influenced the occurrence of disease severity, none of the treatments displayed the large differences in disease severity previously observed between replicates. Notably, large differences in disease severity were seen during these inoculations within control groups and plants treated identically. This caused complications in determining the true influence of the treatment because the controls were so variable. The cause of irreproducibility between experiments was not variable temperature exposures and was a confounding variable in these inoculations. Further

work should be performed to isolate the specific influences of this treatment and the specificity of the effects.

Table 5-1: *Magnaporthe* stocks and strains utilized for experiments

Isolate Name	Host	Year	Type	Place of Isolation	Source
2539 HL	*Oryza sativa*	1988	Wild-Type	Laboratory Strain	H. Leung, bred strain
2539 MH	*Oryza sativa*	2014	Wild-Type	Laboratory Strain	M. Heist, subculture of 2539
2539 FG	*Oryza sativa*	2007	Wild-Type	Laboratory Strain	Subculture of 2539
2539 5.5.01	*Oryza sativa*	2007	Wild-Type	Laboratory Strain	Subculture of 2539
2539 5.5.02	*Oryza sativa*	2007	Wild-Type	Laboratory Strain	Subculture of 2539
2539 ss1	*Oryza sativa*	2014	Mutant	Laboratory Strain	M. Heist, 2539 single spore from lesion of 51583
2539 ss2	*Oryza sativa*	2014	Mutant	Laboratory Strain	M. Heist, 2539 single spore from lesion of 51583
2539 ss3	*Oryza sativa*	2014	Mutant	Laboratory Strain	M. Heist, 2539 single spore from lesion of 51583
2539 ss4	*Oryza sativa*	2014	Mutant	Laboratory Strain	M. Heist, 2539 single spore from lesion of 51583
2539 ss4*	*Oryza sativa*	2015	Mutant	Laboratory Strain	M. Heist, subculture of 2539 ss4
2539 ss5	*Oryza sativa*	2014	Mutant	Laboratory Strain	M. Heist, 2539 single spore from lesion of 51583
Guy11	*Oryza sativa*	1979	Wild-Type	French Guyana	J. Notteghem, field isolation

Table 5-2 Rice Cultivars Utilized for Experiments

Cultivar Name	Type	Year	Origin	Known R genes	Reference Source
51583	*Indica*		USSR		(Leung, et. al, 1988)

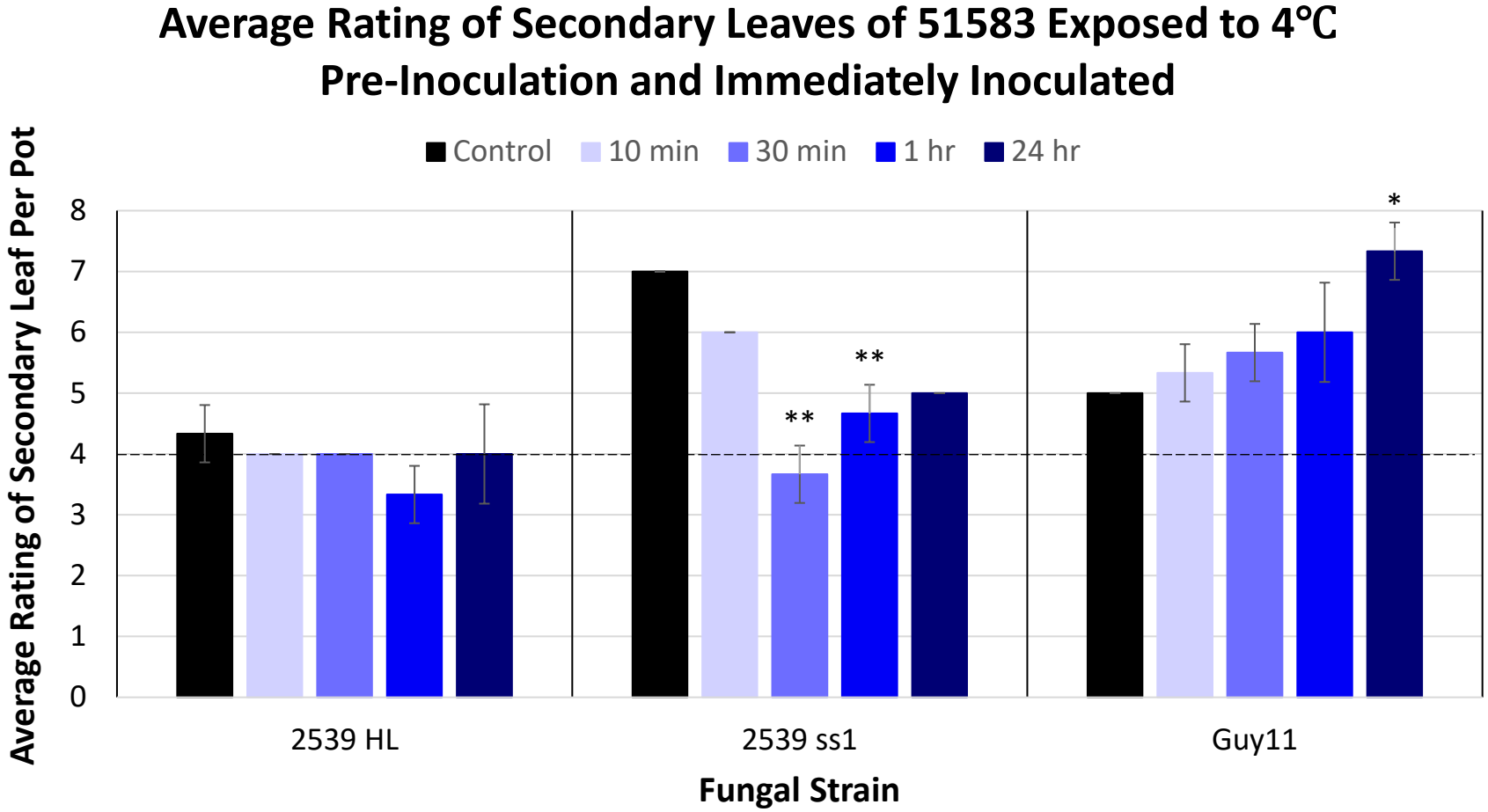


Figure 5-1 Average rating of secondary leaves of 51583 exposed to 4°C pre-inoculation and immediately inoculated Ratings of 4 and above are categorized as virulent while ratings below 4 are categorized as avirulent. One * denote $p<0.05$ and two ** denote $p<0.01$. Each bar represents an approximate n=3.

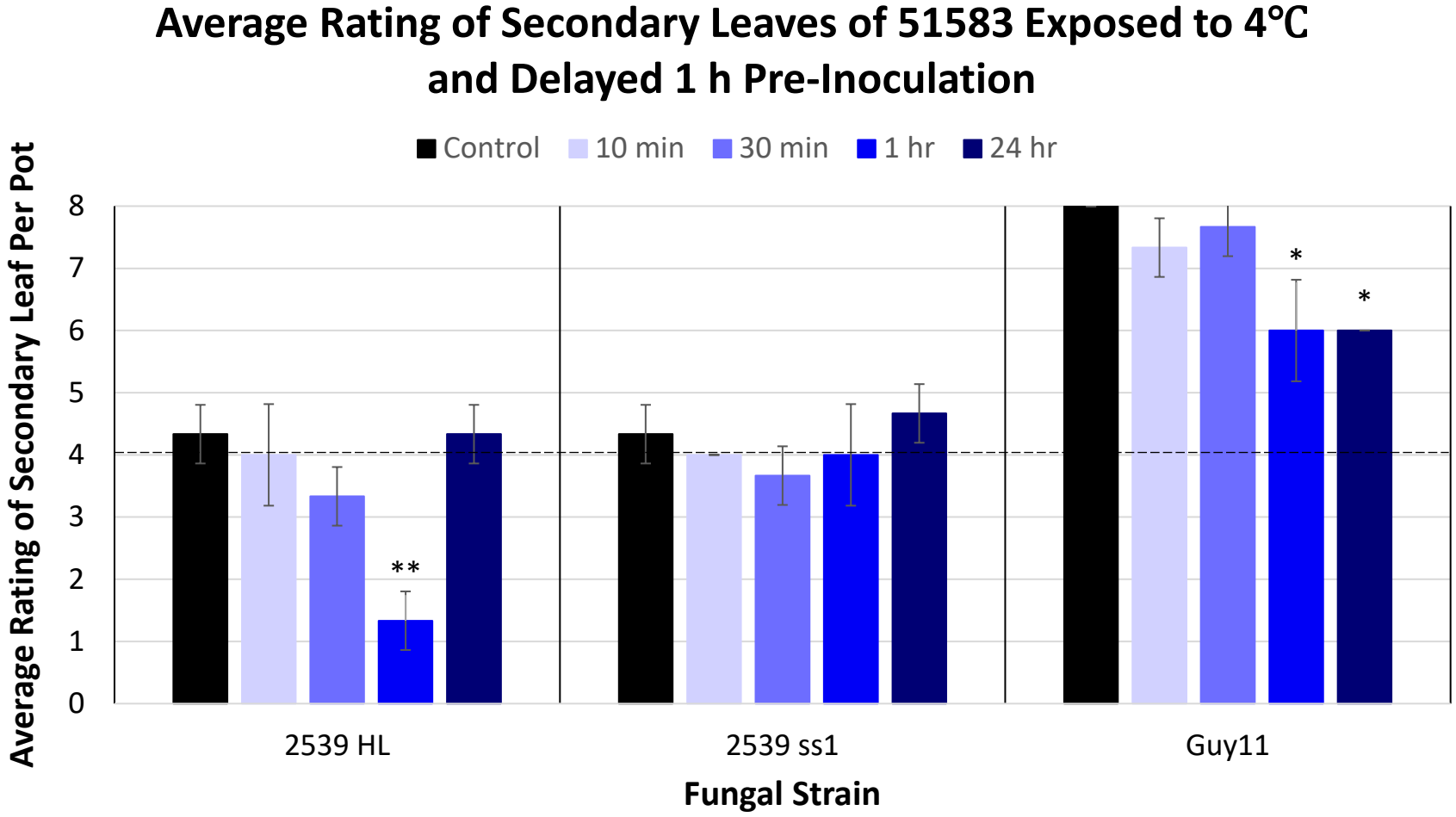


Figure 5-2 Average rating of secondary leaves of 51583 exposed to 4°C and delayed 1 h pre-inoculation Ratings of 4 and above are categorized as virulent while ratings below 4 are categorized as avirulent. One * denote $p<0.05$ and two ** denote $p<0.01$. Each bar represents an approximate n=3.

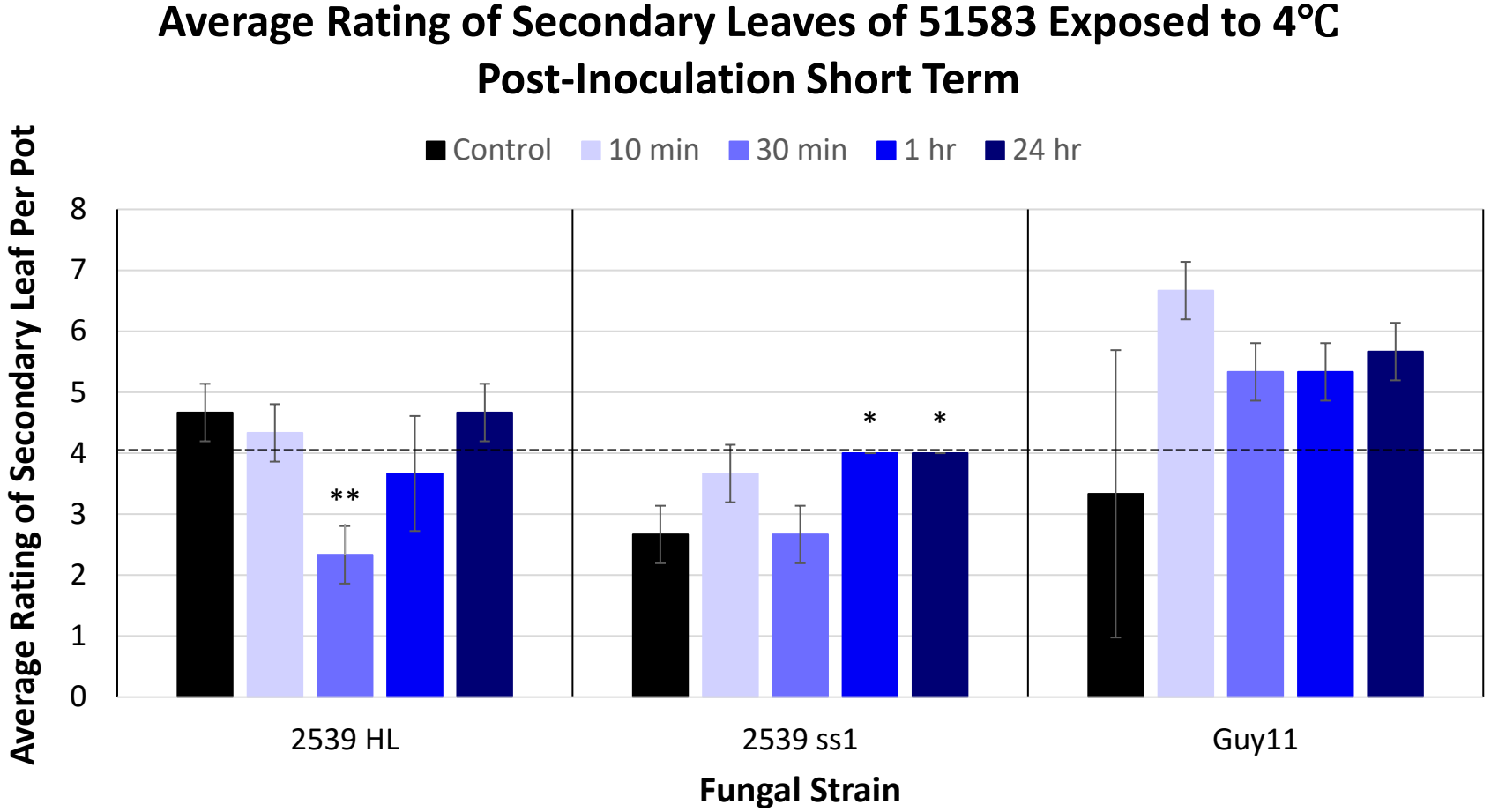


Figure 5-3 Average rating of secondary leaves of 51583 exposed to 4°C post-inoculation short term Ratings of 4 and above are categorized as virulent while ratings below 4 are categorized as avirulent. One * denote $p<0.05$ and two ** denote $p<0.01$. Each bar represents an approximate n=3.

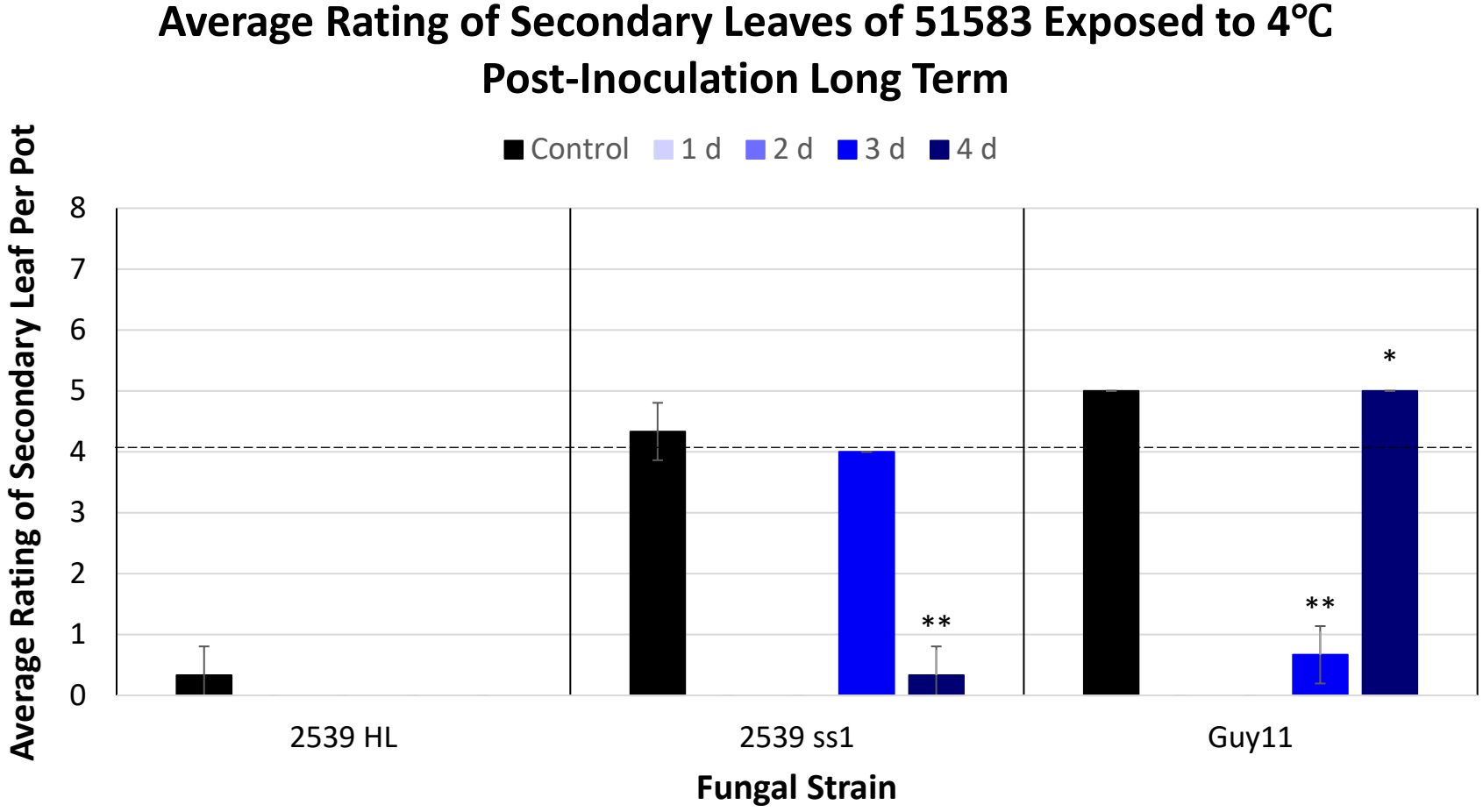


Figure 5-4 Average rating of secondary leaves of 51583 exposed to 4°C post-inoculation long term Ratings of 4 and above are categorized as virulent while ratings below 4 are categorized as avirulent. One * denote $p<0.05$ and two ** denote $p<0.01$. Each bar represents an approximate n=3.

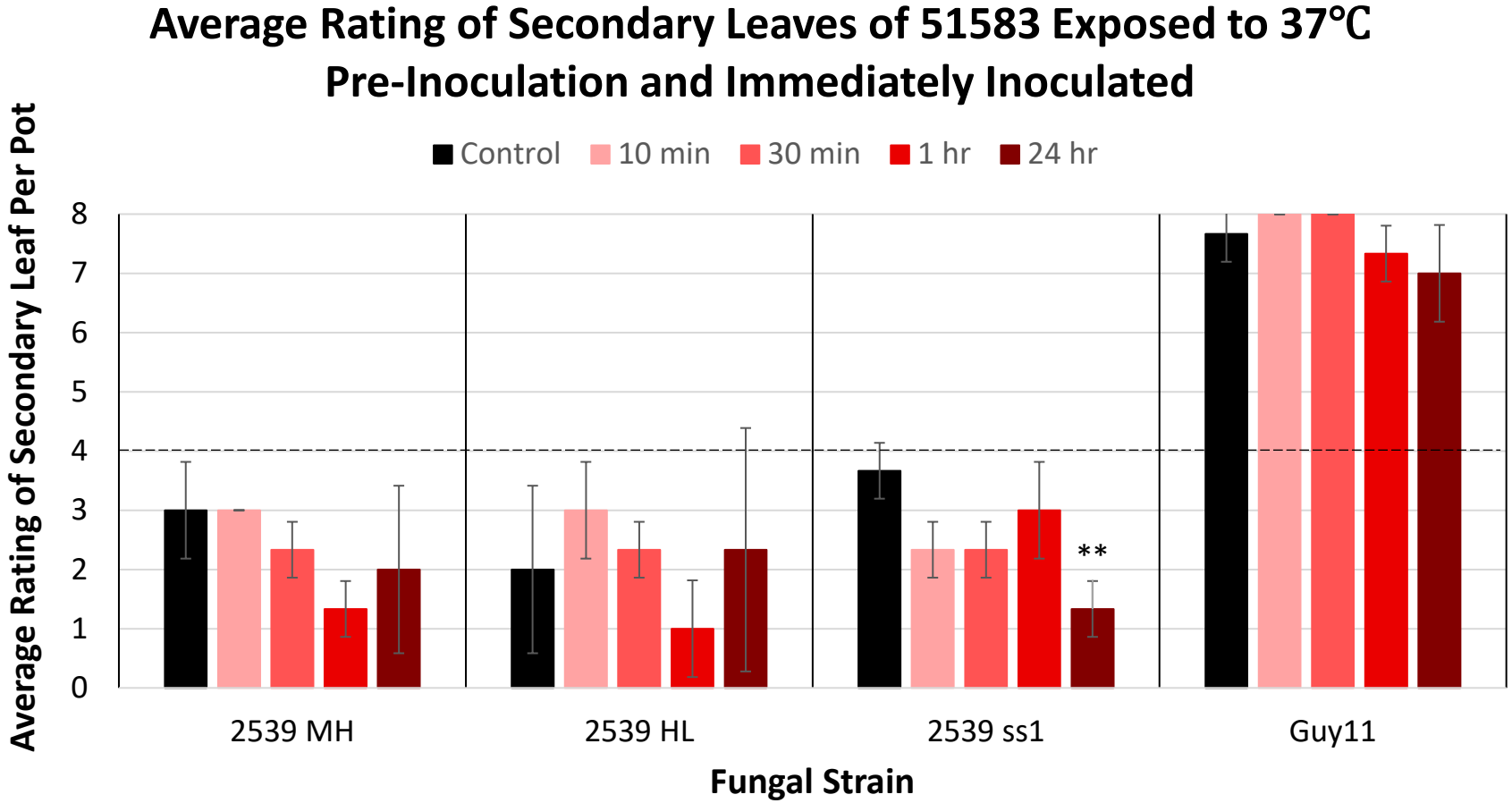


Figure 5-5 Average rating of secondary leaves of 51583 exposed to 37°C pre-inoculation and immediately inoculated Ratings of 4 and above are categorized as virulent while ratings below 4 are categorized as avirulent. One * denote $p<0.05$ and two ** denote $p<0.01$. Each bar represents an approximate n=3.

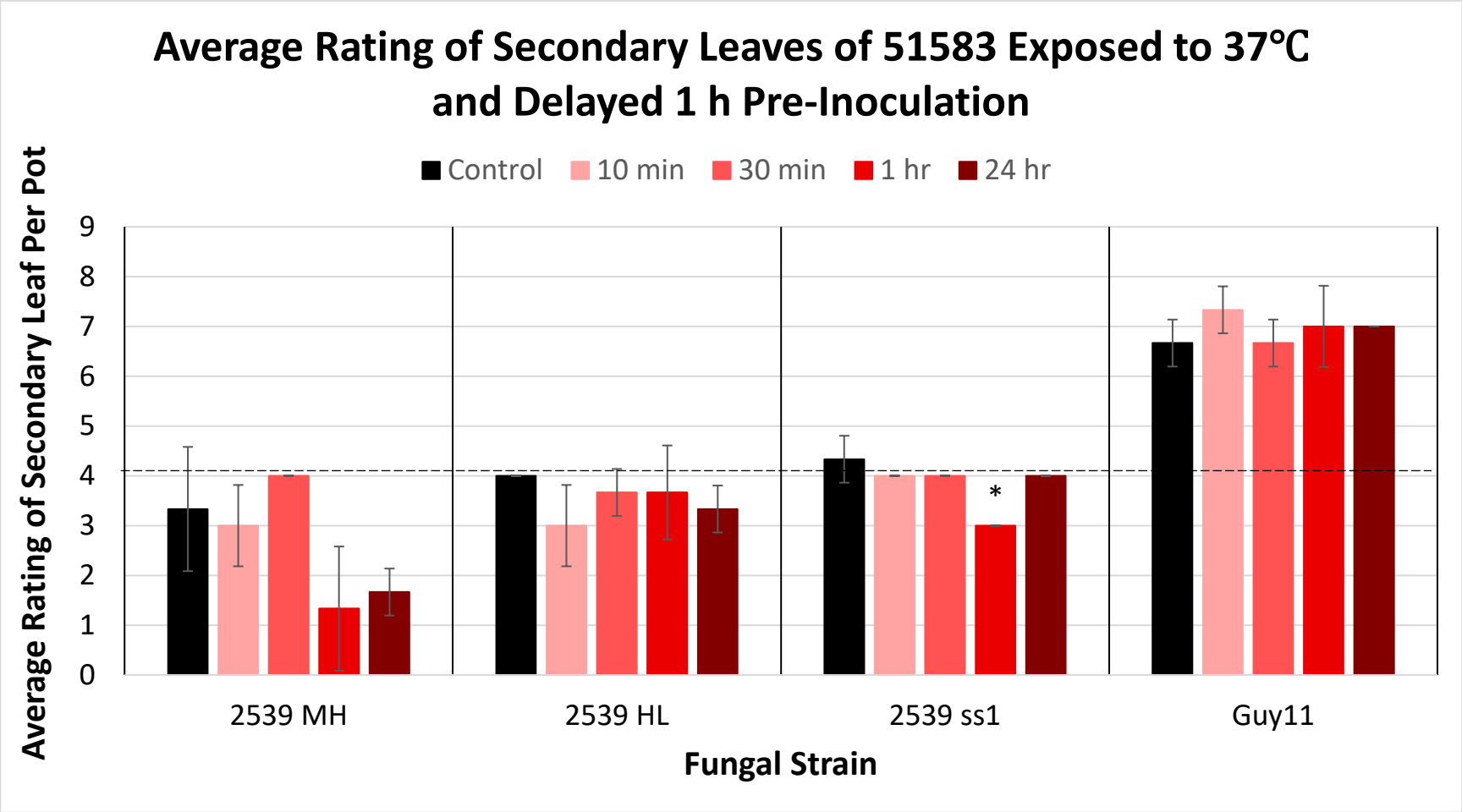


Figure 5-6 Average rating of secondary leaves of 51583 exposed to 37°C and delayed 1 h pre-inoculation Ratings of 4 and above are categorized as virulent while ratings below 4 are categorized as avirulent. One * denote $p<0.05$ and two ** denote $p<0.01$. Each bar represents an approximate n=3.

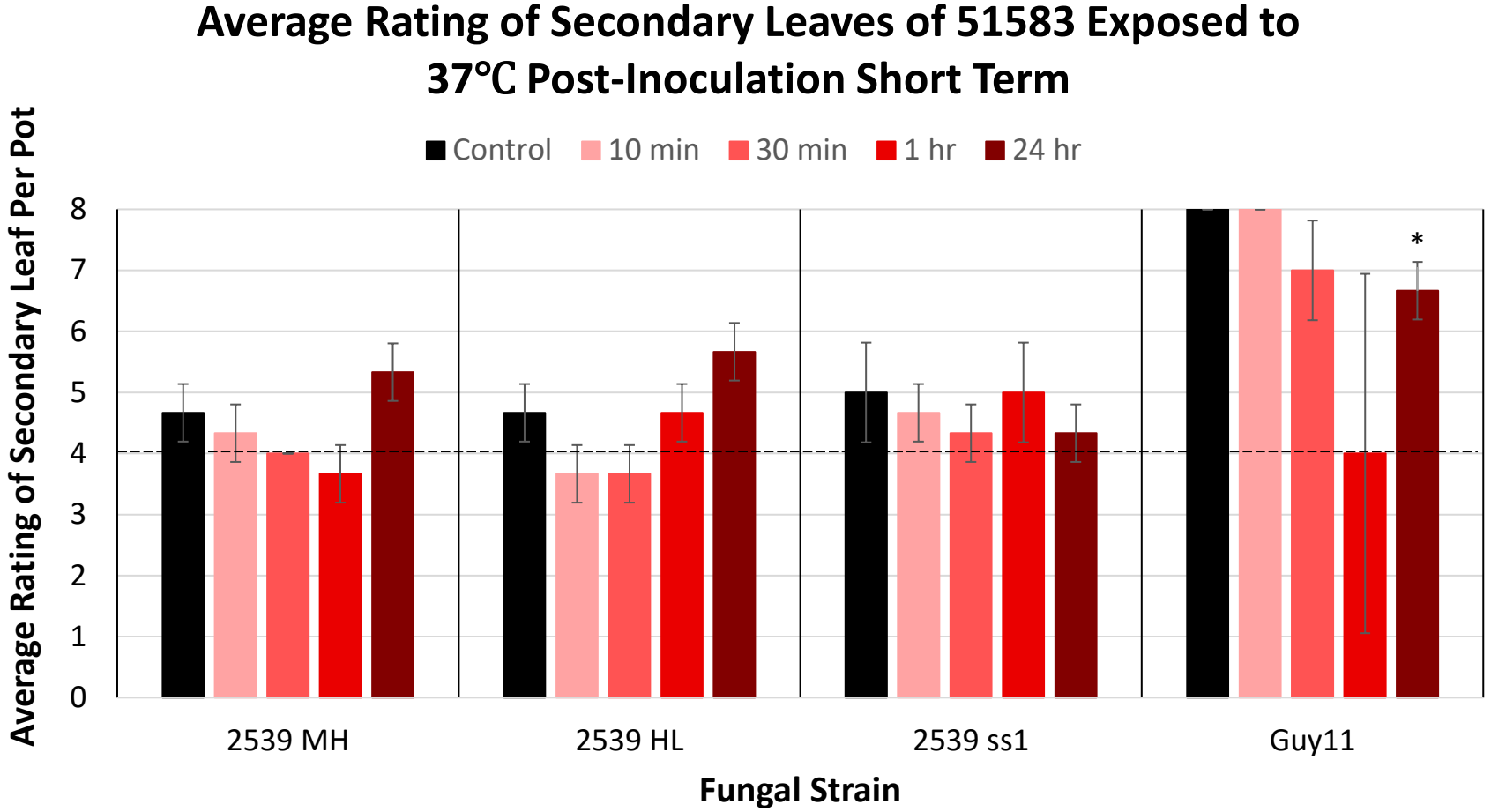


Figure 5-7 Average rating of secondary leaves of 51583 exposed to 37℃ post-inoculation short term Ratings of 4 and above are categorized as virulent while ratings below 4 are categorized as avirulent. One * denote $p<0.05$ and two ** denote $p<0.01$. Each bar represents an approximate n=3.

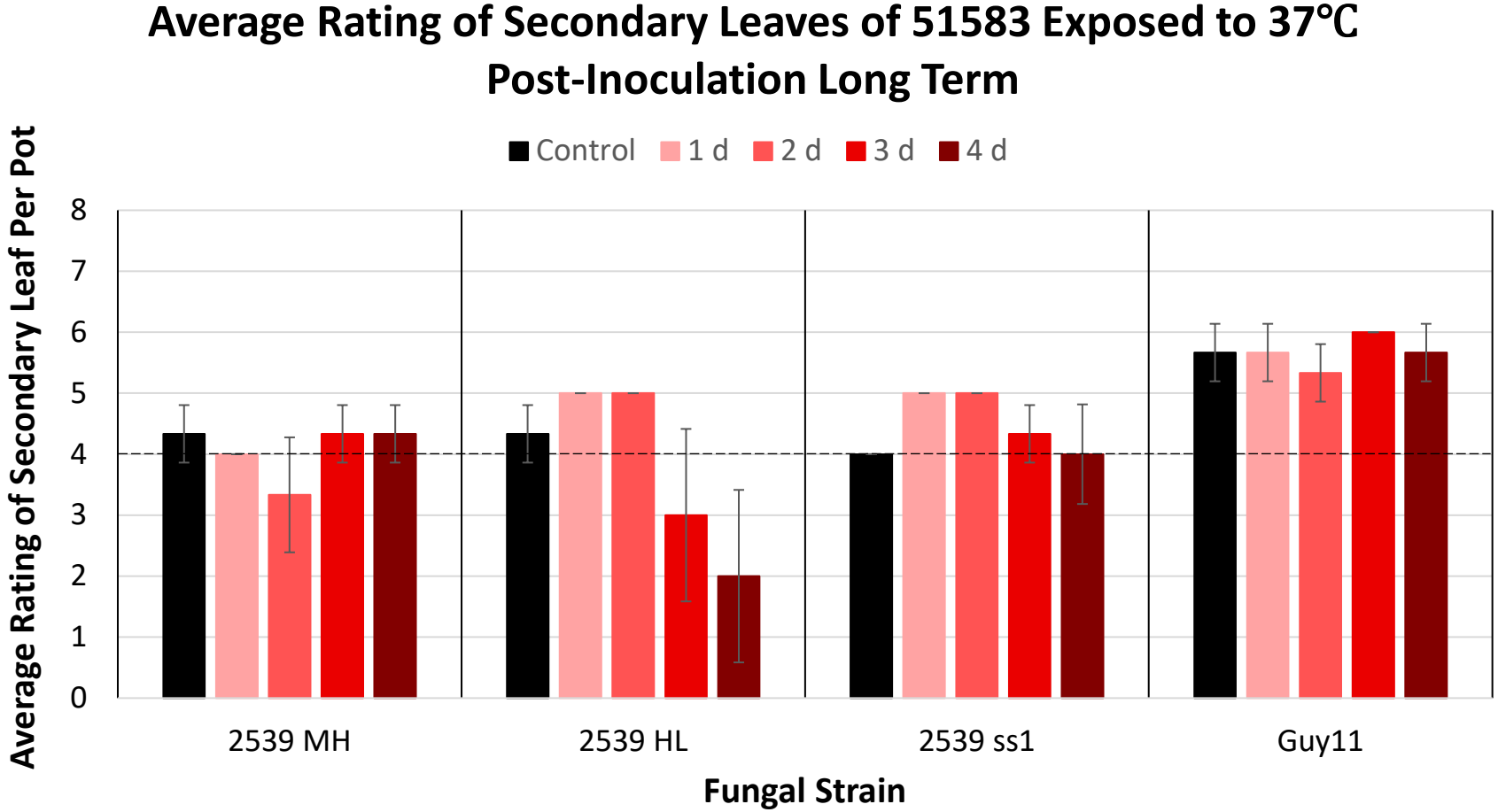


Figure 5-8 Average rating of secondary leaves of 51583 exposed to 37°C post-inoculation long term Ratings of 4 and above are categorized as virulent while ratings below 4 are categorized as avirulent. One * denote $p<0.05$ and two ** denote $p<0.01$. Each bar represents an approximate n=3.

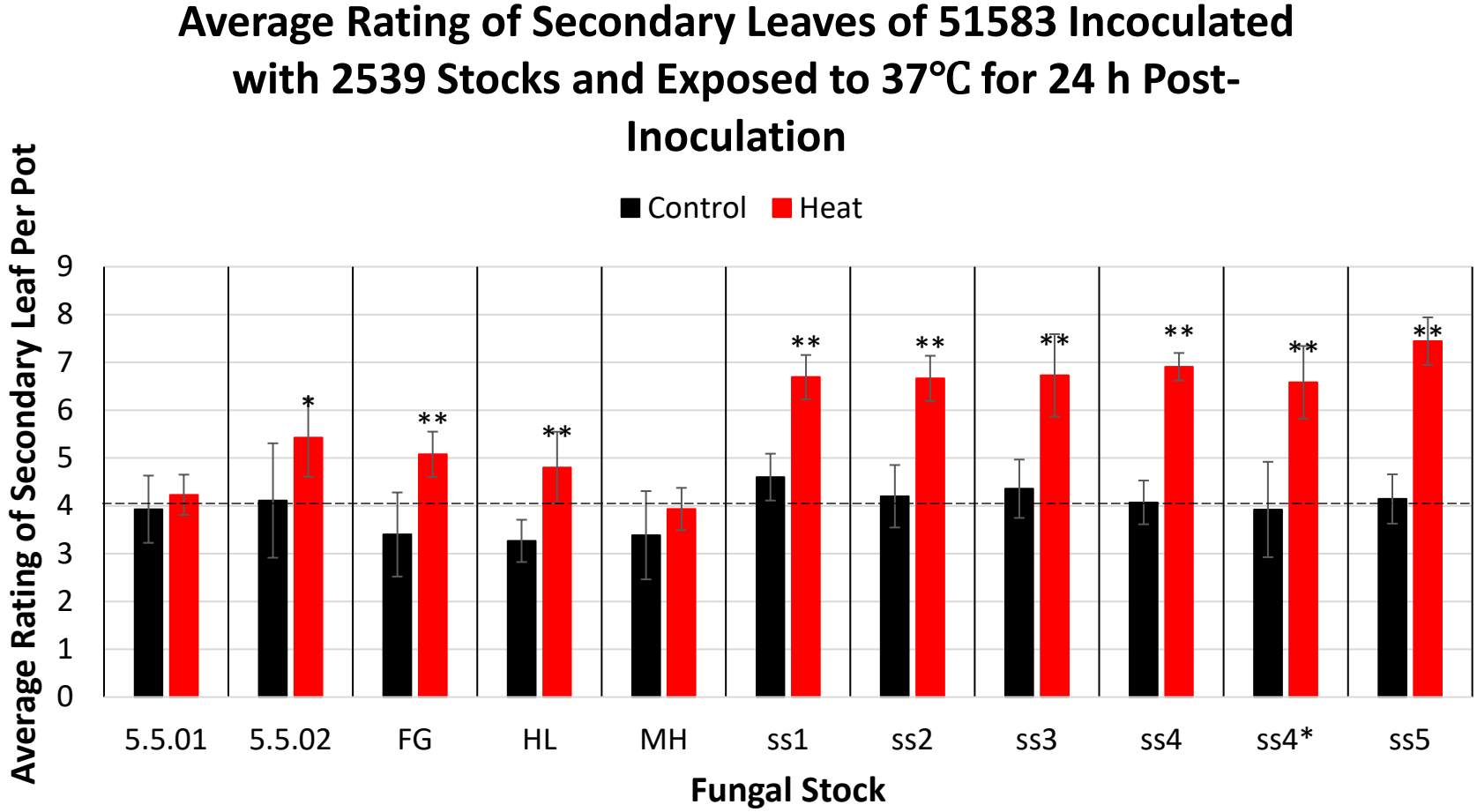


Figure 5-9 Average rating of secondary leaves of 51583 exposed to 37°C post-inoculation long term Ratings of 4 and above are categorized as virulent while ratings below 4 are categorized as avirulent. One * denote $p<0.05$ and two ** denote $p<0.01$. Each bar represents an approximate n=15.

CHAPTER 6: HEAT AS A CRITICAL FACTOR INFLUENCING STRAIN/CULTIVAR INTERACTION PHENOTYPES

6.1 Background

Rice is a drought-tolerant crop produced worldwide, with temperatures in certain planting areas ranging from 14 – 45℃ over the growing season ((MODIS), 2020). Regions where rice is grown as a staple food crop, such as Japan and India, have experienced record-breaking heat waves in 2019, 2020, 2021, and 2022 ((NOAA), 2022). These areas are also predicted to have the most severe heat waves and temperature fluctuations in the coming decades. Current studies within the *M. oryzae* system concerning elevated temperature range from 25 – 30℃ and limited exposure to an average of 1 h or less. This is due to the methodology of inoculation assays, which have shifted from whole plant to point inoculation or leaf sheath assays. Excised leaves or sheaths cannot withstand long exposures to temperatures generally 28℃ and above (Honkura et al., 1995; Laborte et al., 2017; Luo et al., 1998; Qiu et al., 2022; Wuebbles et al., 2017). Responses to elevated temperatures in rice leaves and panicles infected with *M. oryzae* are variable. Two studies found that continuous exposure to 28℃ caused lesion size to increase in both panicle and rice blast compared to 22℃ (Du et al., 2021; Kato & Kozaka, 1974). Other studies have shown decreased infection with continuous exposure to 28℃ and higher of whole plants (Katsantonis et al., 2017; Madhusudhan et al., 2019). Generally, each study has focused on one or two fungal strains and less than six different rice cultivars.

The experiments in this chapter sought to explore the host reaction to elevated temperatures (37℃) during early fungal proliferation. Preliminary results reported in

Chapter 5, showed a stable increase of disease severity in 51583 plants inoculated with 2539. The present goal, therefore, was to determine if the post-inoculation treatment of 37°C for 24 h suppressed host resistance in a general manner or if the effect was at the level of recognition. A separate aim was to clarify the conflicting results within the literature regarding post-inoculation heat treatments using multiple strains and cultivars. Previous studies exploring the effects of higher temperatures in *M. oryzae* were limited due to the lack of diversity among fungal strains and cultivars. Only utilizing certain strains or cultivars would limit the potential AVR/R interactions being tested. This practice would preclude identifying an influence at the level of recognition because a mixed population of AVR/R genes would be necessary for comparison.

6.2 Materials and Methods

6.2.1 Post-inoculation heat exposure

The procedure matched the plant inoculation protocol listed in Chapter 2. After incubating the inoculated plants in the dark at 22°C for 20 h, the control group of plants were maintained in the growth chamber under 25°C conditions, while the treatment groups were temporarily exposed to 37°C for 24 h or 48 h before being returned to normal 25°C growth conditions. A third treatment group was continuously exposed to 37°C for a full 7 d. Stocks utilized in the experiments are listed in Table 6-1. Cultivars utilized in the experiments are listed in Table 6-2.

6.2.2 Ratings and statistical analysis

Disease ratings for secondary leaves were made using the scale shown in Figure 3-1. Ratings were used to determine the severity of the disease. Ratings 0-3 represent resistance levels on the host (avirulent), while ratings 4-8 represent levels of

susceptibility on the host (virulent). Ratings of 4 and above were categorized as virulent, while ratings below 4 were categorized as avirulent. Statistical analysis was performed using an unpaired, unequal variance t-test in order to compared two groups. Analysis of variance was performed for the replications of each stock to ensure that data could be combined.

6.2.3 *Agrobacterium*-mediated fungal transformation

Fungal cultures were activated from frozen stocks by placing fungal paper disks on oatmeal agar and incubating at 25°C under continuous illumination. After 14 d, the plates were flooded with 2 ml of a 0.25% gelatin suspension in water, and the conidia were liberated by massaging the colony surface with a sterilized bacterial cell spreader. The solution was filtered through 0.2 µM Miracloth, the spores were quantified using a hemocytometer, and concentrations were adjusted to 10^5/ml using 0.25% gelatin.

Agrobacterium tumefaciens strain AGL-1 with the desired plasmid was activated from -80°C storage to LB agar supplemented with 50 µg/ml kanamycin (Gold Biotechnology®) 48 h before the experiment. The black filter paper was cut into 1 cm^2 squares, autoclaved, and placed on the surface of the induction medium agar. A sterile pipette tip was used to collect a small amount of *Agrobacterium*, and the sample was placed on the black filter paper on the agar. Then, 100 µl of spore suspension was added to the black filter paper, and a sterilized bacterial cell spreader was used to distribute the bacterial cells and fungal spores across the plate. The plates were inverted and allowed to grow at 25°C under continuous illumination for 48 hrs. Sterile forceps were used to transfer the black filter paper

to selection agar (hygromycin B:300 µg/ml, cefotaxime:200 µg/ml, carbenicillin:250 µg/ml, and 200 µM AS). The co-cultures were maintained on the selection plates at 25℃ for 7 d. Following incubation, sterile toothpicks were used to excise putative transformants and transfer them to oatmeal agar supplemented with 100 µg/ml of hygromycin B antibiotic.

6.3 Results

6.3.1 Influence of 37℃ for 24h post-inoculation on different strain/cultivar combinations

This experiment aimed to determine if the enhanced susceptibility of 51583 to 2539, with exposure to 37℃ for 24 h post-inoculation described in Chapter 5, was due to general suppression of host resistance or if the influence functioned at the level of recognition. If the influence was only a general suppression of host resistance, the expected result would be that all fungal strains on 51583 would display increased disease severity after treatment. Other cultivars would display either an increased disease severity after treatment with all strains or no difference between treatments as an effect of generalized host resistance suppression. However, if elevated temperatures functioned at the level of recognition, then each strain and cultivar pairing would present a unique reaction that could be opposite the reaction of another pairing, including those on 51583.

The results of this experiment supported the hypothesis that exposure to 37℃ for 24 h post-inoculation acts at the level of recognition (AVR/R interaction) because each strain and cultivar paring displayed a unique reaction after treatment. After infection and exposure to the treatment, each fungal strain displayed at least one instance of increased disease severity on one cultivar and decreased disease severity on another. After infection and exposure to the treatment, each cultivar displayed at least one instance of increased

disease severity from a fungal strain and decreased disease severity from another fungal strain, except for ToRide and Kitake. These results were replicated in a secondary set of inoculations focused on the strain/cultivar parings with significant differences, and produced similar results. 2539 HL and SSID116 were utilized in these inoculations; however, the lack of infection on all cultivars from both strains precipitated excluding those results.

Different rice cultivars had variable responses to elevated temperatures following inoculation with strain FR13. FR13 on CO39 exposed to 37℃ for 24 h post-inoculation had an average rating of 4±0 compared to the average rating of 2.3±0.46 in the control, leading to a statistically significant increase in infection with a $p<0.01$. FR13 on Nipponbare exposed to 37℃ for 24 h post-inoculation had an average rating of 4.55±1.5 compared to the average rating of 2.27±2.49 in the control, leading to a statistically significant increase in infection with a $p<0.05$. FR13 on TP-309 exposed to 37℃ for 24 h post-inoculation had an average rating of 2.75±2.13 compared to the average rating of 5.27±1.71 in the control, leading to a statistically significant decrease in infection with a $p<0.01$. FR13 on Yt16 exposed to 37℃ for 24 h post-inoculation had an average rating of 6.64±0.88 compared to the average rating of 4.92±1.71 in the control, leading to a statistically significant increase in infection with a $p<0.01$.These results support an influence of heat at the level of recognition as each cultivar did not display a generalized suppression of host resistance to FR13. CO39, Nipponbare, and Yt16, displayed significant increases in infection and TP-309 displayed significant decreases in infection. All of these results are displayed in Figure 6-1.

Different rice cultivars had variable responses to elevated temperatures following inoculation with strain Guy11. Guy11 on Kitake exposed to 37℃ for 24 h post-inoculation had an average rating of 2.75±1.88, compared to the average rating of 0.23±0.42 in the control, leading to a statistically significant increase in infection with a $p<0.01$. Guy11 on LTH exposed to 37℃ for 24 h post-inoculation had an average rating of 2.33±1.97, compared to the average rating of 5.45±0.5 in the control, leading to a statistically significant decrease in infection with a $p<0.01$. Guy11 on ToRide exposed to 37℃ for 24 h post-inoculation had an average rating of 5.09±1.08, compared to the average rating of 2.38±2.34 in the control, leading to a statistically significant increase in infection with a $p<0.01$. Guy11 on Yt14 exposed to 37℃ for 24 h post-inoculation had an average rating of 1.43±2.03, compared to the average rating of 3.6±1.93 in the control, leading to a statistically significant decrease in infection with a $p<0.01$. These results support an influence of heat at the level of recognition as each cultivar did not display a generalized suppression of host resistance to Guy11. Kitake and ToRide displayed significant increases in infection and LTH and Yt14 displayed significant decreases in infection. All of these results are displayed in Figure 6-2.

Different rice cultivars had variable responses to elevated temperatures following inoculation with strain ML33. ML33 on CO39 exposed to 37℃ for 24 h post-inoculation had an average rating of 8±0 compared to the average rating of 6.17±0.69 in the control, leading to a statistically significant increase in infection with a $p<0.01$. ML33 on Kitake exposed to 37℃ for 24 h post-inoculation had an average rating of 2.31±2.52, compared to the average rating of 0.33±1.25 in the control, leading to a statistically significant increase in infection with a $p<0.05$. ML33 on Yt16 exposed to 37℃ for 24 h post-

inoculation had an average rating of 5.5±1.8, compared to the average rating of 7.25±0.83 in the control, leading to a statistically significant decrease in infection with a $p<0.05$. These results support an influence of heat at the level of recognition as each cultivar did not display a generalized suppression of host resistance to ML33. CO39 and Kitake displayed significant increases in infection and Yt16 displayed significant decreases in infection. All of these results are displayed in Figure 6-3.

6.3.2 Stability of reaction after 24 h of 37°C post-inoculation on specific strain/cultivar pairings

This experiment aimed to establish the stability of the reaction displayed by specific strain/cultivar parings after exposure to 24 h of 37°C post-inoculation. The previous experiments supported the hypothesis that post-inoculation exposure to elevated temperatures has influences at the level of recognition. In order to determine the validity of these results, inoculations were performed to a replication of ten, with strain and cultivar pairings that displayed a trend of increased or decreased disease severity. The expected results were that each strain and cultivar pairing would display a consistent decrease or increase in disease severity after treatment, similar to the previously observed experimental results. The results displayed a stable reaction to treatment for some of the cultivar and fungal pairings, while others displayed extreme variability. This indicated that the influence of heat while acting at the level of recognition also acts quantitatively.

The particular strain and cultivar pairings were selected for these inoculations due to a reaction of increased or decreased disease severity after treatment from preliminary experiments described above. FR13 on CO39 exposed to 37°C for 24 h post-inoculation had a statistically significant increase in infection with a $p<0.01$ for all ten replications. The combined average rating for FR13 on CO39 was 3.99±0.81, compared to the average

rating of 1.44±1.17 in the control. All of these results are displayed in Figure 6-4. Guy11 on LTH exposed to 37℃ for 24 h post-inoculation had a statistically significant decrease in infection with a $p<0.01$ for all ten replications. The combined average rating for Guy11 on LTH was 1.14±1.44, compared to the average rating of 5.47±0.48 in the control. All of these results are displayed in Figure 6-5. ML33 on 51583 exposed to 37℃ for 24 h post-inoculation displayed a statistically significant increase in infection with a $p<0.01$ for two replications and one statistically significant decrease in infection with a $p<0.01$. The averages of ML33 on 51583 could not be combined, as different trends were displayed in different replications. All of these results are displayed in Figure 6-6.

These results support the influence of heat on a quantitative factor involved with recognition. The trend of decreased disease severity with heat indicated that host resistance, or R gene recognition, was not simply being suppressed. General suppression would display either increased disease severity or no change depending on the R genes present. This was not the case for LTH plants inoculated the Guy11 and exposed to treatment.

6.3.2 Influence of post-inoculation 37℃ exposure on fungal strains with a known AVR gene

This experiment aimed to determine if a known AVR gene could be influenced by elevated temperature and if increasing the exposure time would intensify the reaction. This reaction would be the increase or decrease in disease severity after treatment. Guy11 and ML33 had previously been utilized for temperature exposure inoculations with rice cultivar CO39. Guy11 displayed decreased disease severity while ML33 displayed increased disease severity. Neither strain possesses *AVR1-CO39*. *AVR1-CO39* is a major AVR gene proposed to interact directly with plant R proteins including *Pi-CO39*

(Chauhan et al., 2002); thus, it was chosen for these inoculations. Any change in infection phenotype after treatment would be obvious, compared to the completely resistant controls. If the 24 h of 37°C post-inoculation exposure did influence *AVR1-CO39,* the expected results would be an increase in disease severity on the treatment plants compared to the controls.

The results of this experiment displayed increased disease severity in the treatment plants for each exposure time inoculated with fungal strains transformed with *AVR1-CO39* compared to the control. However, none of these differences in severity were statistically significant, nor did any of the treatment plants display a virulent reaction. This would indicate that while post-inoculation exposure to elevated temperatures may influence the *AVR1-CO39/Pi-CO39* interaction, the effect lacks the intensity necessary to overcome the direct recognition conferred by *Pi-CO39.*

The combined average rating across five replicates for Guy11 on CO39 with a 37°C exposure for 24 h was 1.75±0.67, compared to the average rating of 1.78±0.1 in the control. The combined average rating across five replicates for Guy11 on CO39 with a 37°C exposure for 48 h was 1.04±1.13, compared to the average rating of 1.87±0.7 in the control. The combined average rating across five replicates for Guy11 on CO39 with a 37°C exposure for 7 d was 0.17±0.2, compared to the average rating of 1.81±0.39 in the control, leading to a statistically significant decrease in infection with a $p<0.01$. Guy11 was included due to the stable reaction of decreased disease severity seen with this strain on CO39. Guy11 does not possess *AVR1-CO39.*

The combined average rating across five replicates for ML33 on CO39 with a 37°C exposure for 24 h was 6.04±0.5, compared to the average rating of 5.7±0.53 in the

control. The combined average rating across five replicates for ML33 on CO39 with a 37℃ exposure for 48 h was 4.96±1.64, compared to the average rating of 4.72±0.35 in the control. The combined average rating across five replicates for ML33 on CO39 with a 37℃ exposure for 7 d was 4.35±0.44, compared to the average rating of 4.67±0.76 in the control. The combined average rating across five replicates for ML33$^{AVR1\text{-}CO39}$ on CO39 with a 37℃ exposure for 24 h was 1.29±0.61, compared to the average rating of 0.43±0.25 in the control. The combined average rating across five replicates for ML33$^{AVR1\text{-}CO39}$ on CO39 with a 37℃ exposure for 48 h was 0.47±0.23, compared to the average rating of 0.31±0.71 in the control. The combined average rating across five replicates for ML33$^{AVR1\text{-}CO39}$ on CO39 with a 37℃ exposure for 7 d was 0.31±0.39, compared to the average rating of 0.28±0.53 in the control. All of these results are displayed in Figure 6-7. ML33 does not possess *AVR1-CO39* in the wild-type. The decrease in disease severity of the treatment plants inoculated with Guy11 indicated that post-inoculation heat treatment did not simply suppress host recognition in CO39. In contrast, the increase in disease severity of the *AVR1-CO39* transformants indicated a heat influence at the level of recognition specific to the AVR/R interaction.

6.4 Discussion

The experiments described in this chapter were performed to determine if post-inoculation exposure to elevated temperatures suppressed general host recognition or functioned at the recognition level. Different fungal strains inoculated on multiple rice cultivars resulted in distinctive reactions specific to the pairing when treated with a post-inoculation exposure to 37℃ for 24 h. FR13, Guy11, and ML33 inoculated on eleven rice cultivars did not display a generalized suppression of host resistance. After infection and

exposure to the treatment, each fungal strain displayed at least one instance of increased disease severity on one cultivar and decreased disease severity on another. Similarly, after infection and exposure to the treatment, each cultivar displayed at least one instance of increased disease susceptibility from a fungal strain and decreased disease susceptibility from another fungal strain, except for ToRide and Kitake. Rice cultivar LTH displayed a stable and significant decrease in disease severity after inoculation with Guy11 and exposure to treatment. Alternatively, rice cultivar CO39 displayed a stable and significant increase in disease severity after inoculation with FR13 and exposure to treatment. These results indicated that post-inoculation heat exposure was influencing at the recognition level instead of general host resistance.

The suppression of R genes due to elevated heat is well-established in the literature (MacQueen & Bergelson, 2016; Negeri et al., 2013; Zhu et al., 2010). Suppression of R genes could lead to increased disease severity on hosts if the R gene had conveyed resistance due to an interaction with the infecting pathogen (Venkatesh & Kang, 2019). Alternatively, if the R gene could not convey resistance initially, suppression of R genes would lead to no change in disease severity (Zheng et al., 2021; Zhu et al., 2010). The results described in this chapter include trends of decreased disease severity on hosts after exposure to elevated temperatures. This would be unlikely to occur if the R genes were simply suppressed by elevated temperatures (Richard et al., 2020). Fungal recovery culture studies also determined that the fungus was not heat-killed at 37°C (Appendix 5). The results described in this chapter indicate a nuanced temperature influence on the plant-microbe interaction that isolates the optimal performance temperatures for R genes, AVR genes, or both.

R genes possessing different optimal temperatures for functionality have been described, and elevated temperatures have been shown to benefit certain R genes in the rice bacterial blight pathosystem (Webb et al., 2010). Research has been heavily focused on the influences of temperature on R gene function and not the pathogen aspects of the interaction, despite some studies that suggest higher temperatures cause differential effector production in bacterial and fungal systems (Cheng et al., 2013; Meyer et al., 2017; Tao et al., 2020). *M. oryzae* effector expression and secretion is thought to be extremely specific for the infection stages of the fungus and critical for successful host proliferation (Cao et al., 2022; Sharpee et al., 2017; Sornkom et al., 2017). Effectors are known to have specific secretion timings and concentrations (de Wit et al., 2009; Lo Presti et al., 2015; Stergiopoulos & De Wit, 2009). Changes to the expression or timing of effectors due to temperature modulation could result in increased or decreased disease severity depending on the recognition factors in the host and the properties of the effectors. Modulation of both the R genes and AVR genes with temperature could explain the variable reactions, such as ML33 on 51583 described in this chapter. Influencing the expression of effectors or binding of R proteins with elevated temperatures post-inoculation would result in a more quantitative change in infection phenotype that would be variable depending on the strain, cultivar, or even fungal individuals involved in the interaction.

Based on the results in this chapter, it is reasonable to suggest that blast epidemics during a growing season may not simply be due to the fungus overcoming resistance. The plant-microbe interaction has been well-documented to involve environmental factors (Jones & Dangl, 2006). In times of high stress, plants are more

susceptible to infection (Desaint et al., 2021; Janda et al., 2019; Kumar & Verma, 2018; Xiong & Yang, 2003). Although rice plants are drought tolerant, long periods of heat and drought will cause plant decline (Arun K et al., 2020). There is a potential for an increase in severe blast epidemics with rising temperatures.

6.5 Conclusions

The experiments described in this chapter support the influence of brief exposure to higher temperature post-inoculation acting at the level of recognition. Fungal strains Guy11, ML33, and FR13 displayed increased disease severity on at least one rice cultivar and decreased disease severity on at least one other rice cultivar after treatment. Concurrently, rice cultivars 51583, CO39, IRBL9-w, KATY, LTH, Nipponbare, TP-309, Yt14, and Yt16 displayed increased disease severity for at least one fungal strain and decreased disease severity for at least one other fungal strain after treatment. These results indicated that brief exposure to higher temperatures influenced the specific interaction between a strain and cultivar. These results were further validated by replication of inoculations on six cultivars paired with the three fungal strains, which highlighted the effect of the temperature influence. Further inoculations involving fungal strains with the addition of *AVR1-CO39* displayed an influence of brief heat treatment post-inoculation on the AVR gene, but not enough to be significant. Future work should be performed to understand the underlying molecular mechanisms involved with the treatment of 37℃ for 24 h post-inoculation. AVR genes, in addition to *AVR1-CO39*, should also be studied cytologically to determine specific heat influences on the secretion timing and sequestration concentration.

Table 6-1: Magnaporthe stocks and strains utilized for experiments

Isolate Name	Host	Year	Type	Place of Isolation	Source
2539 HL	Oryza sativa	1988	Wild-Type	Laboratory Strain	H. Leung, bred strain
ML33	Oryza sativa	1986	Wild-Type	Mali	D. Tharreau, field isolation
Guy11	Oryza sativa	1979	Wild-Type	French Guyana	J. Notteghem, field isolation
SSID116	Oryza sativa	2013	Wild-Type	United States	Y. Jia, field isolation
FR13	Oryza sativa	1988	Wild-Type	France	D. Tharreau, field isolation
ML33-2$^{AVR1\text{-}CO39}$	Oryza sativa	2006	AVR Gene	Laboratory Strain	R. Peyyala, transformant of ML33 with AVR1-CO39

Table 6-2: Rice Cultivars Utilized for Experiments

Cultivar Name	Type	Year	Origin	Known R genes	Reference Source
51583	Indica		USSR		(Leung, et. al, 1988)
CO39	Indica		India	Pi-CO39, Pi-a	(Miah, et al., 2013), (Bryan, et al., 2000), (Tsunematsu, et al., 2000)
M202	Japonica	1985	United States		(Costanzo and Jia, 2010), (Wang, et al., 2007)
Lijiangxintuanheigu (LTH)	Japonica	2001	China		(Tsunematsu, et al., 2000)
Yt14				Pi-ta	(Valent, et al., 2000)
Yt16				Pt-r	(Bryan, et al., 2000), (Zhao, et al., 2018)
Taipei 309 (TP-309)	Japonica			Pi-54	(Singh, et al., 2020)
ToRide				Pi-zt, Pi-9, Pi-sh, Piz-5	(Miah, et al., 2013), (Tsunematsu, et al., 2000), (Tacconi, et al., 2010)
Nipponbare	Japonica		Japan	Pi-t, Pi-sh	(Hayashi, et al., 2010), (Costanzo and Jia, 2010), (Wang, et al., 2007), (Sharma, et al., 2012)
Kitake	Japonica	1997	Japan	Pi-km	(Costanzo and Jia, 2010)
KATY	Japonica	1989	United States	Pi-km, Pi-ta, Pi-ta2, Pt-r	(Costanzo and Jia, 2010), (Meng, et al., 2020)
IRBL9-w	Japonica	2000	Philippines	Pi-9	(Singh, et al., 2015), (Tsunematsu, et al., 2000)

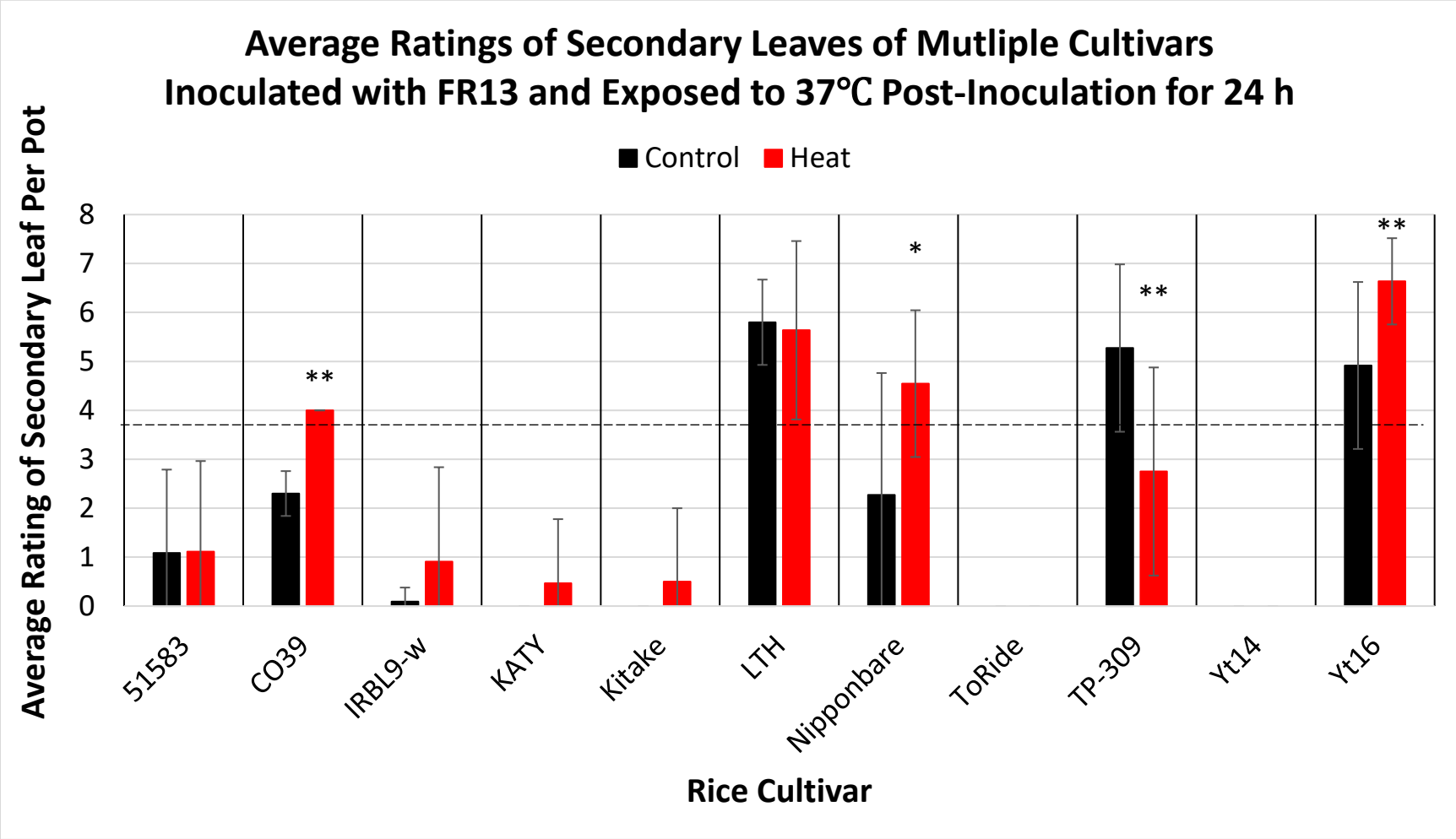


Figure 6-1 Average rating of secondary leaves of multiple cultivars inoculated with FR13 and exposed to 37℃ post-inoculation Ratings of 4 and above are categorized as virulent while ratings below 4 are categorized as avirulent. One * denote $p<0.05$ and two ** denote $p<0.01$. Each bar represents an approximate n=15.

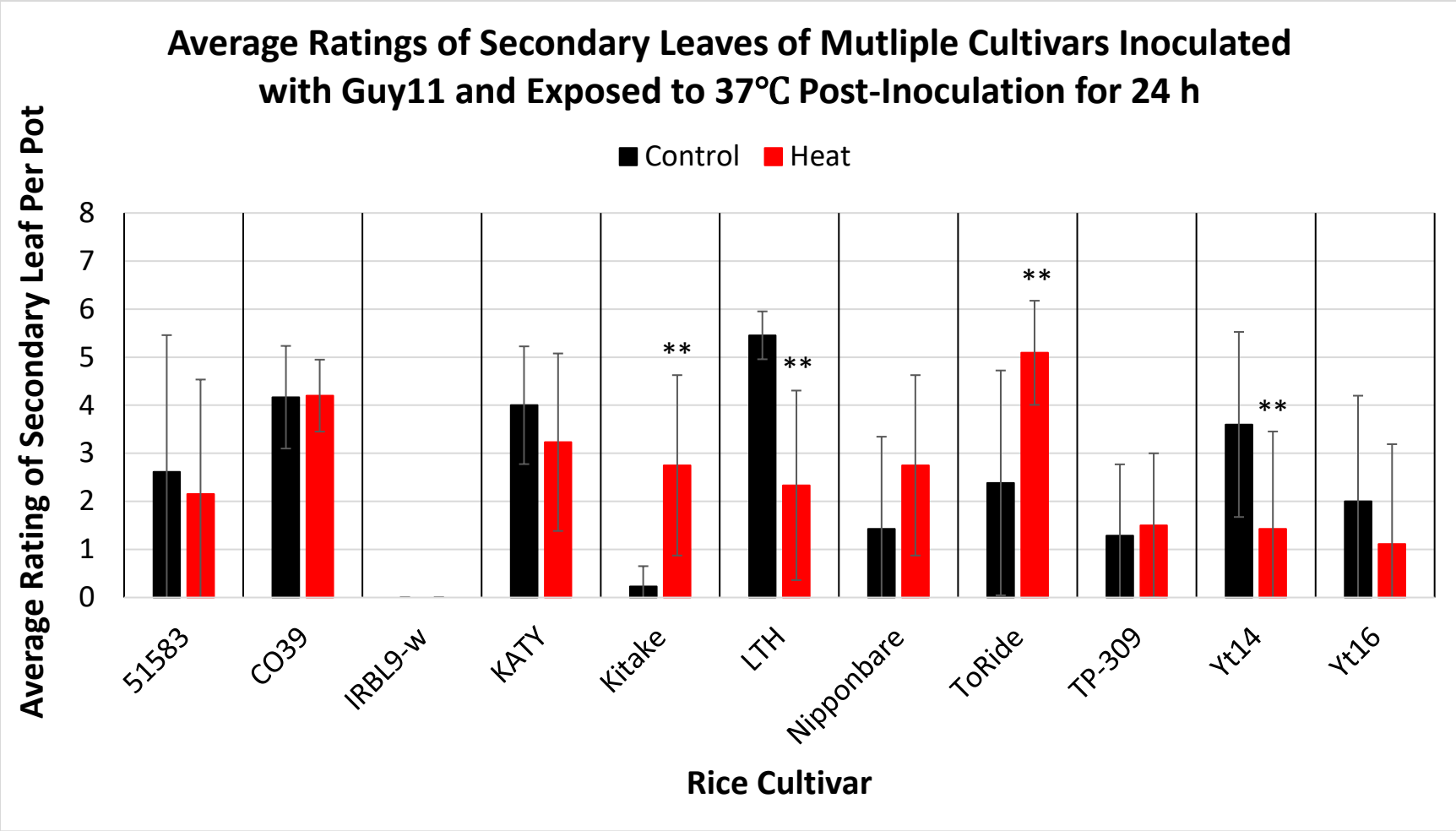


Figure 6-2 Average rating of secondary leaves of multiple cultivars inoculated with Guy11 and exposed to 37℃ post-inoculation Ratings of 4 and above are categorized as virulent while ratings below 4 are categorized as avirulent. One * denote $p<0.05$ and two ** denote $p<0.01$. Each bar represents an approximate n=15.

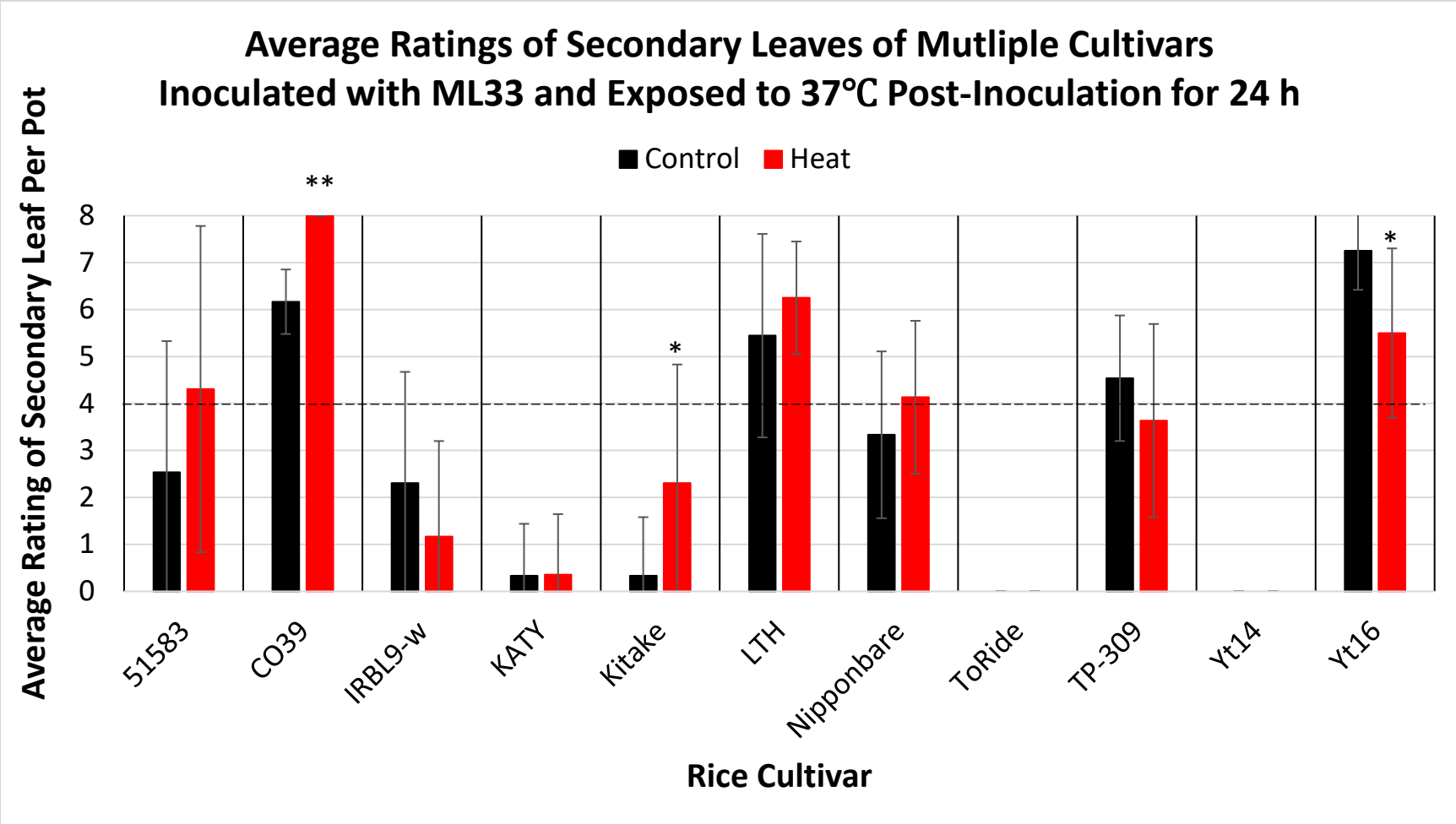


Figure 6-3 Average rating of secondary leaves of multiple cultivars inoculated with ML33 and exposed to 37℃ post-inoculation Ratings of 4 and above are categorized as virulent while ratings below 4 are categorized as avirulent. One * denote $p<0.05$ and two ** denote $p<0.01$. Each bar represents an approximate n=15.

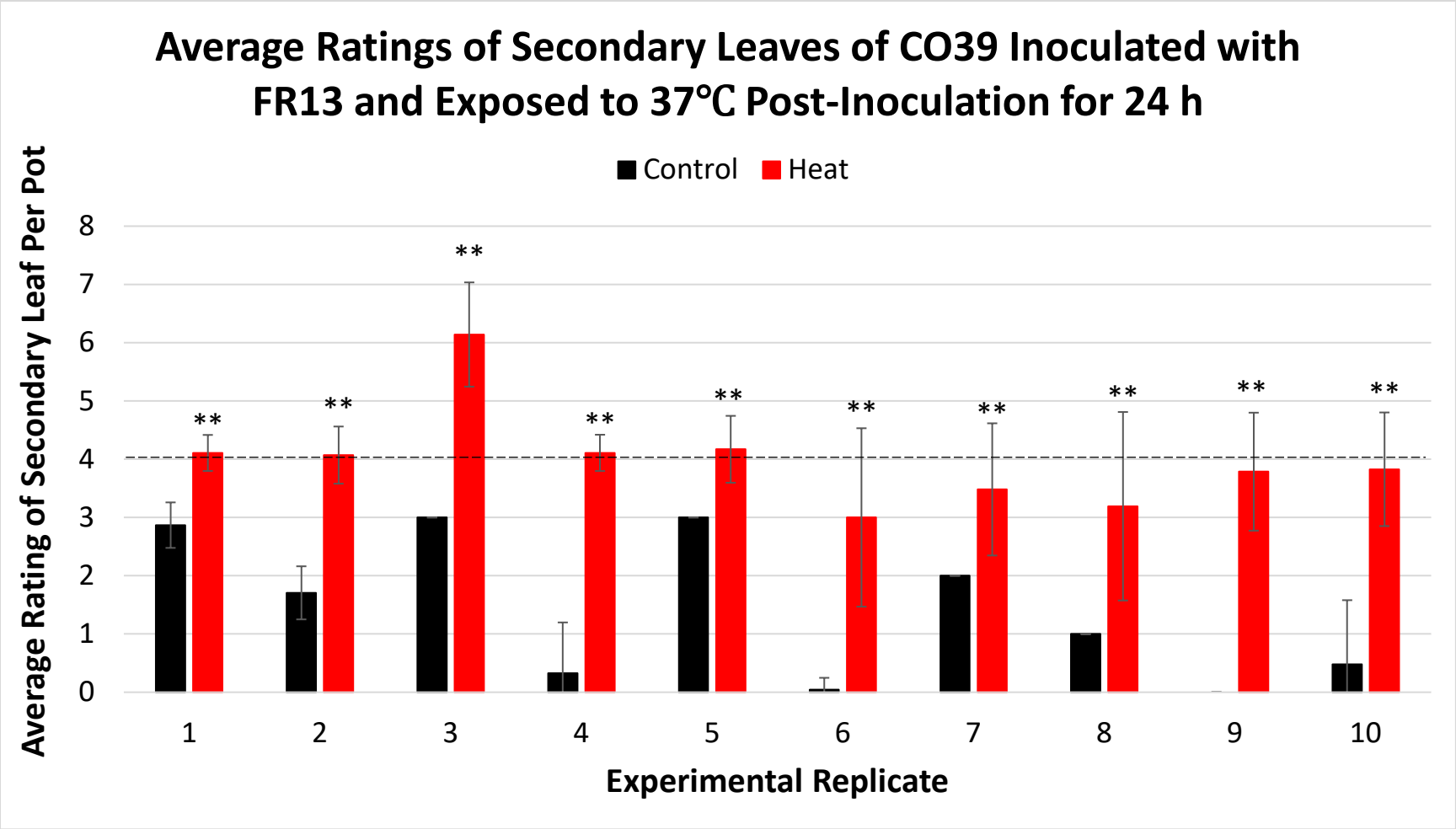


Figure 6-4 Average rating of secondary leaves of CO39 inoculated with FR13 and exposed to 37°C post-inoculation Ratings of 4 and above are categorized as virulent while ratings below 4 are categorized as avirulent. One * denote $p<0.05$ and two ** denote $p<0.01$. Each bar represents an approximate n=60.

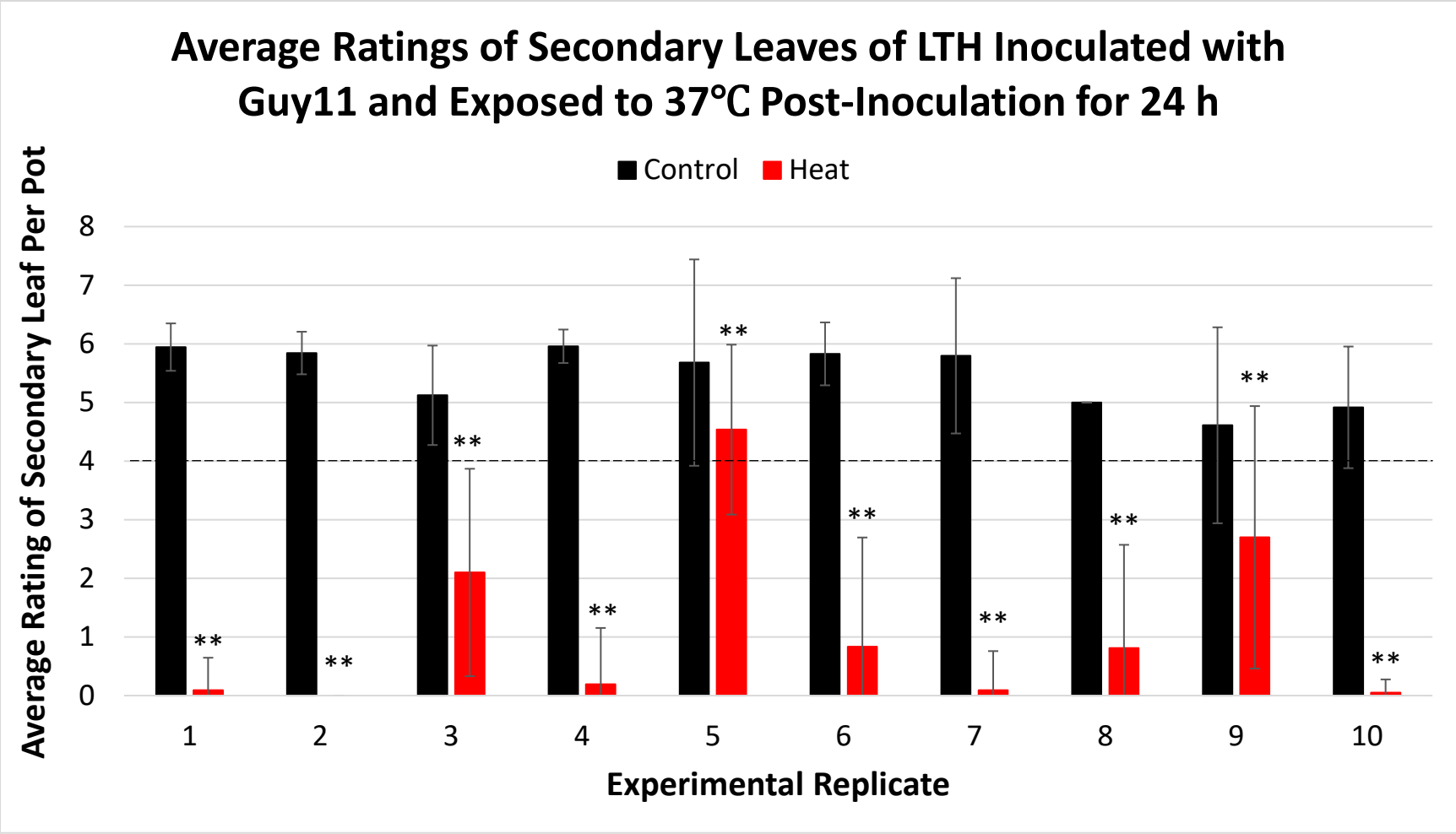


Figure 6-5 Average rating of secondary leaves of LTH inoculated with Guy11 and exposed to 37℃ post-inoculation Ratings of 4 and above are categorized as virulent while ratings below 4 are categorized as avirulent. One * denote $p<0.05$ and two ** denote $p<0.01$. Each bar represents an approximate n=60.

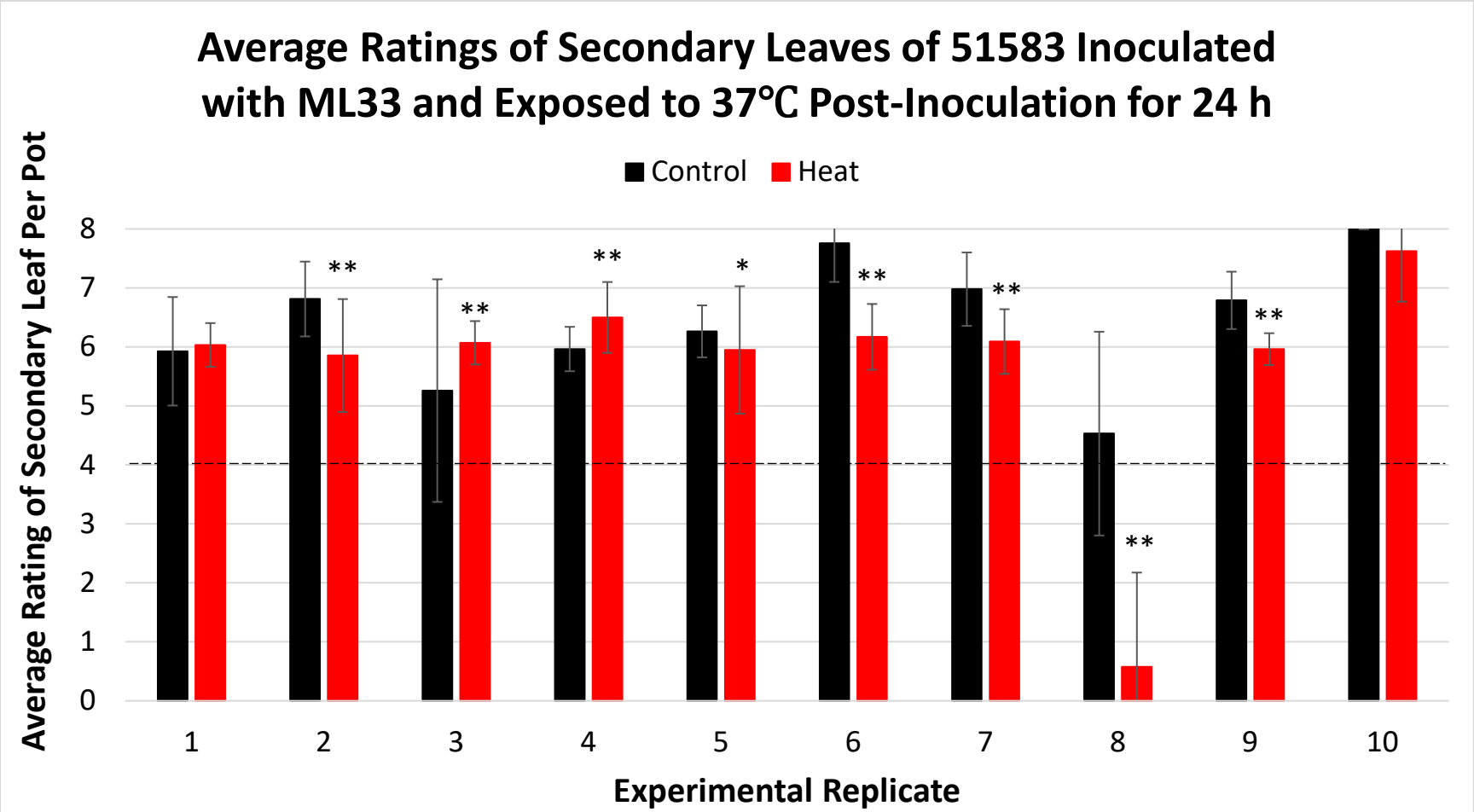


Figure 6-6 Average rating of secondary leaves of 51583 inoculated with ML33 and exposed to 37°C post-inoculation Ratings of 4 and above are categorized as virulent while ratings below 4 are categorized as avirulent. One * denote $p<0.05$ and two ** denote $p<0.01$. Each bar represents an approximate n=60.

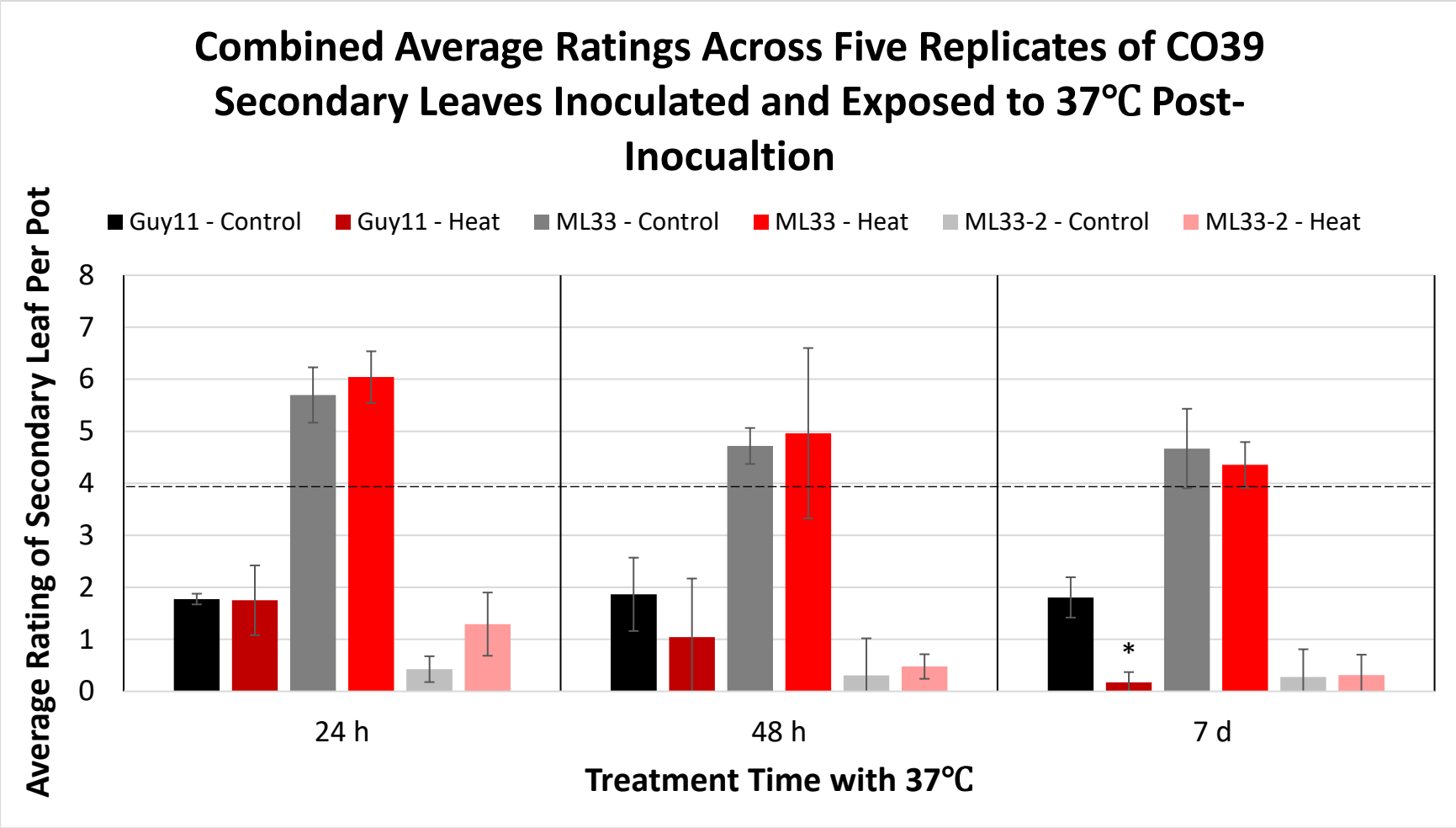


Figure 6-7 Combined average rating across five replicates of CO39 secondary leaves inoculated and exposed to 37℃ post-inoculation Ratings of 4 and above are categorized as virulent while ratings below 4 are categorized as avirulent. One * denote $p<0.05$ and two ** denote $p<0.01$. Each replicate had an approximate n=30.

CHAPTER 7: CLONAL VARIATION IN FUNGAL STOCKS REPRESENTED BY IN VIVO PHENOTYPES

7. 1 Background

Asexual reproduction in fungi is thought to result in clonal progeny that are genetically identical to the parent (Taylor et al., 2015). Studies have shown that clonal lineages of fungi can have differences in genotypic and phenotypic traits (Talbot et al., 1993). This would indicate that fungal individuals can differ even when they are asexual progeny from the same parent. Clonal variation of individuals is well described in yeast systems and accounts for the ability of different individuals to overcome stressors (Kosheleva & Desai, 2018; Lang et al., 2013). Fungal cultures with clonal variation could lead to the selection of individuals with differing phenotypic traits. These individuals may give the appearance of a change in phenotype or interaction of the strain or species. Fungal individuals with differing levels of effector expression could create variable infection patterns and irreproducibility between replications (Meyer et al., 2017; Phan et al., 2016).

The experiments described in this chapter aimed to explore the possibility that clonal variation among supposedly genetically identical fungal paper stocks was a confounding variable leading to irreproducibility between experiments. Factors of light exposure, temperature variability, age of plants, and time of day at inoculation have already been discussed and rejected as the source of irreproducibility between experiments. The only notable variable between experiments that had not been explored was the different fungal paper stocks used to activate fungal strains for inoculations. Fungal paper stocks were created from single-source fungal cultures grown from a single-

spore and were theoretically genetically identical individuals. However, the potential presence of clonal variation in the fungal cultures suggested that paper stocks made from certain regions of the fungal culture could be genetically or phenotypically distinct from others. This would introduce variability in inoculation replications when a different fungal stock was utilized.

7.2 Materials and Methods

7.2.1 Plant inoculation utilizing single-source fungal paper stocks

A single germinated spore was isolated and placed in the center of an oatmeal agar plate. Ten sterile paper disks were placed at equal distances from the germling and from one another. The cultures were grown at 25°C under continuous illumination for 14 d. The paper disks were then removed and transferred to individual oatmeal agar plates, resulting in ten separate fungal stocks from a single-spore culture. These ten stocks were then used individually as the inoculum for each group of plants in a pot (Figure 7-1). The procedure matched the plant inoculation protocol listed in Chapter 2. This experiment was repeated four times, maintaining the same preparation protocols and timings for each inoculation. Stocks utilized in the experiments are listed in Table 7-1. Cultivars utilized in the experiments are listed in Table 7-2.

7.2.2 Ratings and statistical analysis

Disease ratings for secondary leaves were made using the scale shown in Figure 3-1. Ratings were used to determine the severity of the disease. Ratings 0-3 represent resistance levels on the host (avirulent), while ratings 4-8 represent levels of susceptibility on the host (virulent). Ratings of 4 and above were categorized as virulent, while ratings below four were categorized as avirulent. Statistical analysis was performed

using an unpaired, unequal variance t-test. Analysis of variance was performed for the replications of each stock to ensure that data could be combined.

7.2.3 Leaf sheath assays

Fungal cultures were activated from frozen stocks by placing fungal paper disks on oatmeal agar supplemented with 100 µg/ml of ampicillin (Goldbiotechnology®). Cultures were then grown at 25℃ under continuous illumination. After 14 d, the plates were flooded with 2 ml of a 0.25% gelatin suspension in water. The colony's surface was massaged with a sterilized bacterial cell spreader to liberate the conidia. The solution was filtered through Miracloth, and the spores were quantified using a hemocytometer. Concentrations were adjusted to 10^5/ml using sterile water.

Plants grown in the greenhouse for approximately two months were cut at the base of the panicle, and the top leaves were removed. The individual sheaths were then peeled away, keeping the third or fourth oldest sheaths based on the amount of free space on the inside of the sheath. The top taper of the sheath and bottom fibrous areas were removed, creating sheath sections about 3 cm long. After mixing the spore solution, 100 µl was injected into the free space inside each sheath. The sheaths were placed on top of overturned PCR (Thermo Fisher Scientific©) tubes in Petri plates with wet paper towels to keep the sheath level. The sheaths were placed so that the spore suspension would rest on the epidermal layer of cells. The Petri plates were sealed with parafilm and placed in a dark incubator at 21℃. The sheaths were incubated for 72 h, removed from the petri plate, and rinsed with sterile water. A scalpel with a number 11 blade was used to

trim the sheath so that only a 1-2 cell layer would be left of the epidermal layer at an approximate length of 2 cm. The sheath sections were placed on glass slides in sterile water with a coverslip.

Sheaths with transformed fungal strains expressing fluorescence were imaged on an Olympus FV3000 confocal at 20X. Pictures were taken by manually scanning across the sheath. Sheaths with untransformed fungal strains were imaged on Zeiss Specs bright field at 10X. Pictures were taken by manually scanning across the sheath. Counts were taken of five different infection factors on the sheaths: appressoria, single HR, multiple HR, single infected cells, and multiple infected cells. Factors were not double counted. Single, isolated infected cells were counted separately from a group of infected cells. HR cells were not counted as infected cells.

7.2.2 Statistical analysis

Infection factors for each stock were individually analyzed using an unpaired, unequal variance t-test. Analysis of variance was performed for the replications of each stock to ensure that data could be combined. Average means and standard deviations were determined using Excel. The t-tests and analysis of variance were performed in Excel.

7.3 Results

7.3.1 Plant inoculations utilizing single-source fungal paper stocks

This experiment aimed to determine if the specific fungal paper stocks utilized in each inoculation influenced the irreproducibility observed between experiments. Fungal paper stocks were created from one fungal culture grown from a single-spore. Theoretically, each paper stock taken from a single-source fungal culture plate should be clonal and behave similarly. However, if the fungal culture plate were to consist of

variable fungal individuals, the paper stocks made from the plate would also be variable and could behave differently on the host plants. If the paper stocks represented different fungal individuals, the expected result from this experiment would be that each stock could display distinct levels of disease severity on hosts. The results of this experiment supported a variation in the fungal populations on individual paper stocks taken from a single-source culture.

Ten different FR13 stocks were created from a single-spore on one agar plate. FR13-1 had an average rating of 6.5±0.49 across the four replications on LTH. FR13-2 had an average rating of 6.29±0.63 across the four replications on LTH. FR13-3 had an average rating of 6.5±0.21 across the four replications on LTH. FR13-4 had an average rating of 6.29±0.48 across the four replications on LTH. FR13-5 had an average rating of 6.63±0.45 across the four replications on LTH. FR13-6 had an average rating of 6.8±0.09 across the four replications on LTH. FR13-7 had an average rating of 6.17±0.53 across the four replications on LTH. FR13-8 had an average rating of 7.2±0.70 across the four replications on LTH. FR13-9 had an average rating of 7±0.48 across the four replications on LTH. FR13-10 had an average rating of 7.13±1 across the four replications on LTH. All of these results are displayed in Figure 7-2. These results indicate that the FR13 paper stocks do not display statistically different disease severity on LTH. This suggests that the FR13 paper stocks behave as one individual with minimal variability among the stocks.

Ten different ML33 stocks were created from a single-spore on one agar plate. ML33-1 had an average rating of 1.98±1.57 across the four replications on 51583. ML33-2 had an average rating of 1.37±0.84 across the four replications on 51583. ML33-3 had an average rating of 2.3±1.32 across the four replications on 51583. ML33-4 had an

average rating of 3.28±1.39 across the four replications on 51583. ML33-5 had an average rating of 3.5±0.62 across the four replications on 51583. ML33-6 had an average rating of 3.96±0.5 across the four replications on 51583. ML33-7 had an average rating of 3.48±0.43 across the four replications on 51583. ML33-8 had an average rating of 4.1±0.46 across the four replications on 51583. ML33-9 had an average rating of 3.91±0.83 across the four replications on 51583. ML33-10 had an average rating of 3.65±0.78 across the four replications on 51583. ML33-2 has a statistically significant difference in average rating compared to stocks ML33-4 through ML33-9 to a $p<0.01$ and stocks ML33-3 and ML33-9 to a $p<0.05$. All of these results are displayed in Figure 7-3. These results indicate that while most ML33 paper stocks behave as a single individual with slight variability between stocks, ML33-2 is an outlier. One paper stock from a single-source culture displayed significantly different disease severity on 51583 compared to nine other paper stocks made from the same plate.

7.3.2 Leaf sheath assays utilizing single-source fungal paper stocks

This experiment aimed to determine whether the differences in fungal paper stock infection discussed above would be reflected microscopically in leaf sheath cells. The previous experiment supported the rare occurrence of a fungal paper stock behaving differently than all other stocks from the same single-source culture. Counts were taken of five different infection factors on the sheaths: appressoria, single HR, multiple HR, single infected cells, and multiple infected cells. If each fungal paper stock produced an inoculum of variable spores, the expected results from this experiment would be variable infection factors at each infection site in rice cells. Differences in the number of factors between each stock would also be expected. The results indicated that the fungal paper

stocks taken from a single-source culture produced variable spores that resulted in significant differences between fungal stocks for each infection factor. Additionally, there was high variability for a single stock inoculated on sheaths from different plants, indicating clonal variation in spores from the same stock. Alternatively, this could indicate variation in the resistance of individual host cells.

FR13-1 had an average count of 26.36±18.66 appressoria, 8.62±4.27 single HR cells, 26.89±16.05 multiple HR cells, 3.05±1.81 single infected cells, and 22.23±15.42 multiple infected cells. FR13-5 had an average count of 0.43±0.49 appressoria, 0.67±0.36 single HR cells, 0.3±0.32 multiple HR cells, 0.32±0.19 single infected cells, and 0.76±0.91 multiple infected cells. FR13-9 had an average count of 17.81±21.44 appressoria, 4.24±3.36 single HR cells, 24.05±30.53 multiple HR cells, 1.01±1.01 single infected cells, and 34.9±46.17 multiple infected cells. All of the FR13-1 factor counts were significantly different compared to the FR13-5 counts to a $p<0.05$. The FR13-5 multiple of HR cells count was significantly different compared to the FR13-9 count to a $p<0.05$. All of these results are displayed in Figure 7-4. The difference in the average factor counts between the fungal paper stocks indicates that each paper stock has a variable infection, specifically from the spores formed from the paper stocks. The large standard deviations for some factor counts within a paper stock may indicate clonal variation from a single paper stock or that resistance in host cells is variable.

ML33-2 had an average count of 30.59±12.76 appressoria, 0.27±0.24 single HR cells, 1.66±2.71 multiple HR cells, 0.46±0.4 single infected cells, and 67.41±59.76 multiple infected cells. ML33-6 had an average count of 39.25±15.05 appressoria, 1.67±0.63 single HR cells, 8.17±5.66 multiple HR cells, 0.68±0.6 single infected cells,

and 30.51±18.12 multiple infected cells. ML33-10 had an average count of 70.12±33.62 appressoria, 5.38±6.54 single HR cells, 5.38±6.54 multiple HR cells, 0.9±0.62 single infected cells, and 73.17±60.54 multiple infected cells. The ML33-2 appressoria significantly differed from the ML33-10 counts to a $p<0.05$. The ML33-2 single HR cells and multiple HR cell counts were significantly different compared to the ML33-6 counts to a $p<0.05$. All of these results are displayed in Figure 7-5. The difference in the average factor counts between the fungal paper stocks indicates that each paper stock has a variable infection. The large standard deviations for some of the factor counts within a paper stock indicate clonal variation from a single paper stock or that resistance in host cells is variable.

7.4 Discussion

The experiments described in this chapter were performed to determine if the fungal paper stocks utilized were a source of irreproducibility between experiments. The FR13 fungal paper stock whole plant inoculations did not support the hypothesis that the paper stocks were a source of variability due to a lack of significant difference in disease severity on 51583 between paper stocks. The ML33 fungal paper stock whole plant inoculations did support the hypothesis that the paper stocks were a source of variability due to a significant difference in disease severity on 51583 between one of the paper stocks compared to the other paper stocks. This indicates that the use of paper stock in fungal inoculation could introduce variability in infection phenotypes. The FR13 fungal paper stock leaf sheaths supported clonal variation from a single paper stock or variable resistance in host cells due to significant differences in infection factor counts between the stocks. The ML33 fungal paper stock leaf sheaths supported clonal variation from a

single paper stock or variable resistance in host cells due to significant differences in infection factor counts between the stocks.

Storage of fungal stocks on dry filter paper at -20°C is an accepted method of preservation for *Magnaporthe spp.* as well as some other fungal genera (Gupta et al., 2020; Hiruma & Saijo, 2016; Jia, 2009). Using paper stocks in freezers to store fungi is generally thought to keep the fungus in stasis, thereby preventing mutations or changes to the fungal stock (Singh et al., 2018). In *M. oryzae,* paper stocks created from a single-source fungal culture are assumed to be clonal since the fungus, almost exclusively, reproduces asexually (Fong et al., 2000; Talbot, 2003). The results described in this chapter do not support the assumption that paper stocks taken from a single-source plate are clonal. Although rare, paper stocks from a fungal plate can behave differently than all other stocks made on that plate, as observed with ML33-2 on 51583. This would imply clonal variability among fungal individuals sourced from the same plate, resulting in a unique paper stock.

Effector expression in fungi and *M. oryzae,* specifically, is contingent upon the type of AVR genes within the genome of a particular strain and the timing of infection (Rafiqi et al., 2012; Selin et al., 2016; Wang et al., 2017; Wang et al., 2014). If there is clonal variability amongst fungal individuals taken from a single-source plate, the expression of effectors may differ between individuals. Phenotypic and genetic differences in subsequent clonal spores have been described in arbuscular mycorrhizal fungi (Ehinger et al., 2012). Differences in effector expression in spores have been observed in *M. oryzae* (Gong et al., 2015). If a region of fungal growth on a plate had a different effector expression and this region had a paper stock placed in it, the paper stock

would isolate the fungal individual with the differing effector expression. This could explain the apparent lesser ability of ML33-2 to infect 51583 compared to the other stocks. Clonal variation of spores would also explain the differences in individual infection factors noted in the leaf sheath assays.

An alternative or congruent hypothesis to explain the variability observed with the fungal paper stocks is mosaic host cells. Studies in mammalian and plant models have shown that the same cell types can have differing gene expression and phenotypes (Araújo et al., 2017; Elowitz et al., 2002; Smith & Grima, 2018). This stochastic gene expression can have major implications on the immune defense in the individual cells (Hagai et al., 2018). Expression of phytohormones within host cells may change depending on the age of the plant, time of day, and infection (Bai et al., 2017; Hadizadeh et al., 2022; Michael et al., 2008). It may be possible, though undocumented, that R gene expression is also stochastic in individual cells, leading to different levels of susceptibility across the same tissue type. This could explain the large variability in the paper stocks on different leaf sheaths. The stocks may be able to infect certain rice cells and not others due to R gene recognition or lack thereof. Depending on the cellular makeup of a particular rice leaf, this could result in an almost completely resistant reaction. This was displayed by the failure of FR13-5 to cause infection on leaf sheaths when whole plant inoculations had confirmed the ability of the stock to infect LTH.

7.5 Conclusions

The results described in this chapter support the presence of clonal variation in fungal paper stocks. Although rare, one stock out of multiple taken from a single plate can be phenotypically different from the other stocks. This difference can be observed in

the ability of the stocks to infect hosts. Leaf sheath assays also indicated that clonal variation of spores or variability of host cells could greatly influence the inconsistency between individual infection sites. Future work should focus on the effectors in individual spores produced from a single paper stock to determine if differences in expression occur. Additionally, future studies should also explore the variability between host cells and the potential resistance levels therein.

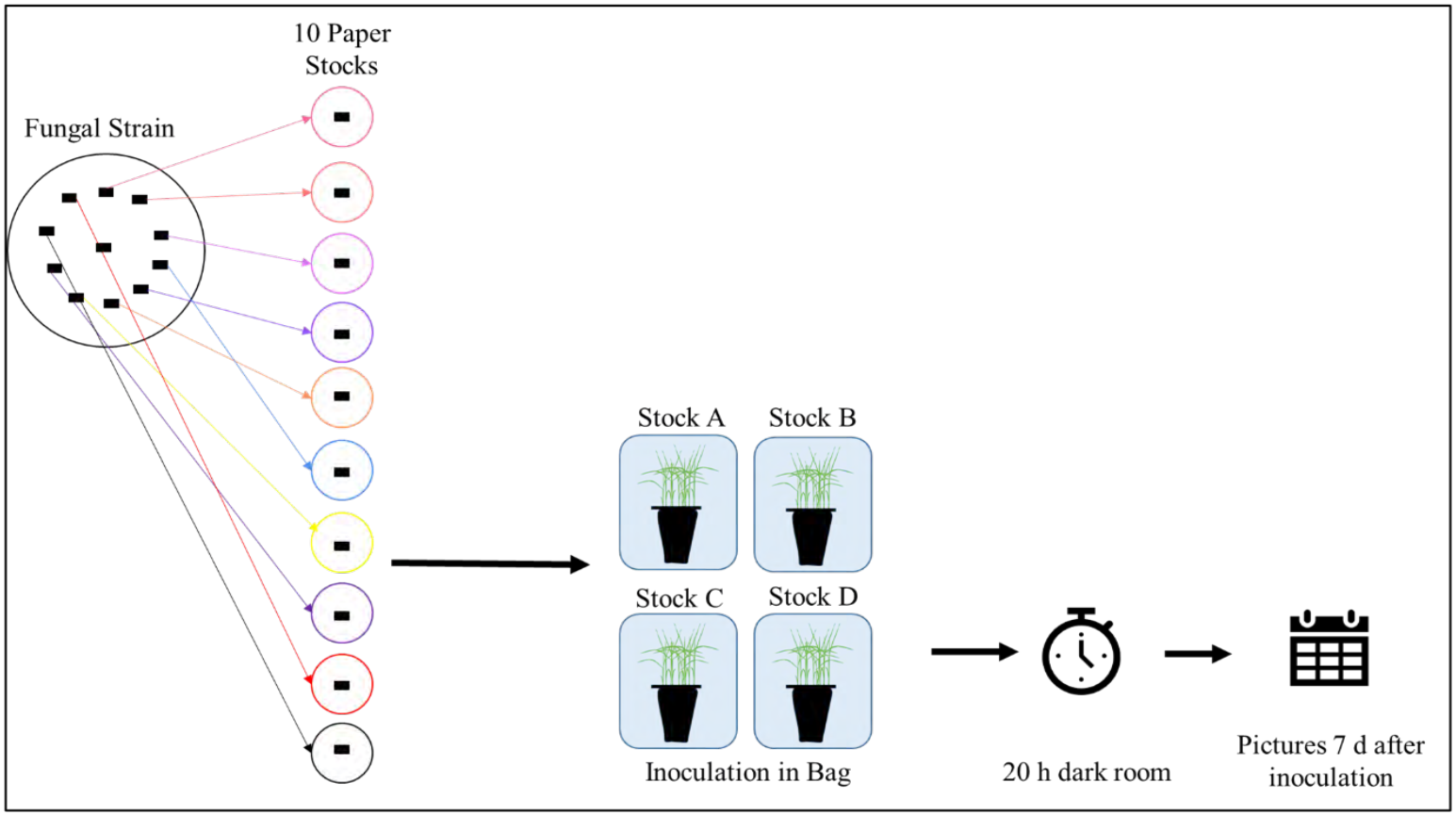


Figure 7-1 Schematic of Clonal Variation in Fungal Stocks Inoculations The figure depicts a simplification of the protocol followed for the Clonal Variation of Fungal Stocks Inoculations. One original paper stock placed in the center of a plate was used to create ten paper stocks for inoculations.

Table 7-1 Magnaporthe Strains and Stocks Utilized for Experiments

Isolate Name	Host	Year	Type	Place of Isolation	Source
FR13 1-10	Oryza sativa	2023	Wild-Type	Laboratory Strain	FR13 1st generation subcultures from a single spore
ML33 1-10	Oryza sativa	2023	Wild-Type	Laboratory Strain	ML33 1st generation subcultures from a single spore
FR13	Oryza sativa	1988	Wild-Type	France	D. Tharreau, field isolation
ML33	Oryza sativa	1986	Wild-Type	Mali	D. Tharreau, field isolation

Table 7-2 Rice Cultivars Utilized for Experiments

Cultivar Name	Type	Year	Origin	Known R genes	Reference Source
51583	Indica		USSR		(Leung, et. al, 1988)

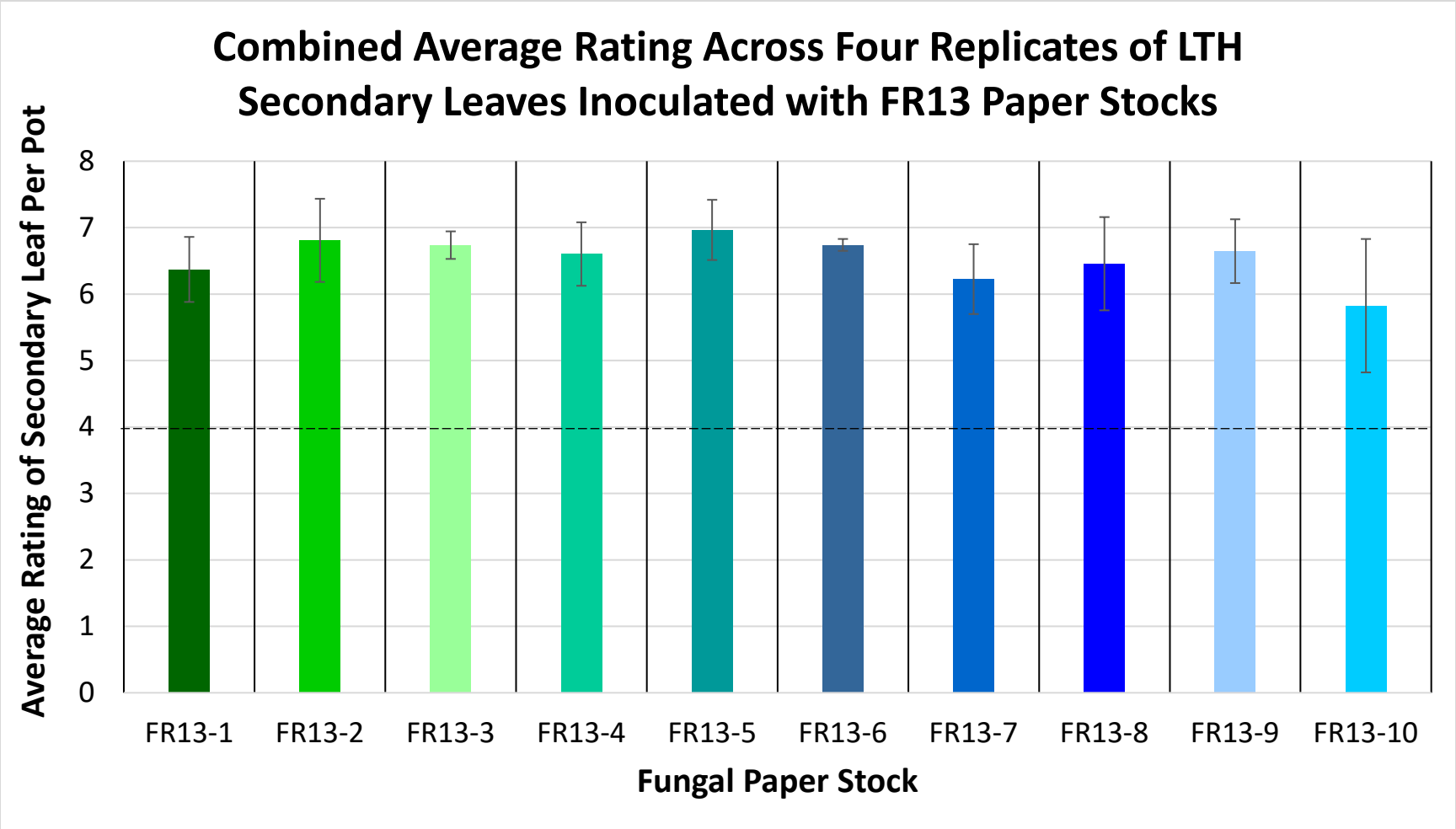


Figure 7-2 Combined Average Ratings Across Four Replicates of LTH Secondary Leaves Inoculated with FR13 Paper Stocks Ratings of 0-3 are categorized as avirulent and show levels of resistance. Ratings of 4-8 are categorized as virulent and shows levels of susceptibility. None of the compared stocks displayed statistical significance.

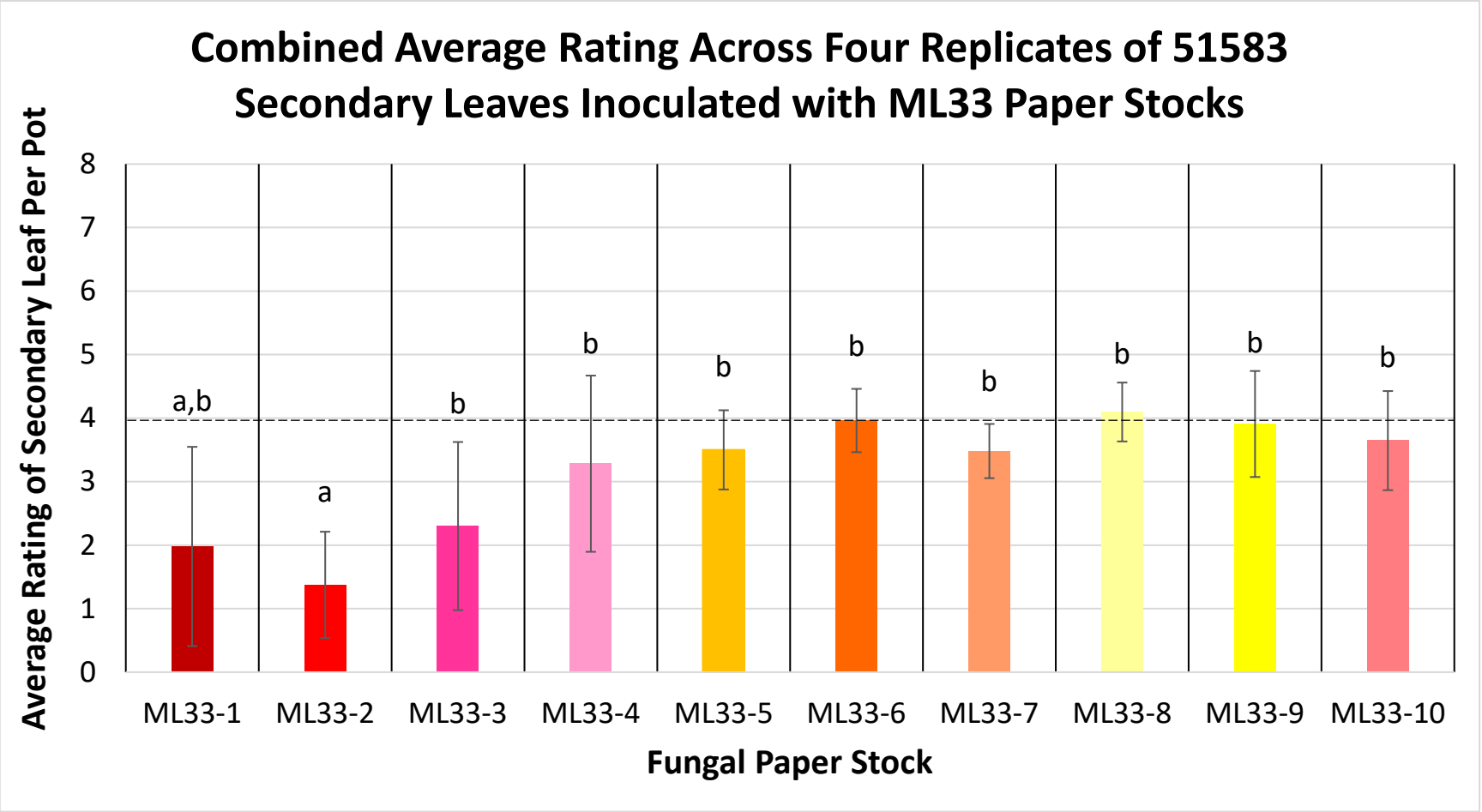


Figure 7-3 Combined Average Ratings Across Four Replicates of 51583 Secondary Leaves Inoculated with ML33 Paper Stocks Ratings of 0-3 are categorized as avirulent and show levels of resistance. Ratings of 4-8 are categorized as virulent and shows levels of susceptibility.

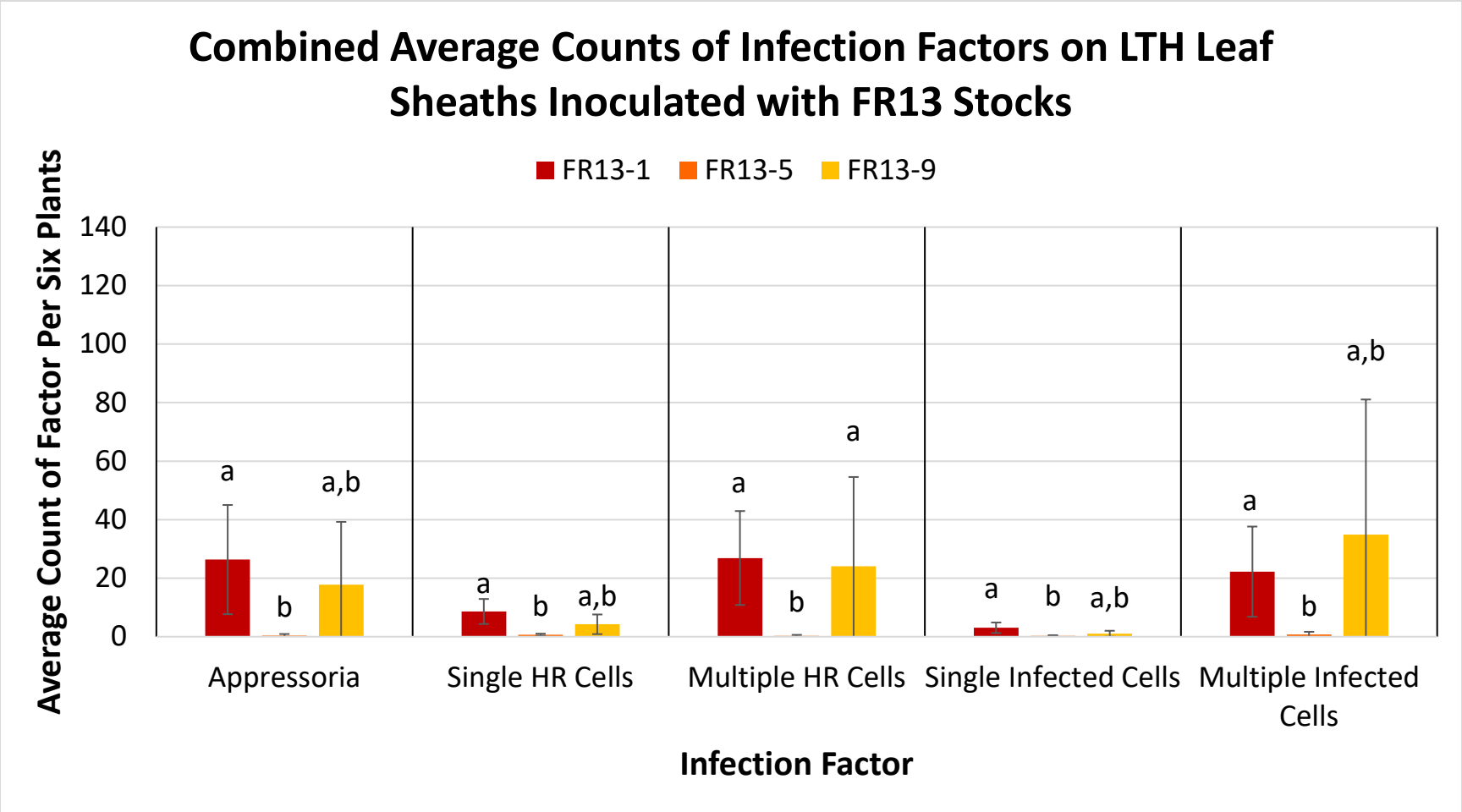


Figure 7-4 Combined Average Counts of Infection Factors on LTH Leaf Sheaths Inoculated with FR13 Paper Stocks
Ratings of 0-3 are categorized as avirulent and show levels of resistance. Ratings of 4-8 are categorized as virulent and shows levels of susceptibility.

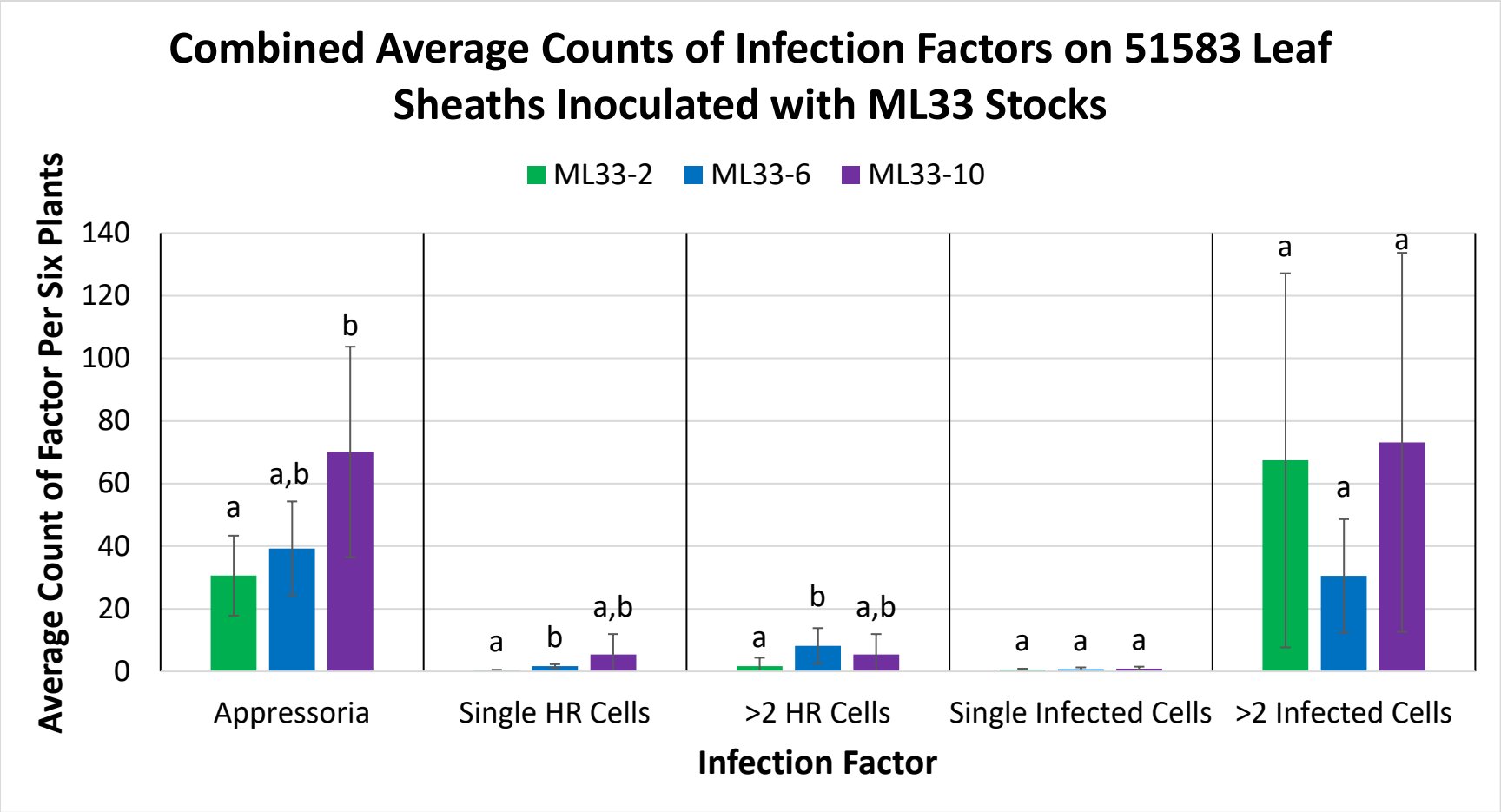
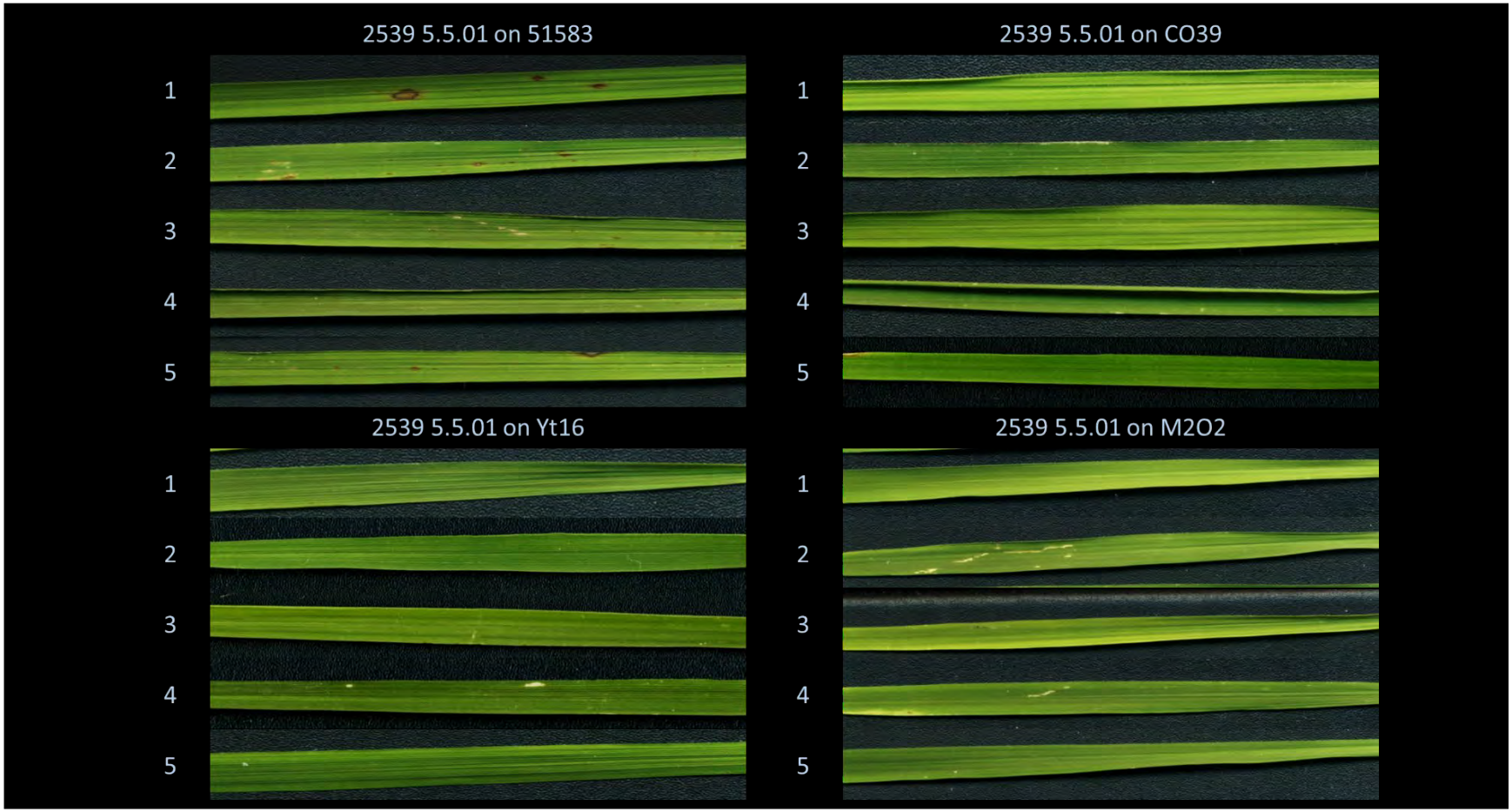
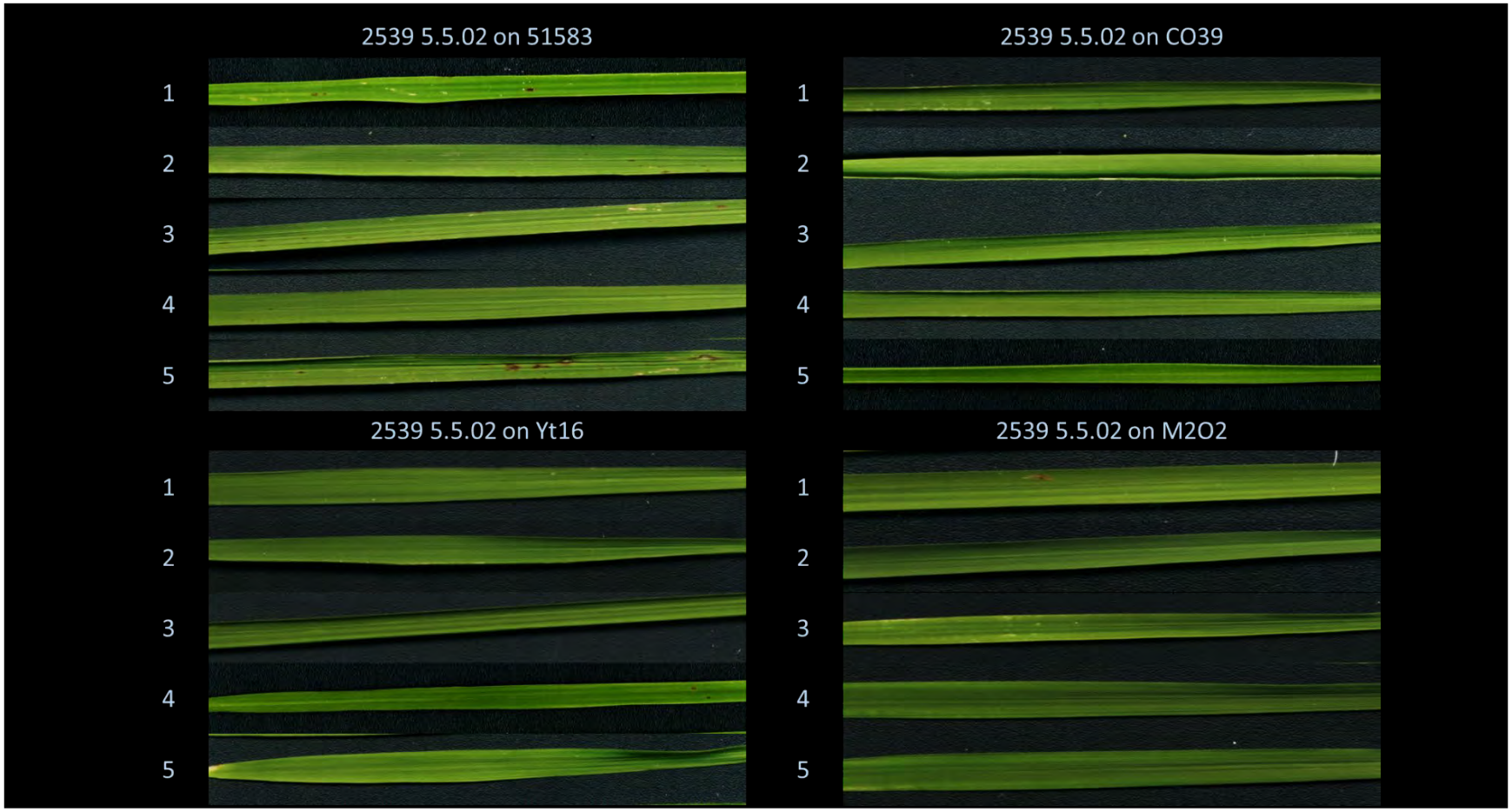


Figure 7-5 Combined Average Counts of Infection Factors on 51583 Leaf Sheaths Inoculated with ML33 Paper Stocks
Ratings of 0-3 are categorized as avirulent and show levels of resistance. Ratings of 4-8 are categorized as virulent and shows levels of susceptibility.

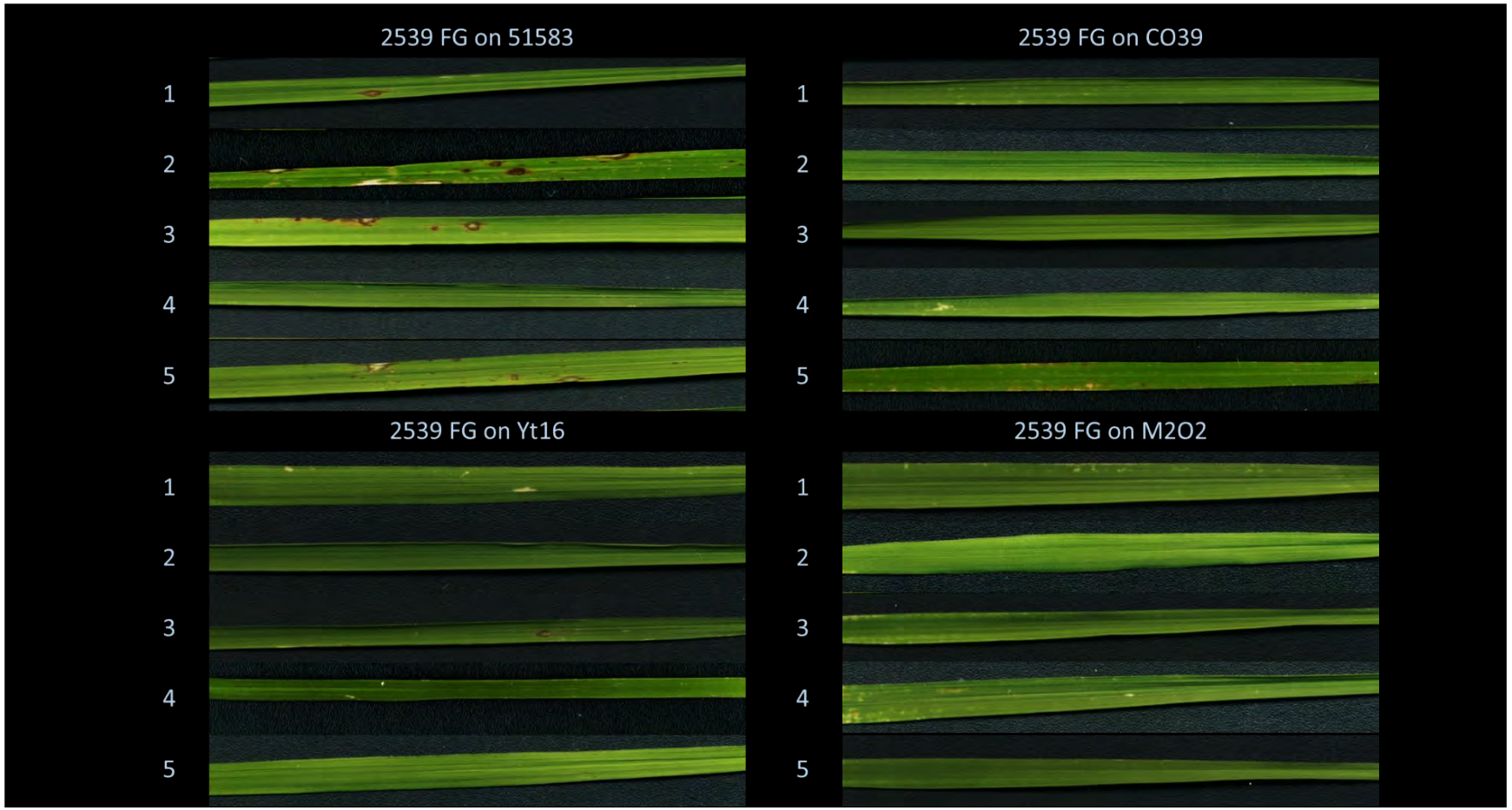
APPENDIX 1: Supplementary Figures for Chapter 3



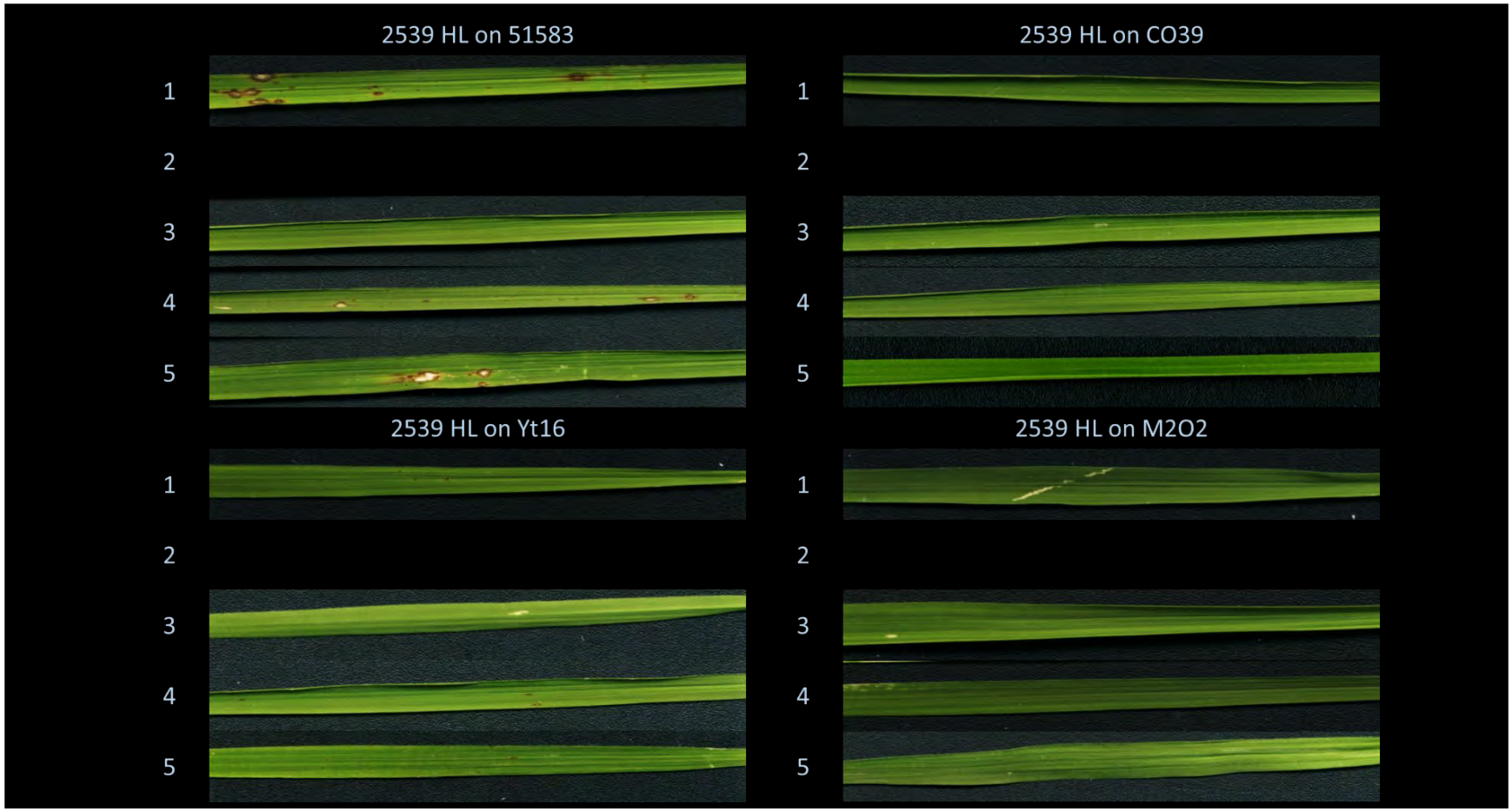
Secondary leaf scans of 51583 inoculated with 2539 5.5.01 for five replications. Leaves shown are a representative example of the entire pot of plants for each inoculation.



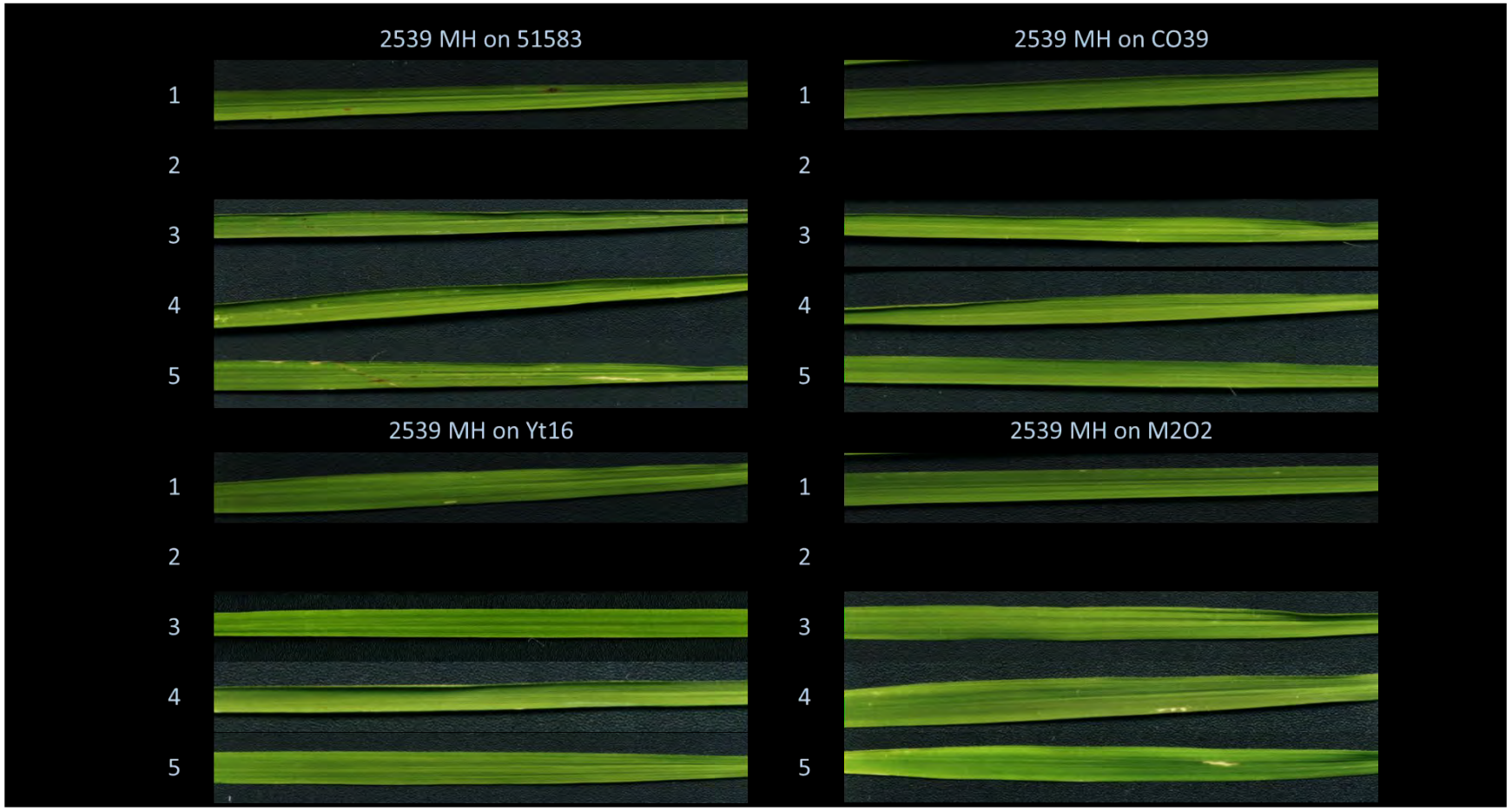
Secondary leaf scans of 51583 inoculated with 2539 5.5.02 for five replications. Leaves shown are a representative example of the entire pot of plants for each inoculation.



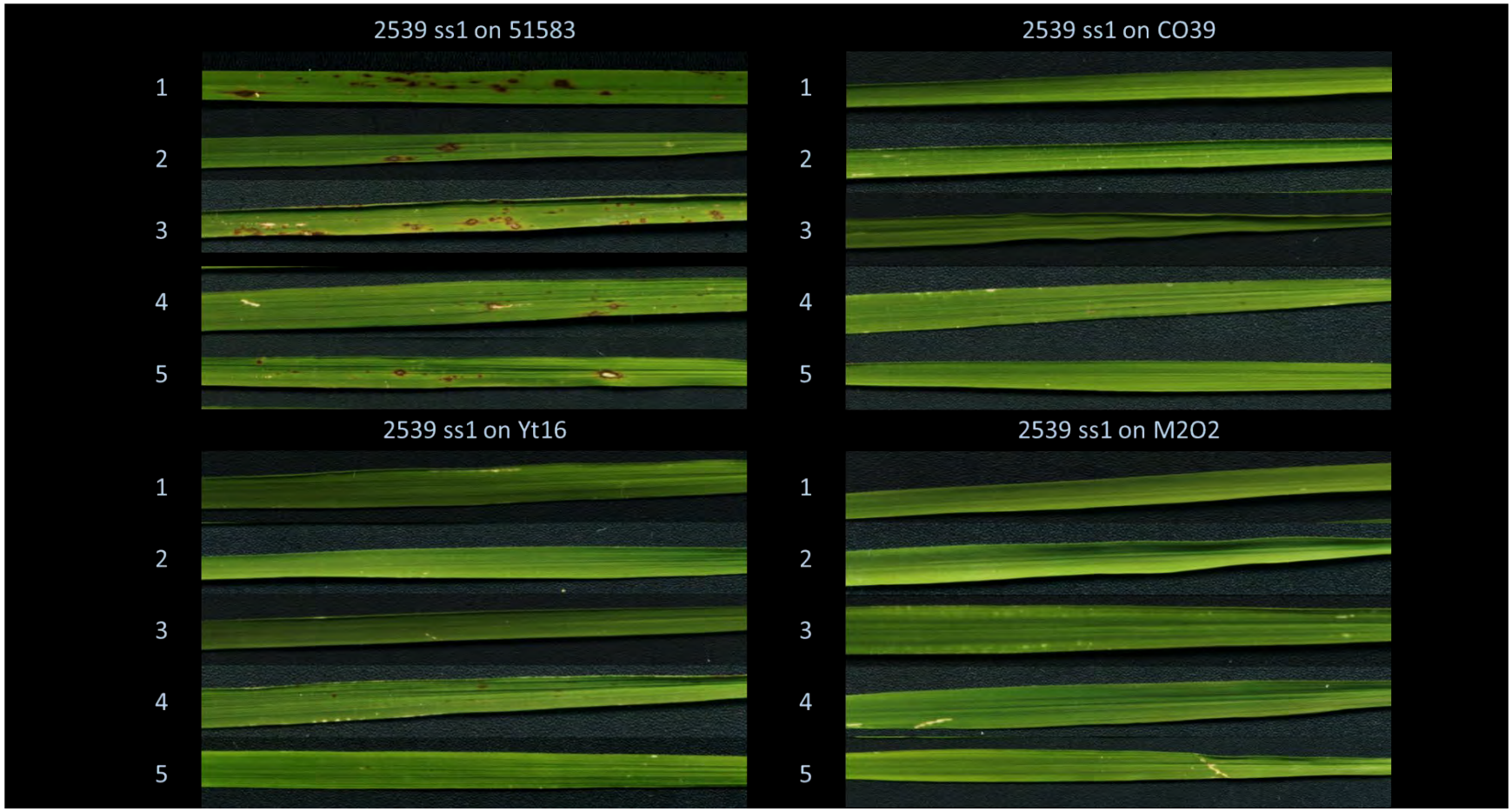
Secondary leaf scans of 51583 inoculated with 2539 FG for five replications. Leaves shown are a representative example of the entire pot of plants for each inoculation.



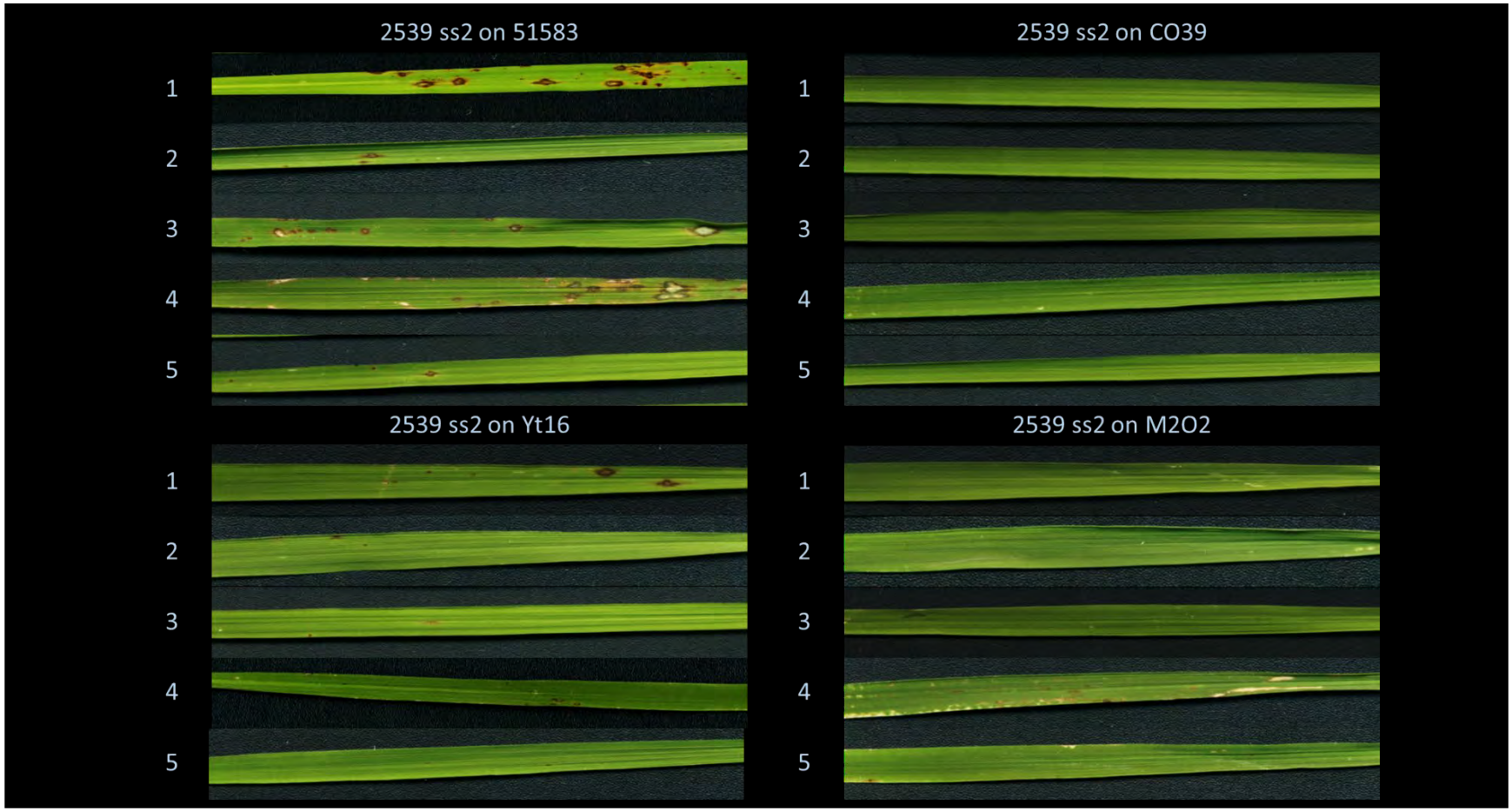
Secondary leaf scans of 51583 inoculated with 2539 HL for five replications. Leaves shown are a representative example of the entire pot of plants for each inoculation.



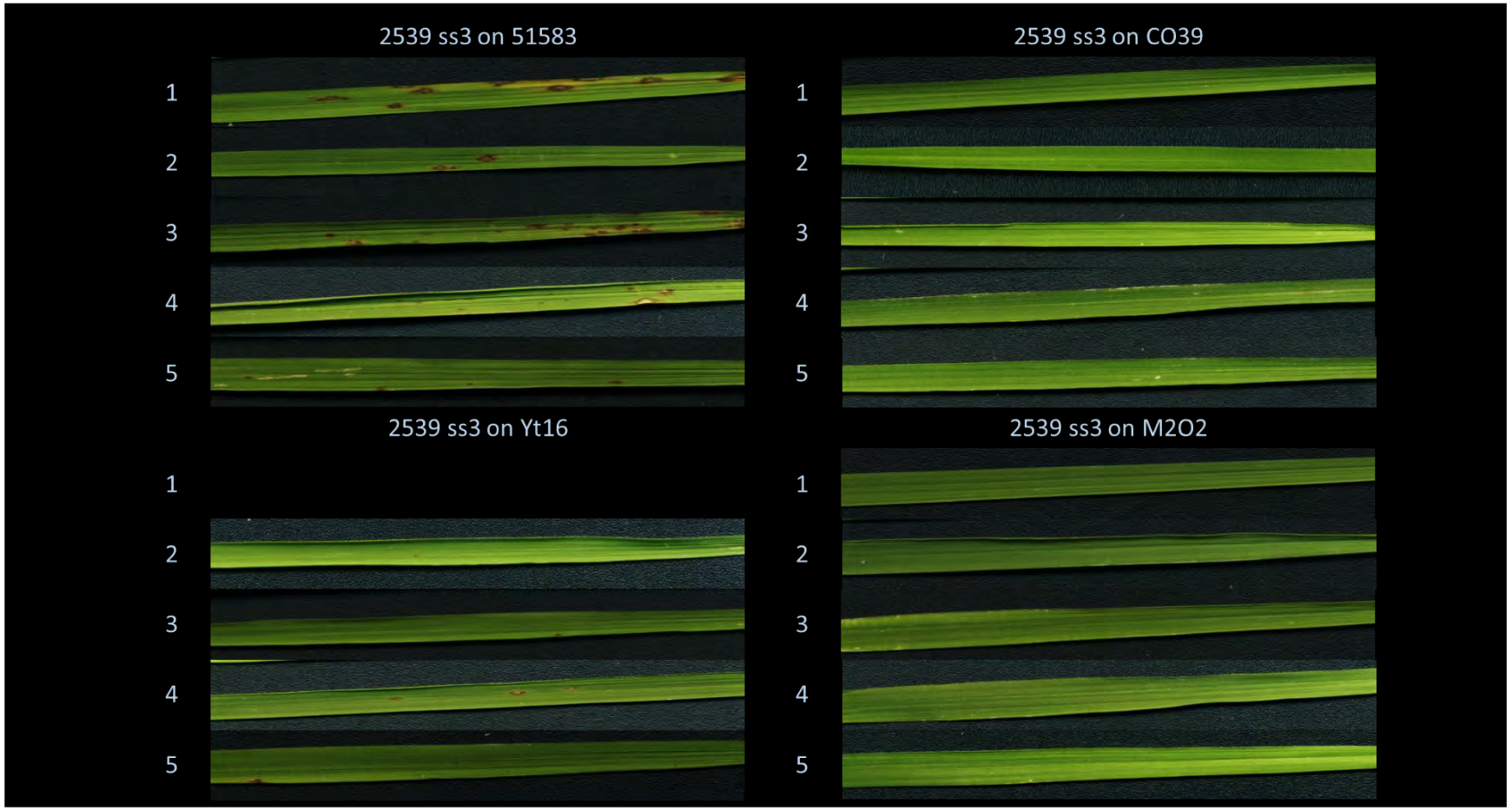
Secondary leaf scans of 51583 inoculated with 2539 MH for five replications. Leaves shown are a representative example of the entire pot of plants for each inoculation.



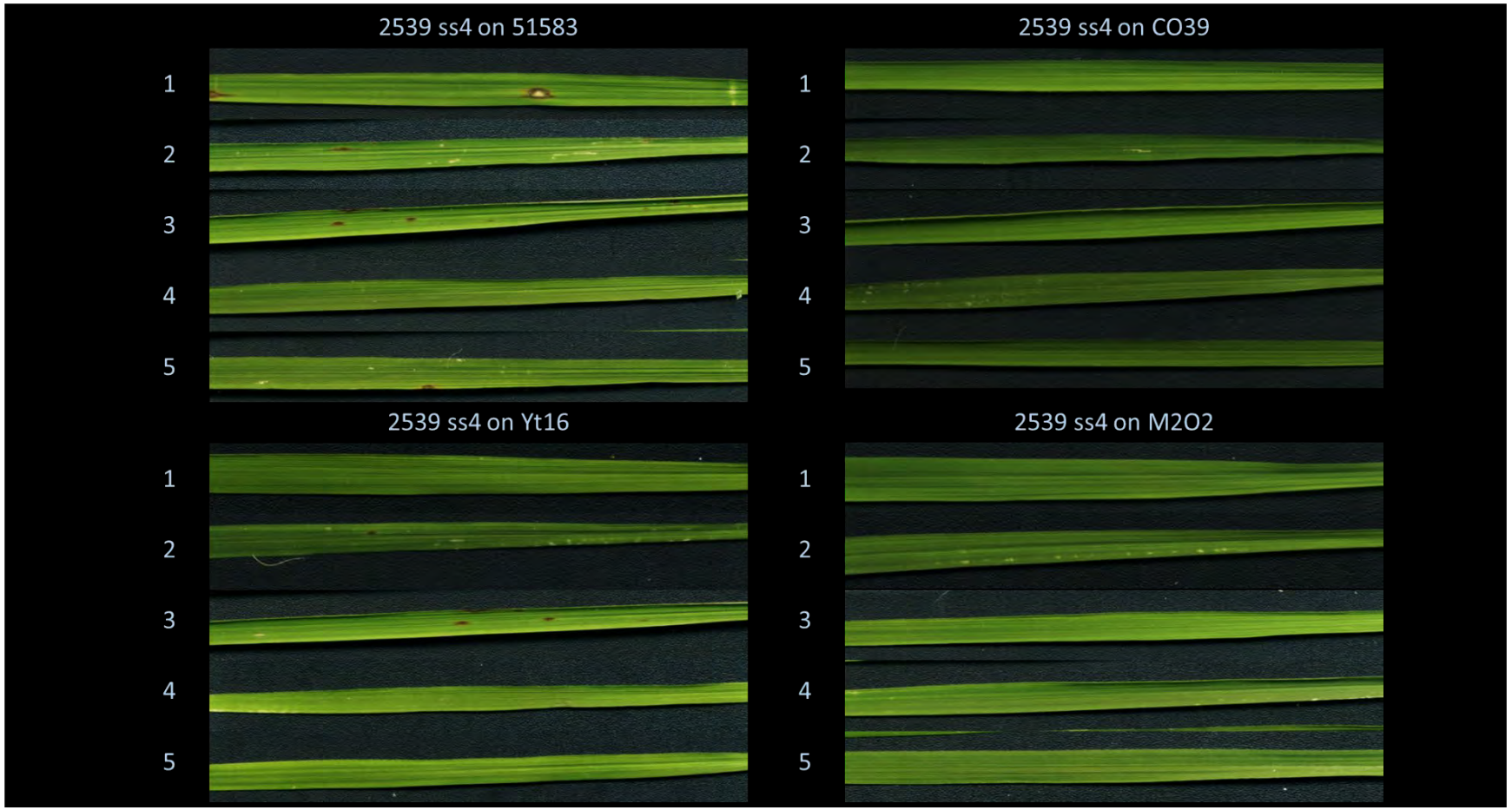
Secondary leaf scans of 51583 inoculated with 2539 ss1 for five replications. Leaves shown are a representative example of the entire pot of plants for each inoculation.



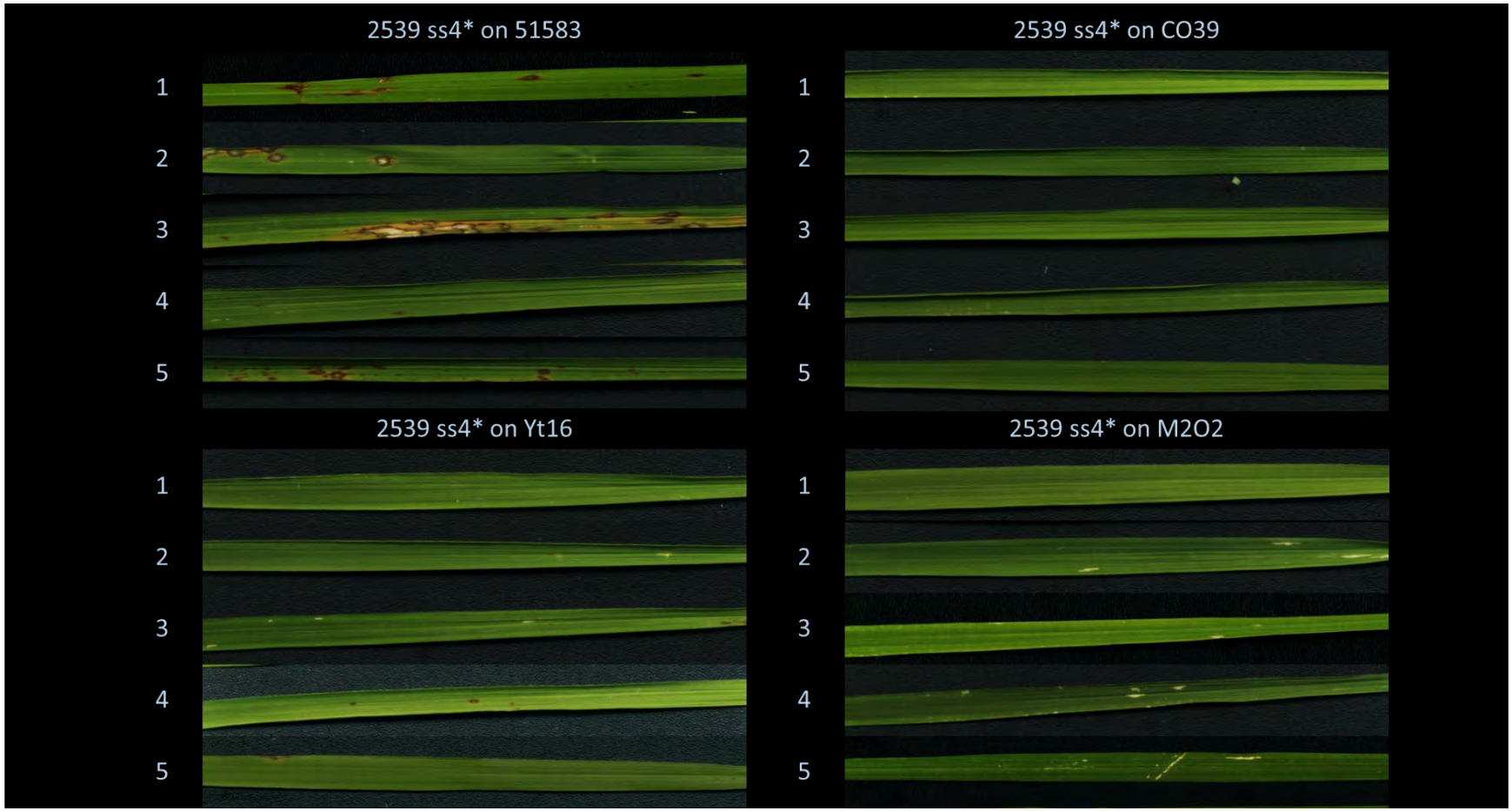
Secondary leaf scans of 51583 inoculated with 2539 ss2 for five replications. Leaves shown are a representative example of the entire pot of plants for each inoculation.



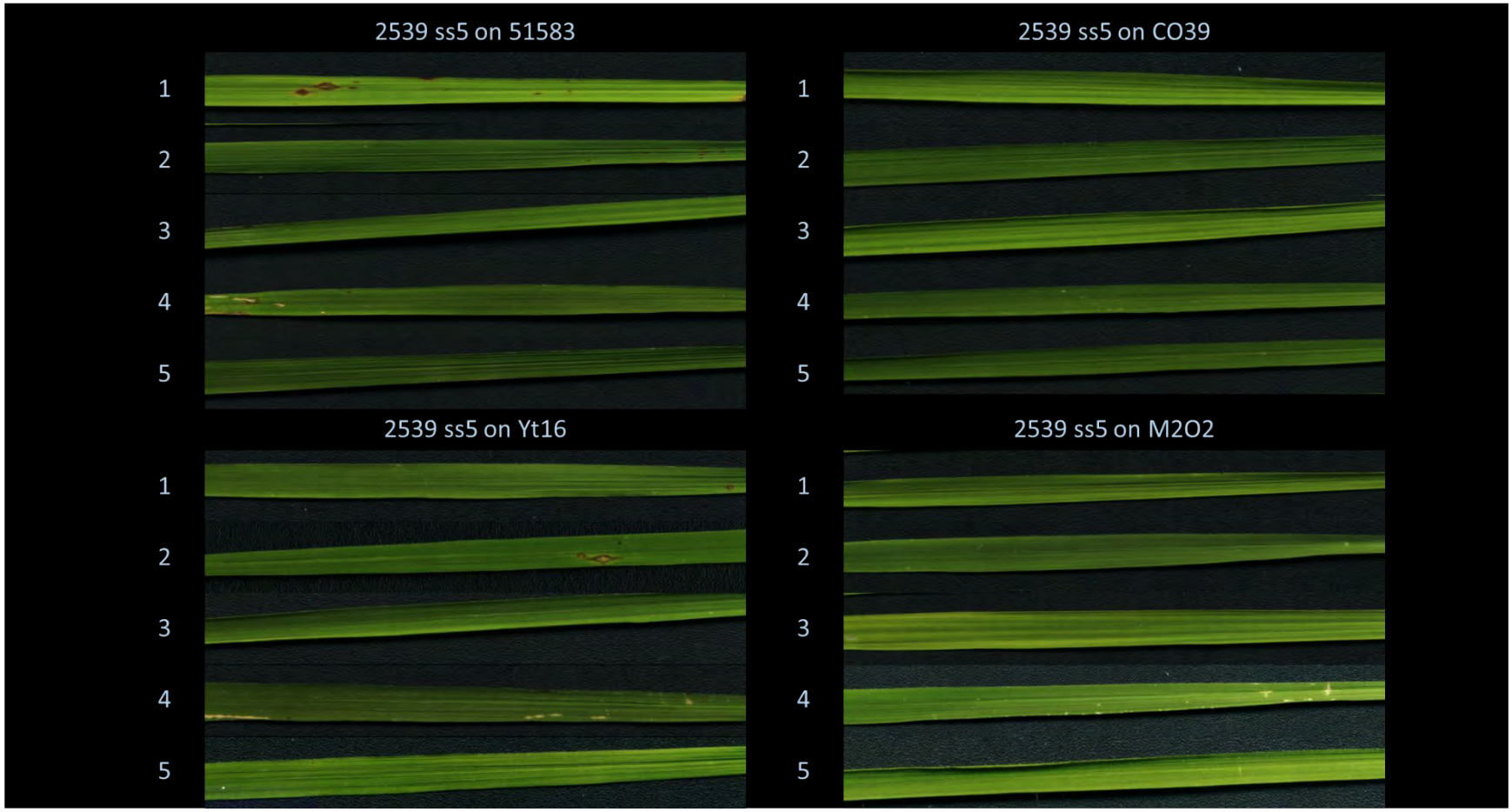
Secondary leaf scans of 51583 inoculated with 2539 ss3 for five replications. Leaves shown are a representative example of the entire pot of plants for each inoculation.



Secondary leaf scans of 51583 inoculated with 2539 ss4 for five replications. Leaves shown are a representative example of the entire pot of plants for each inoculation.

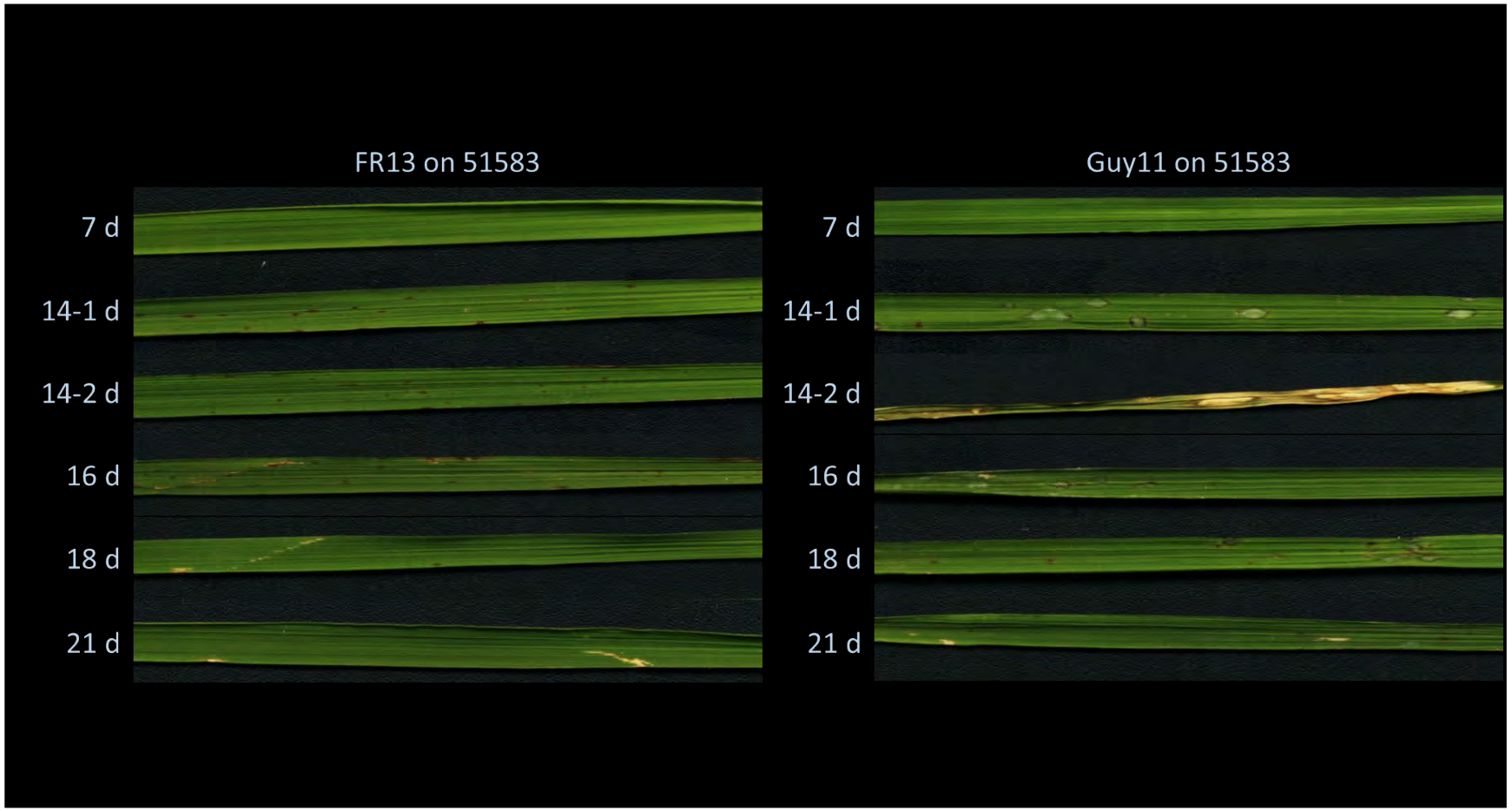


Secondary leaf scans of 51583 inoculated with 2539 ss4* for five replications. Leaves shown are a representative example of the entire pot of plants for each inoculation.

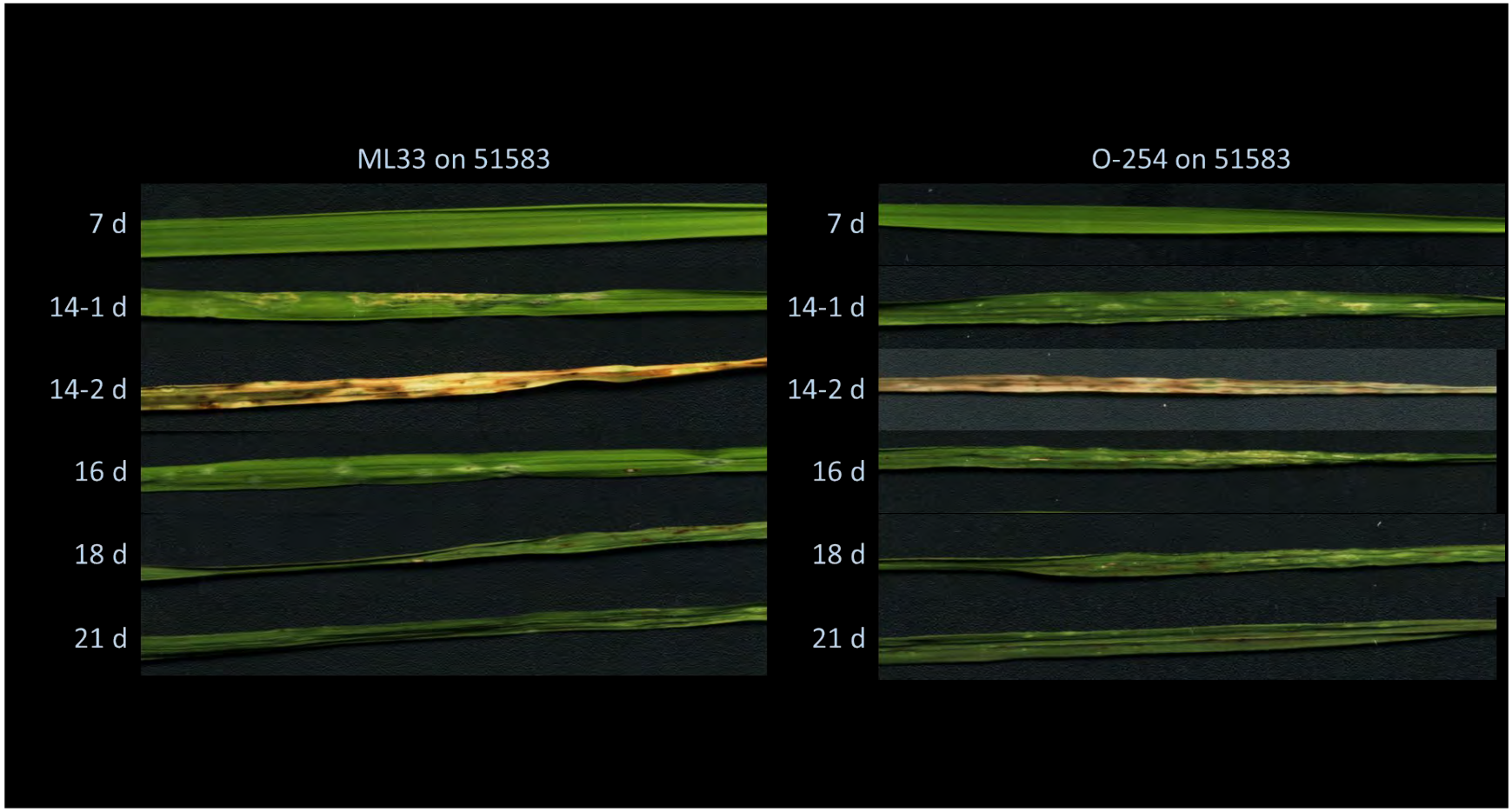


Secondary leaf scans of 51583 inoculated with 2539 ss5 for five replications. Leaves shown are a representative example of the entire pot of plants for each inoculation.

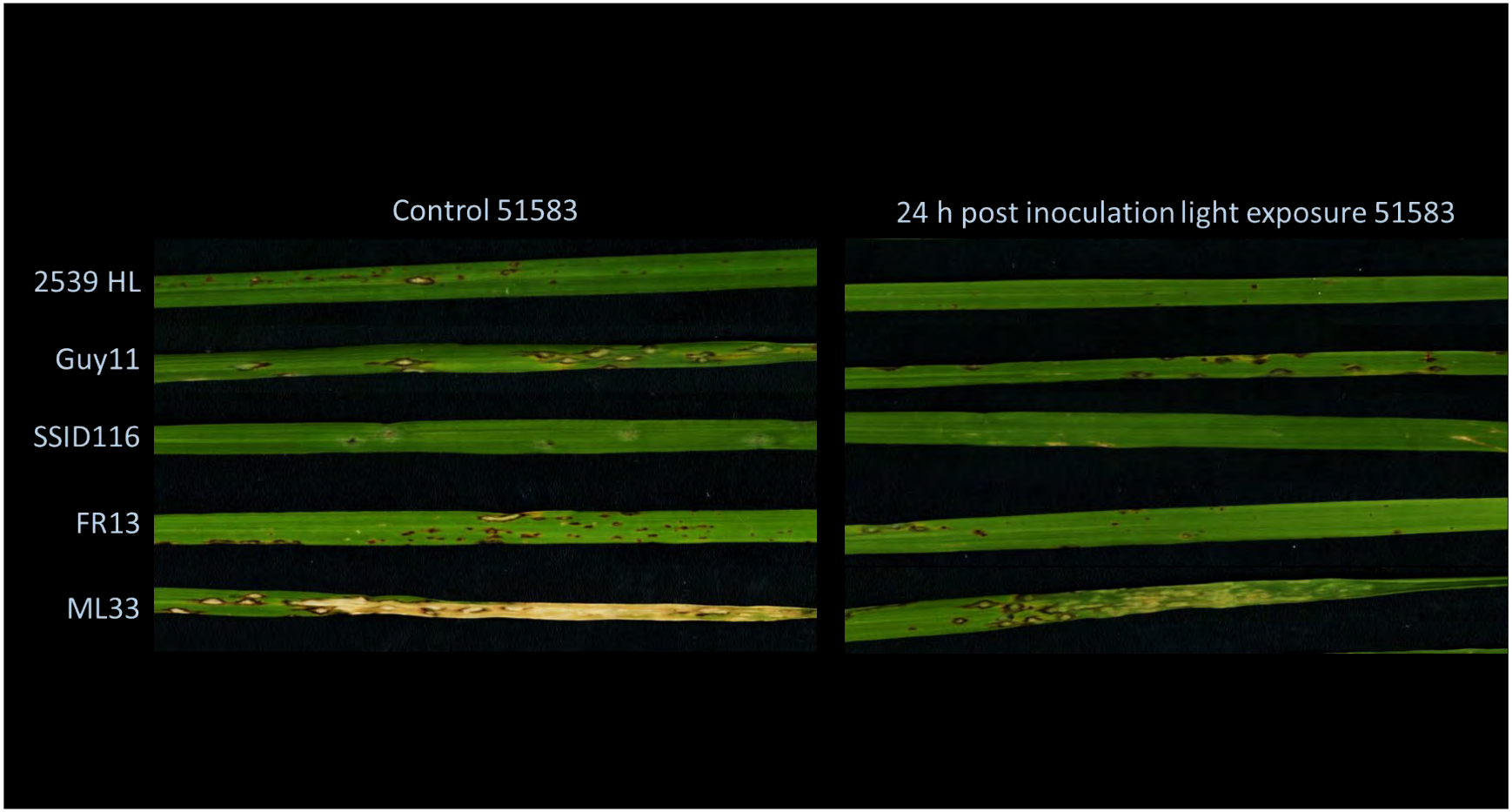
APPENDIX 2: Supplementary Figures for Chapter 4



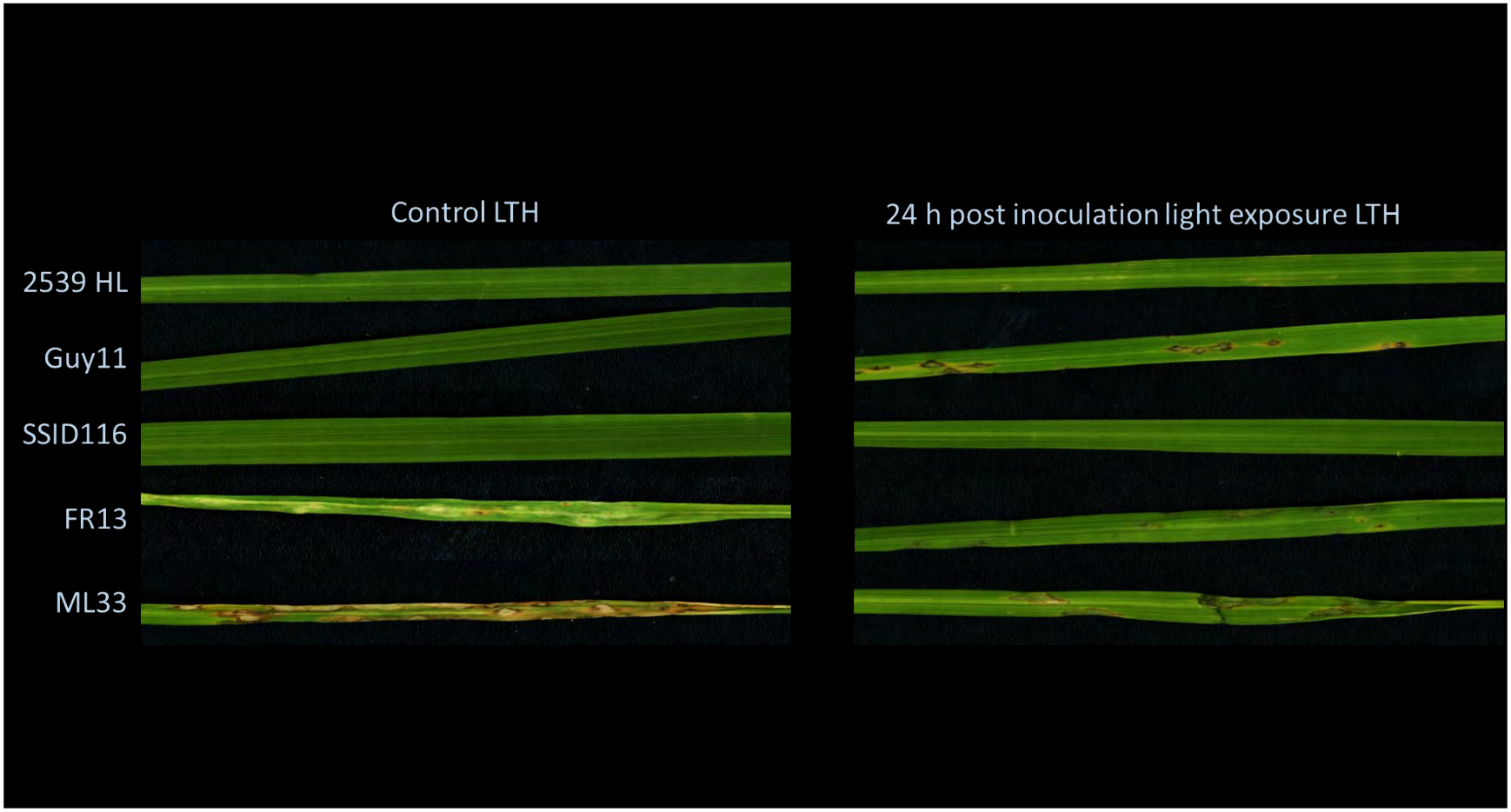
Secondary leaf scans of 51583 inoculated at different ages. Leaves shown are a representative example of the entire pot of plants for each inoculation.



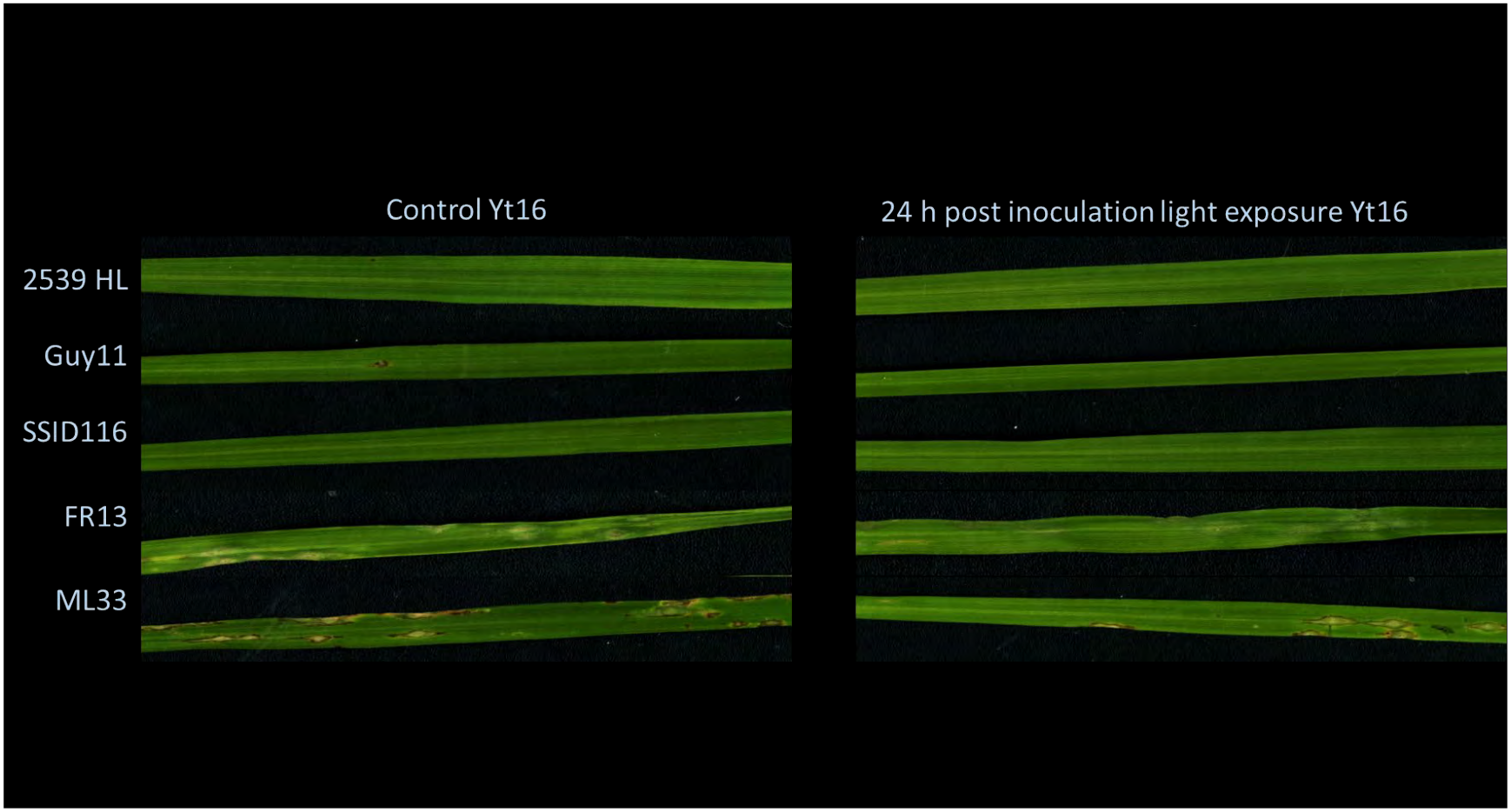
Secondary leaf scans of 51583 inoculated at different ages. Leaves shown are a representative example of the entire pot of plants for each inoculation.



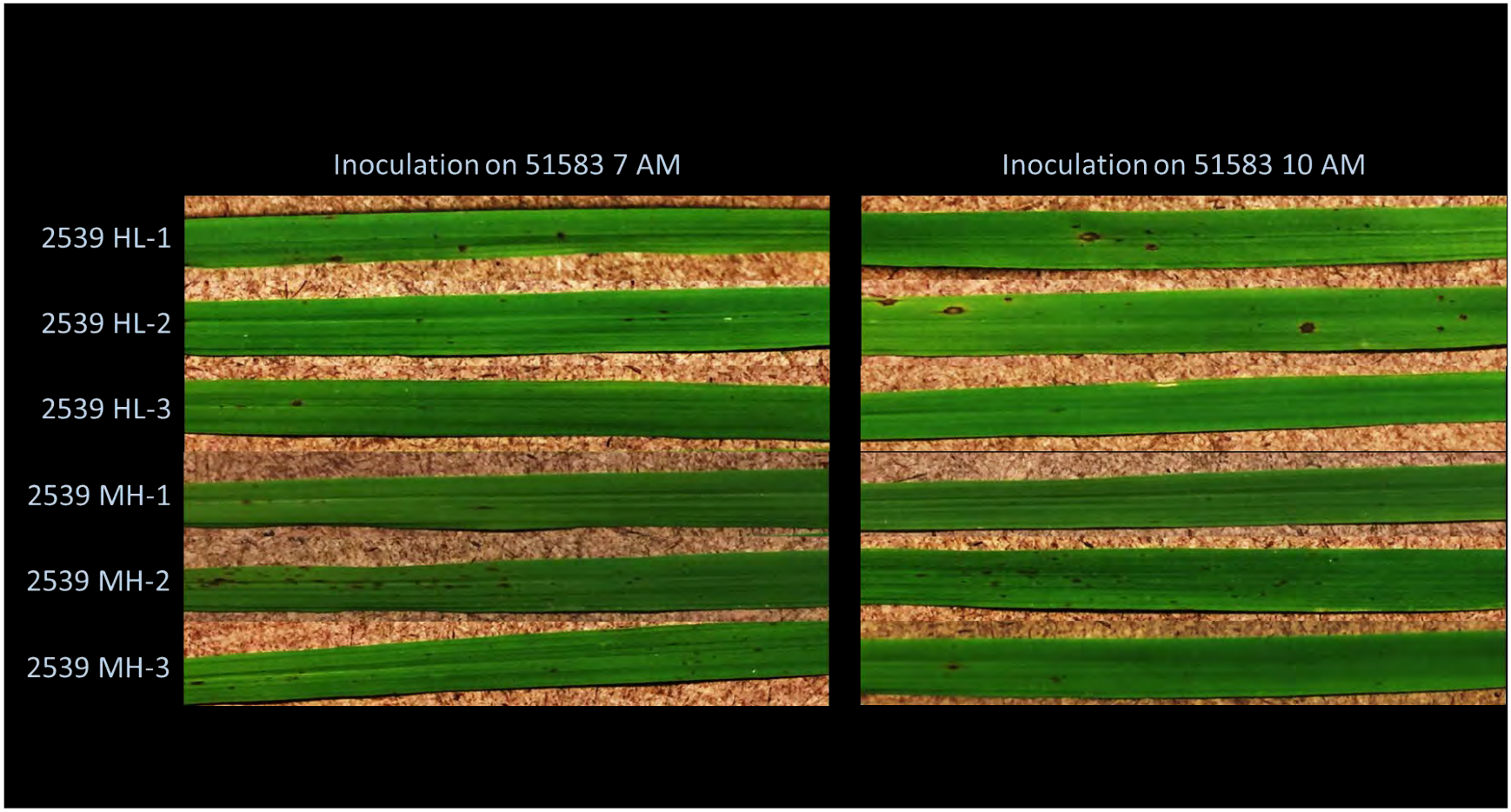
Secondary leaf scans of 51583 inoculated and exposed to 24 h light. Leaves shown are a representative example of the entire pot of plants for each inoculation.



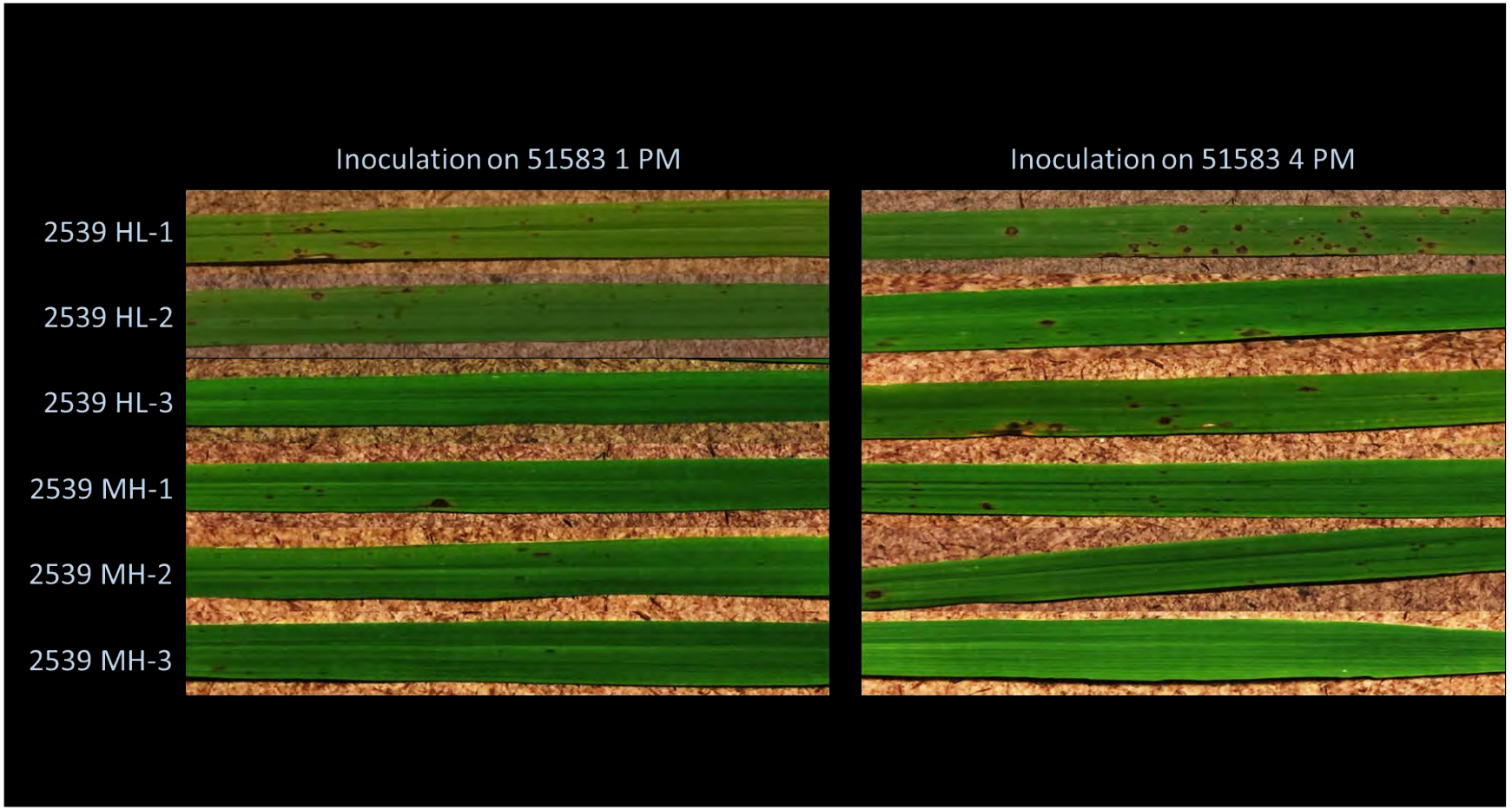
Secondary leaf scans of LTH inoculated and exposed to 24 h light. Leaves shown are a representative example of the entire pot of plants for each inoculation.



Secondary leaf scans of Yt16 inoculated and exposed to 24 h light. Leaves shown are a representative example of the entire pot of plants for each inoculation.

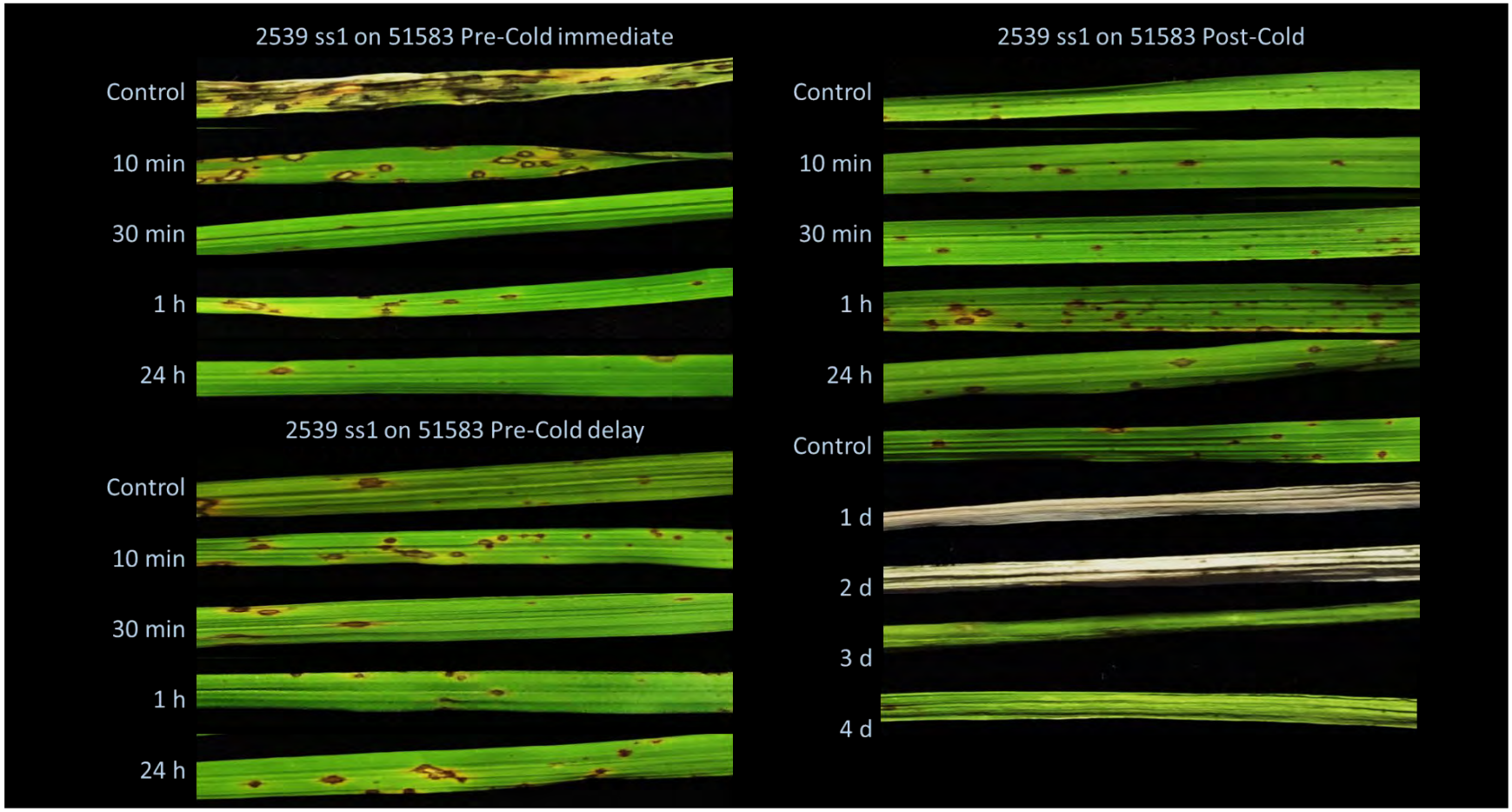


Secondary leaf scans of 51583 inoculated at different times in the day. Leaves shown are a representative example of the entire pot of plants for each inoculation.

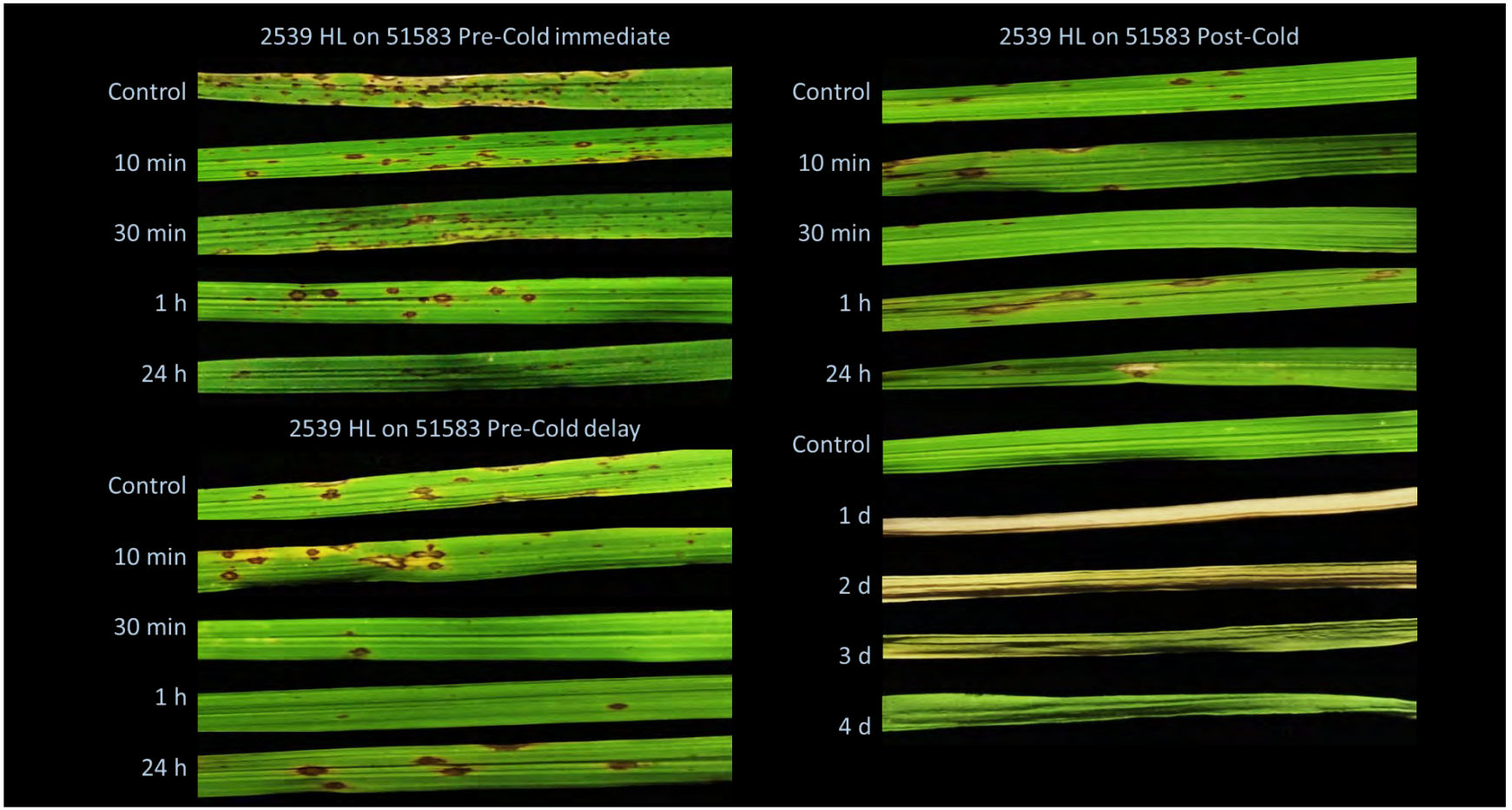


Secondary leaf scans of 51583 inoculated at different times in the day. Leaves shown are a representative example of the entire pot of plants for each inoculation.

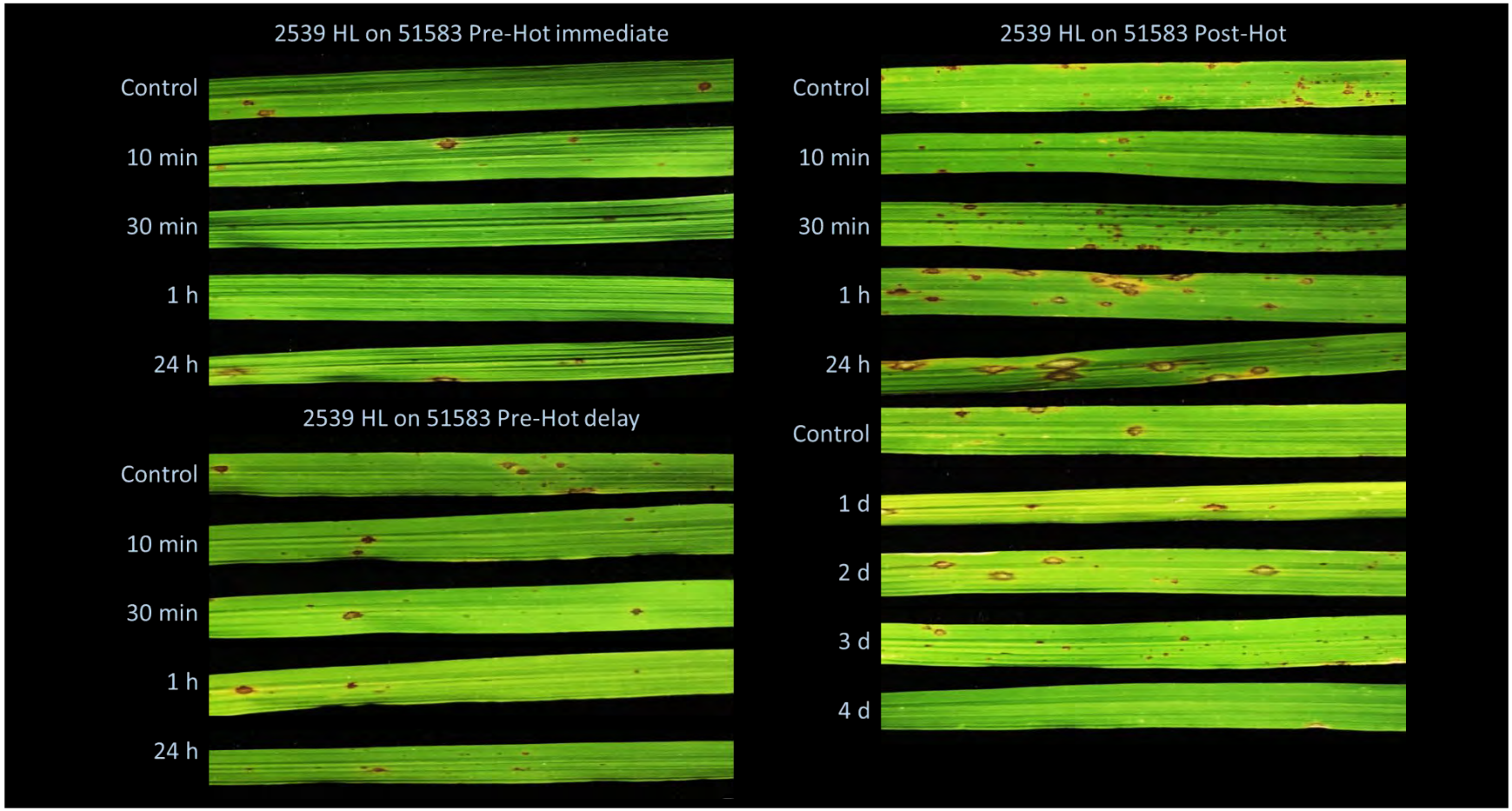
APPENDIX 3: Supplementary Figures for Chapter 5



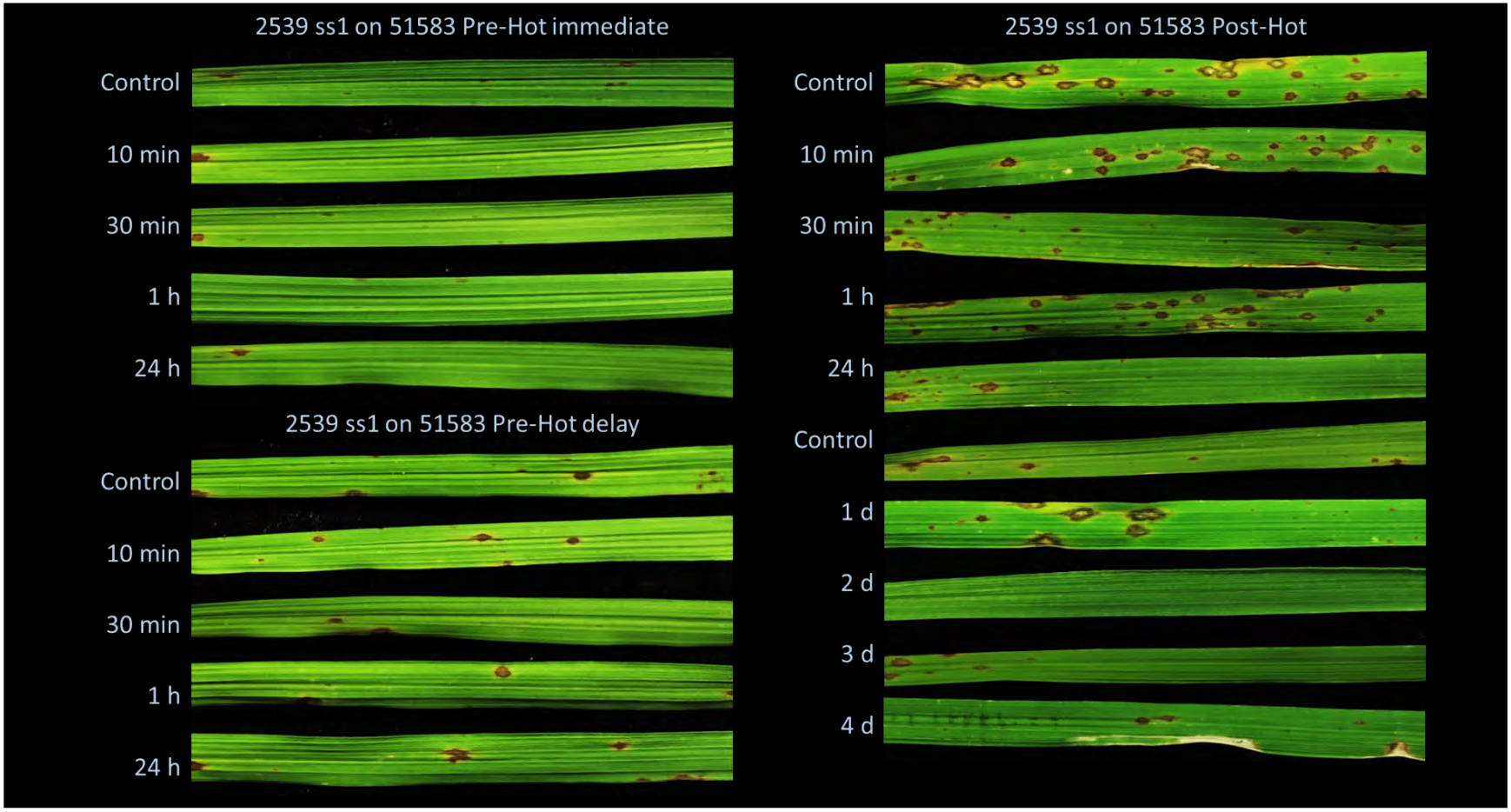
Secondary leaf scans of 51583 inoculated with 2539 ss1 under different temperature conditions. Leaves shown are a representative example of the entire pot of plants for each inoculation.



Secondary leaf scans of 51583 inoculated with 2539 HL under different temperature conditions. Leaves shown are a representative example of the entire pot of plants for each inoculation.

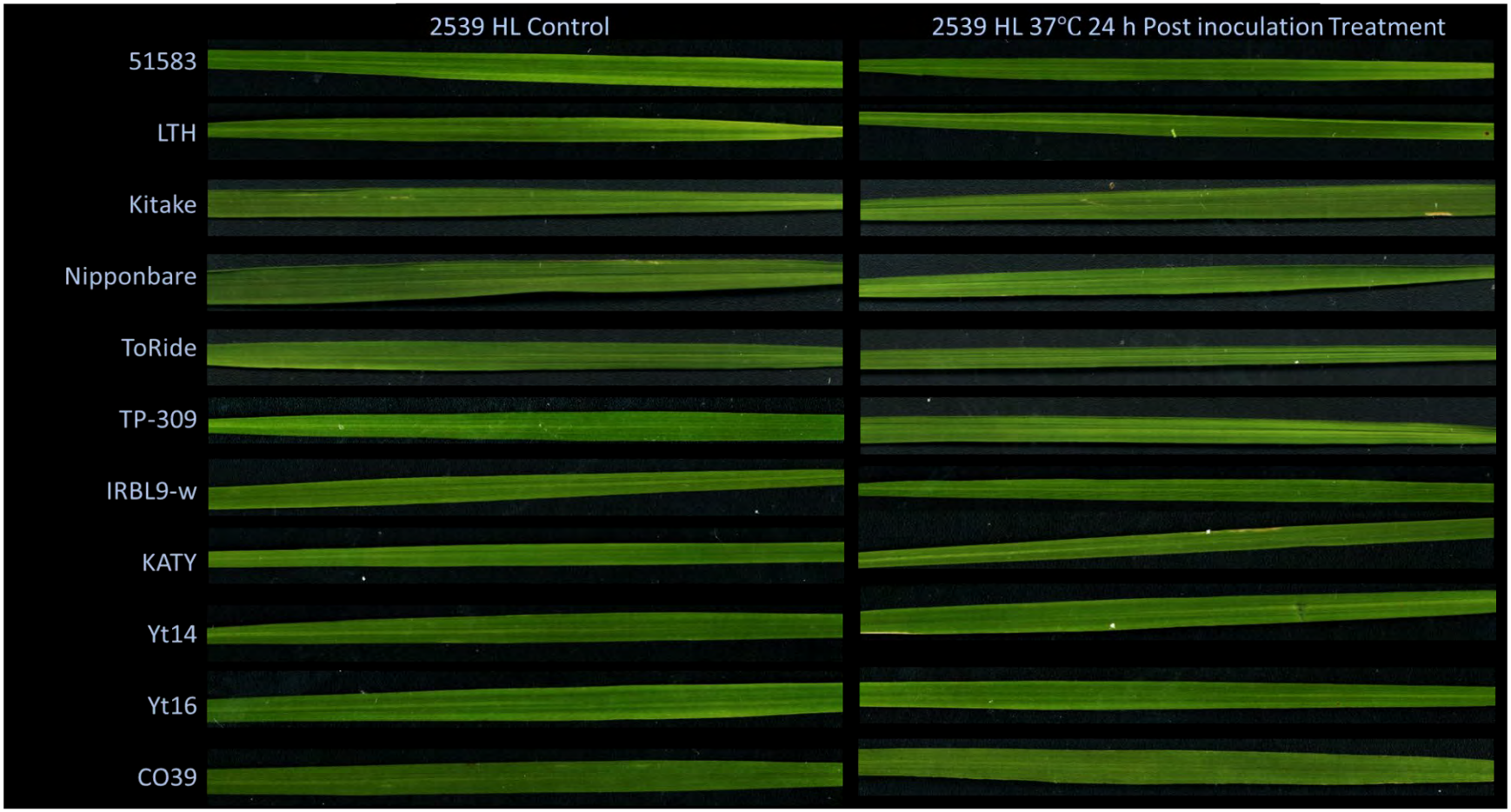


Secondary leaf scans of 51583 inoculated with 2539 HL under different temperature conditions. Leaves shown are a representative example of the entire pot of plants for each inoculation.

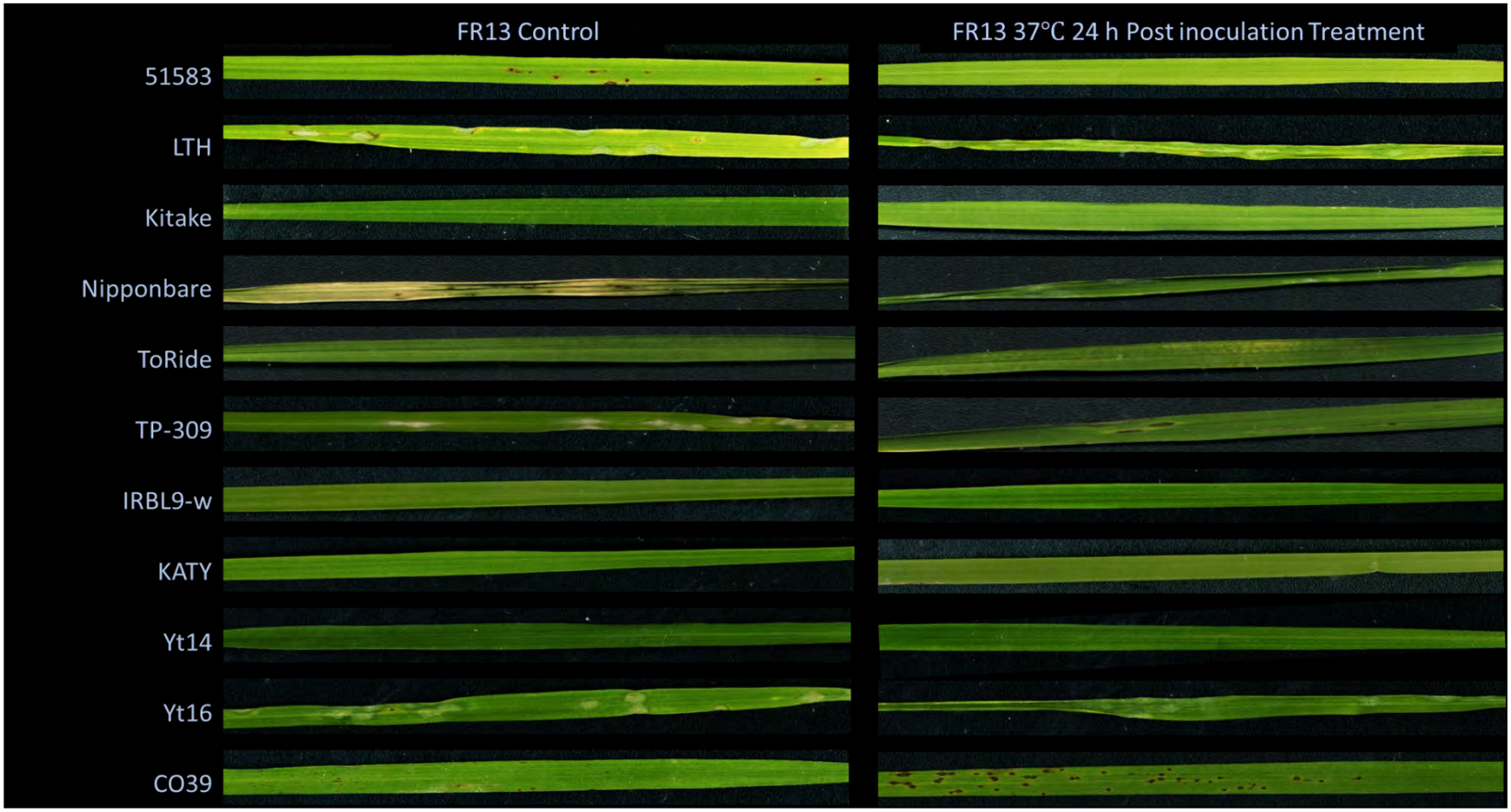


Secondary leaf scans of 51583 inoculated with 2539 ss1 under different temperature conditions. Leaves shown are a representative example of the entire pot of plants for each inoculation.

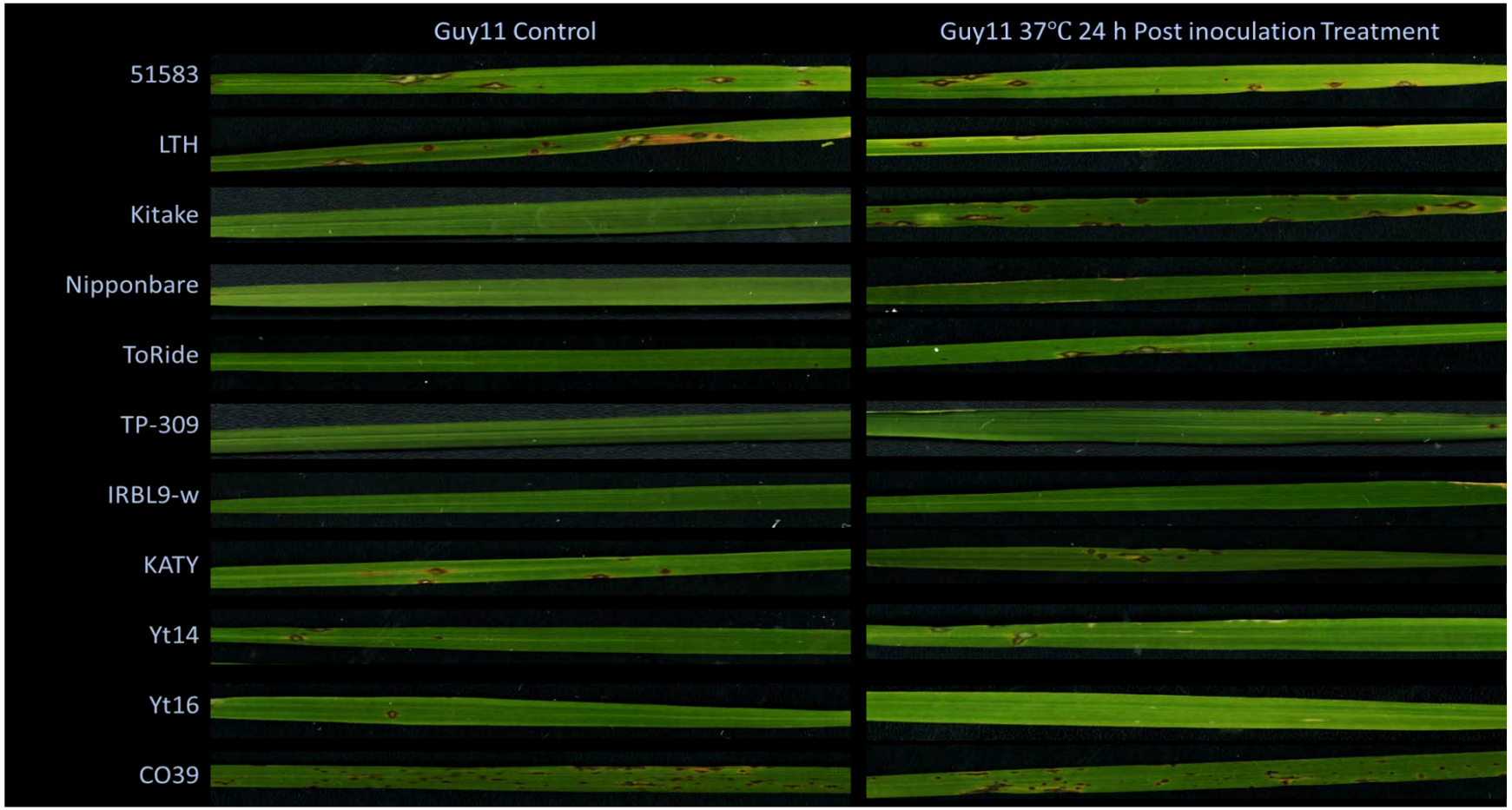
APPENDIX 4: Supplementary Figures for Chapter 6



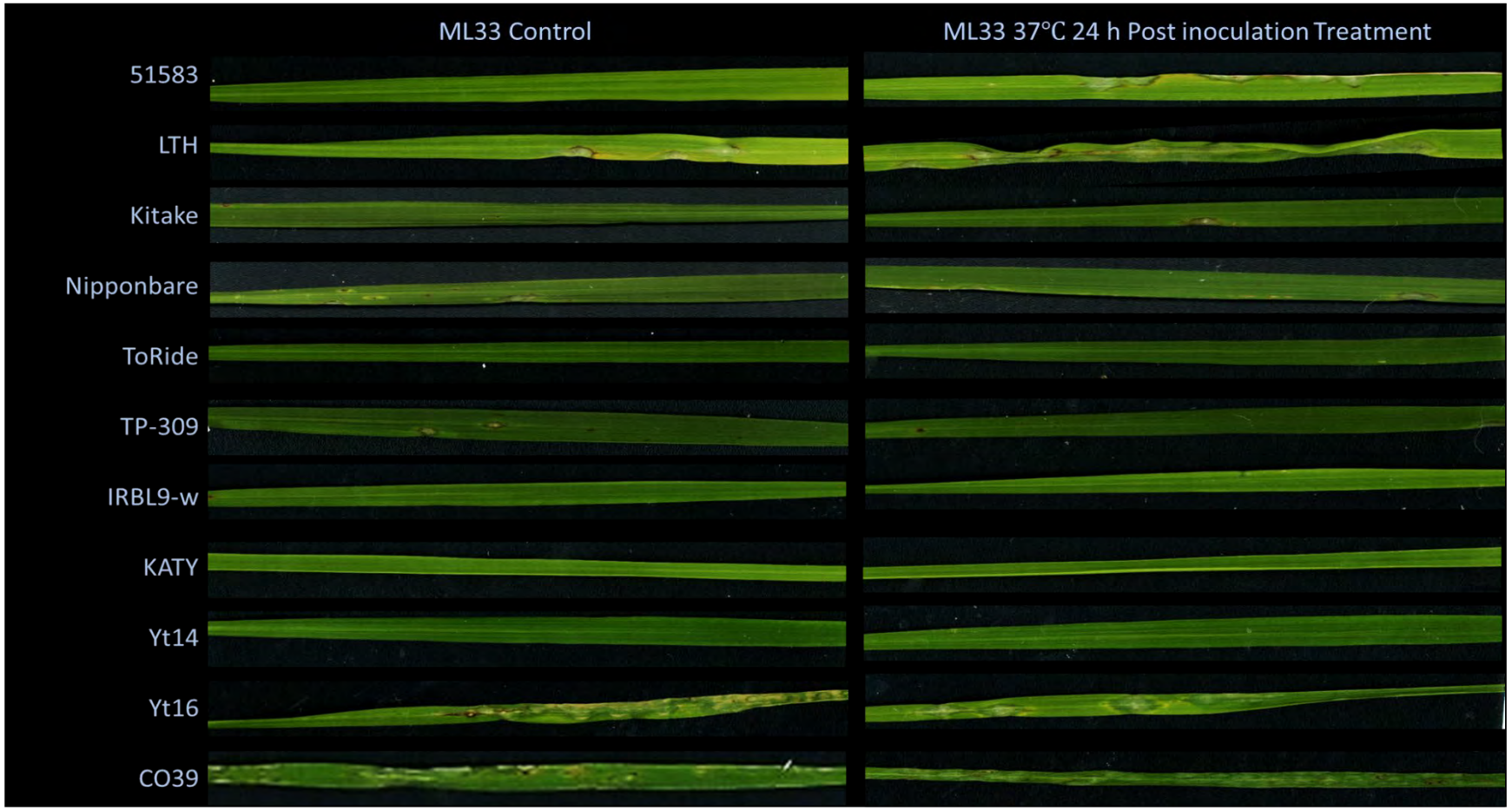
Secondary leaf scans of eleven rice cultivars inoculated with 2539 HL. Leaves shown are a representative example of the entire pot of plants.



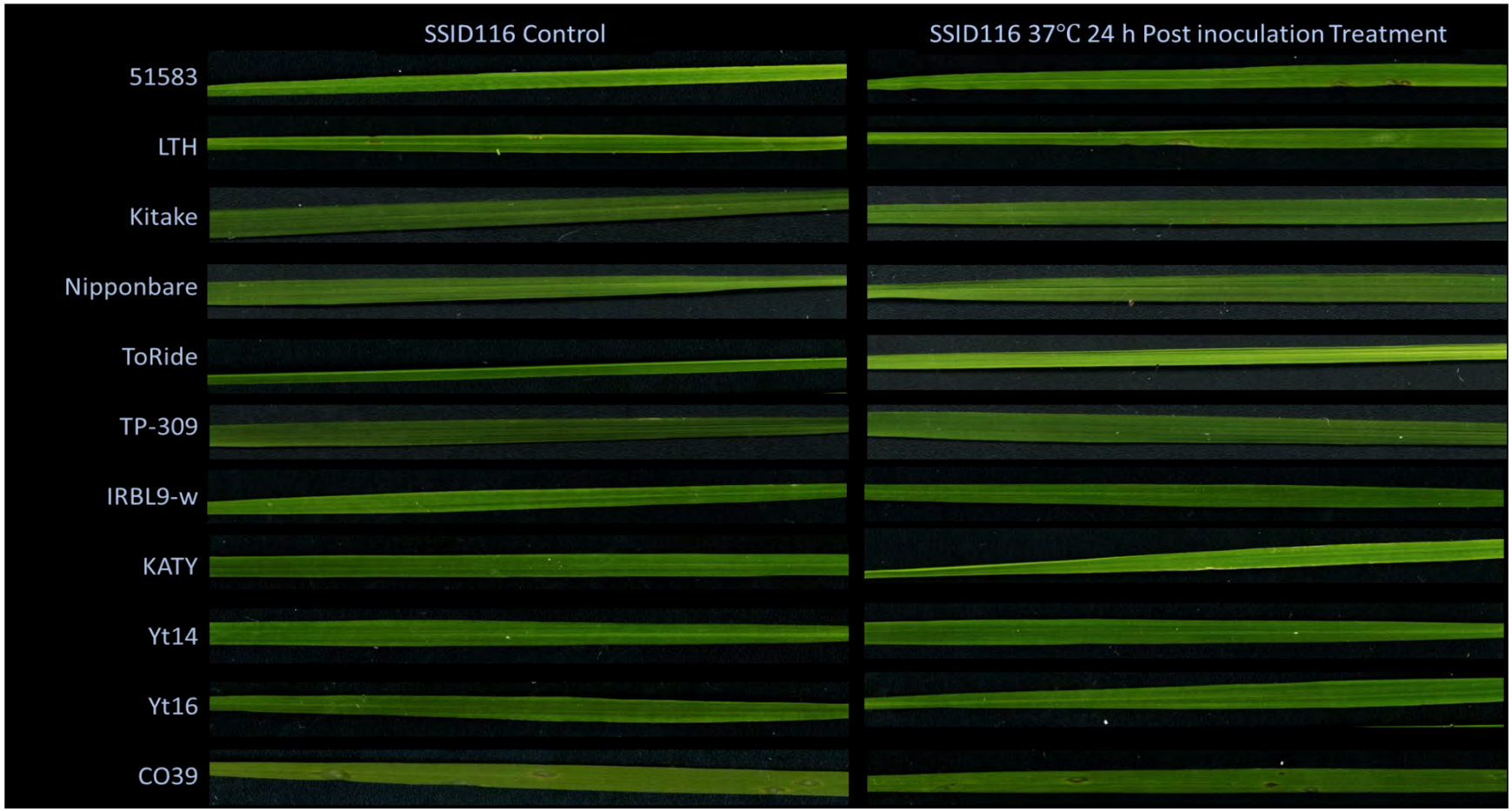
Secondary leaf scans of eleven rice cultivars inoculated with FR13. Leaves shown are a representative example of the entire pot of plants.



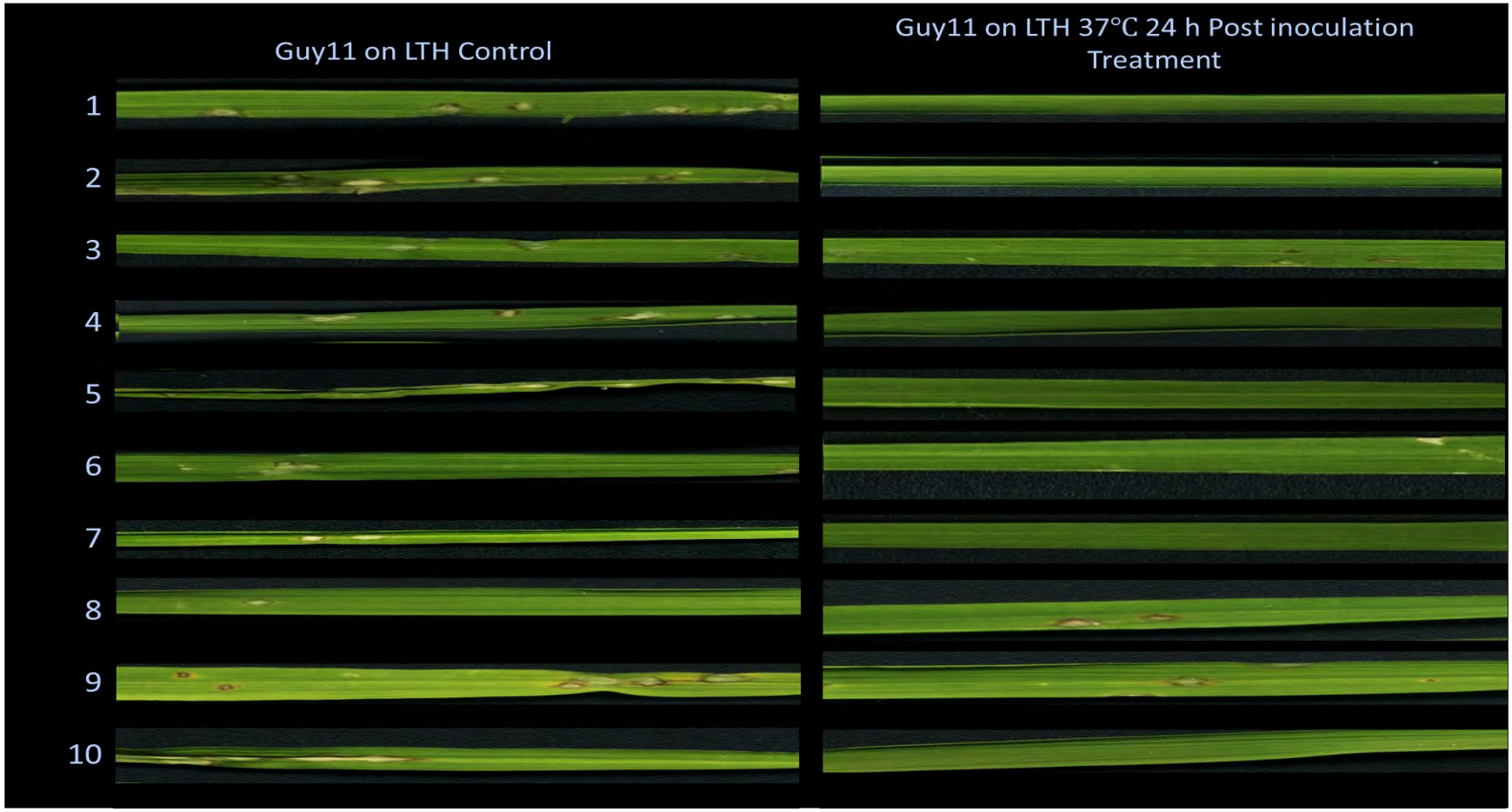
Secondary leaf scans of eleven rice cultivars inoculated with Guy11. Leaves shown are a representative example of the entire pot of plants.



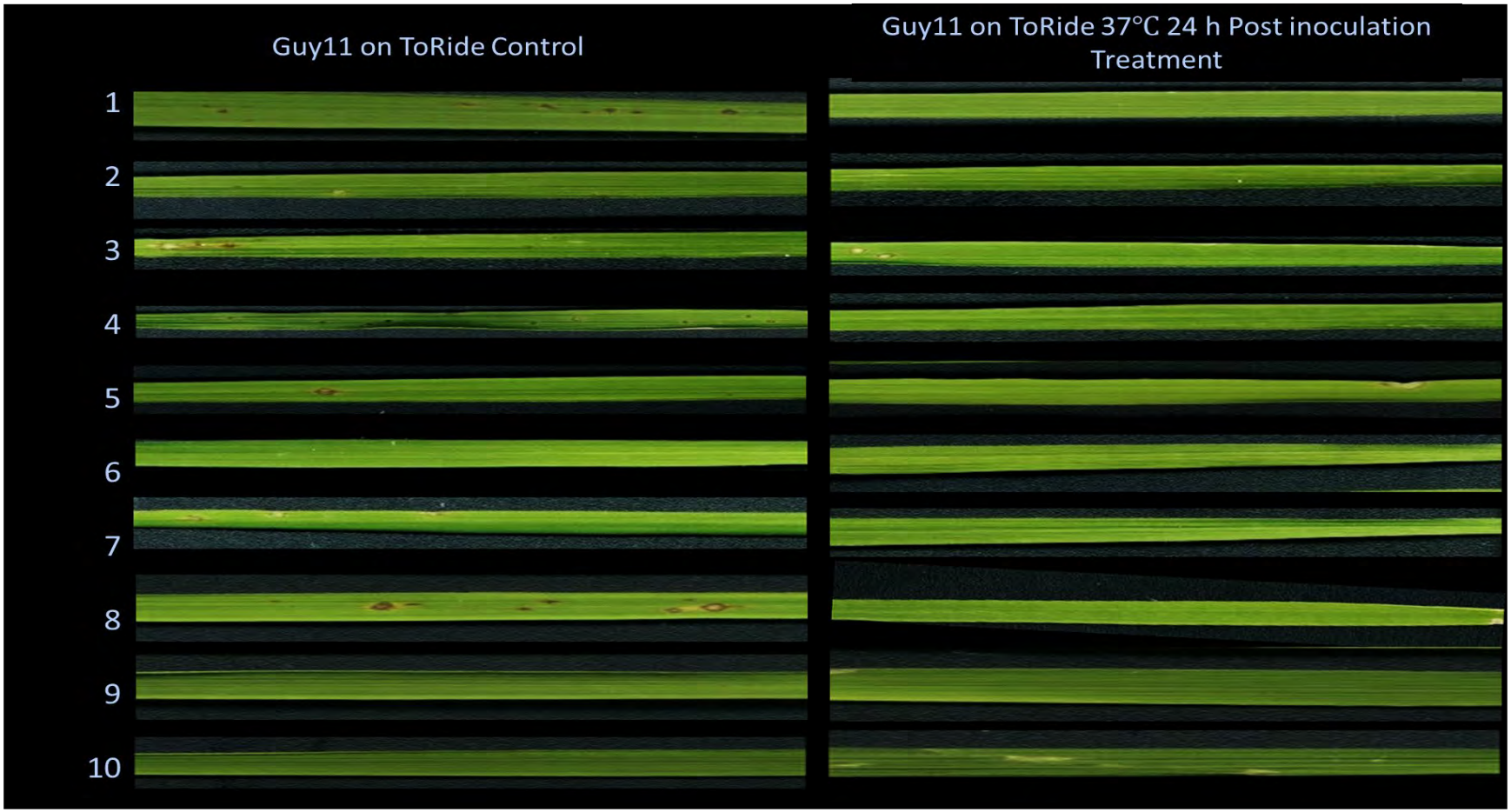
Secondary leaf scans of eleven rice cultivars inoculated with ML33. Leaves shown are a representative example of the entire pot of plants.



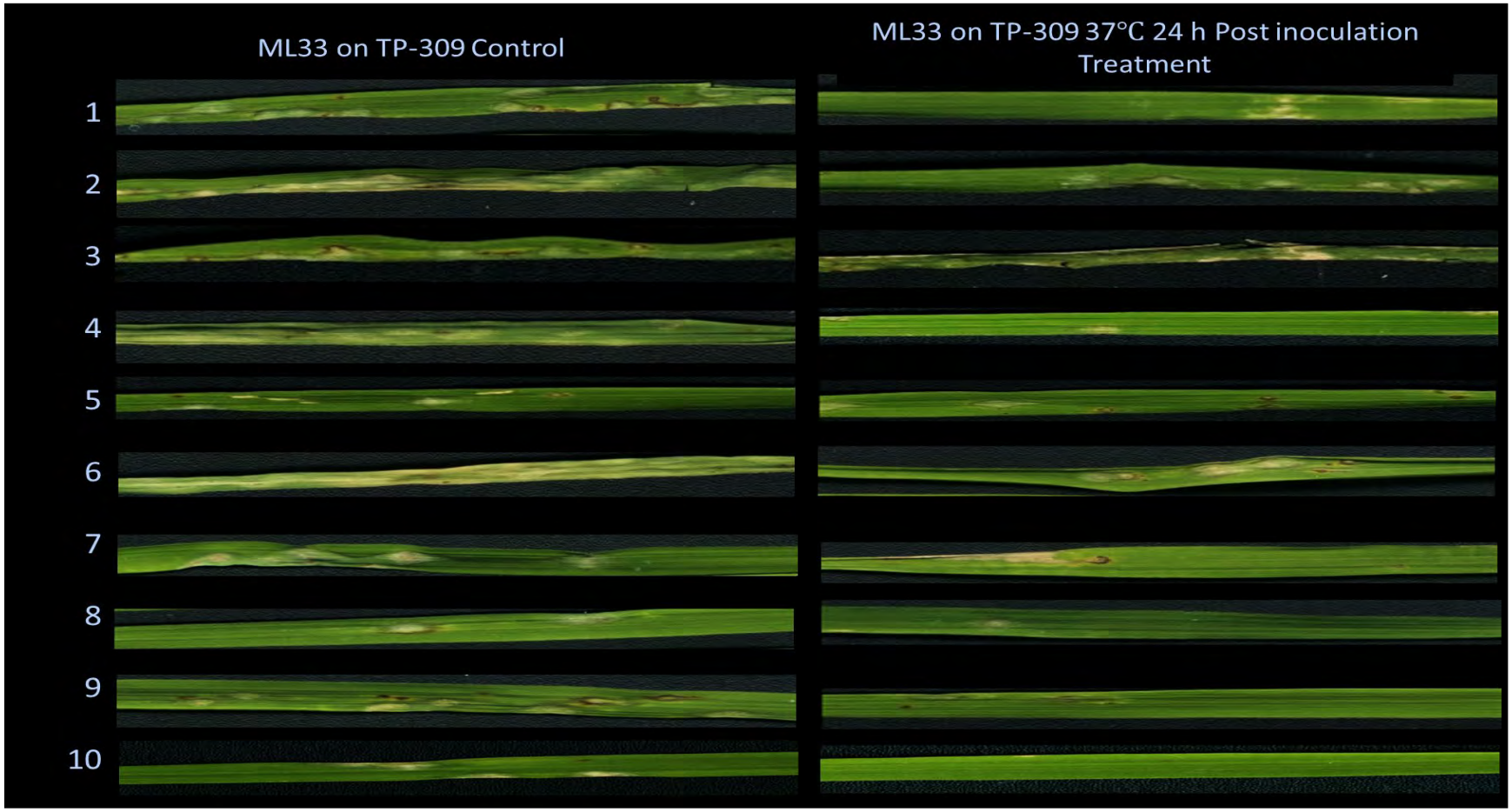
Secondary leaf scans of eleven rice cultivars inoculated with SSID116. Leaves shown are a representative example of the entire pot of plants.



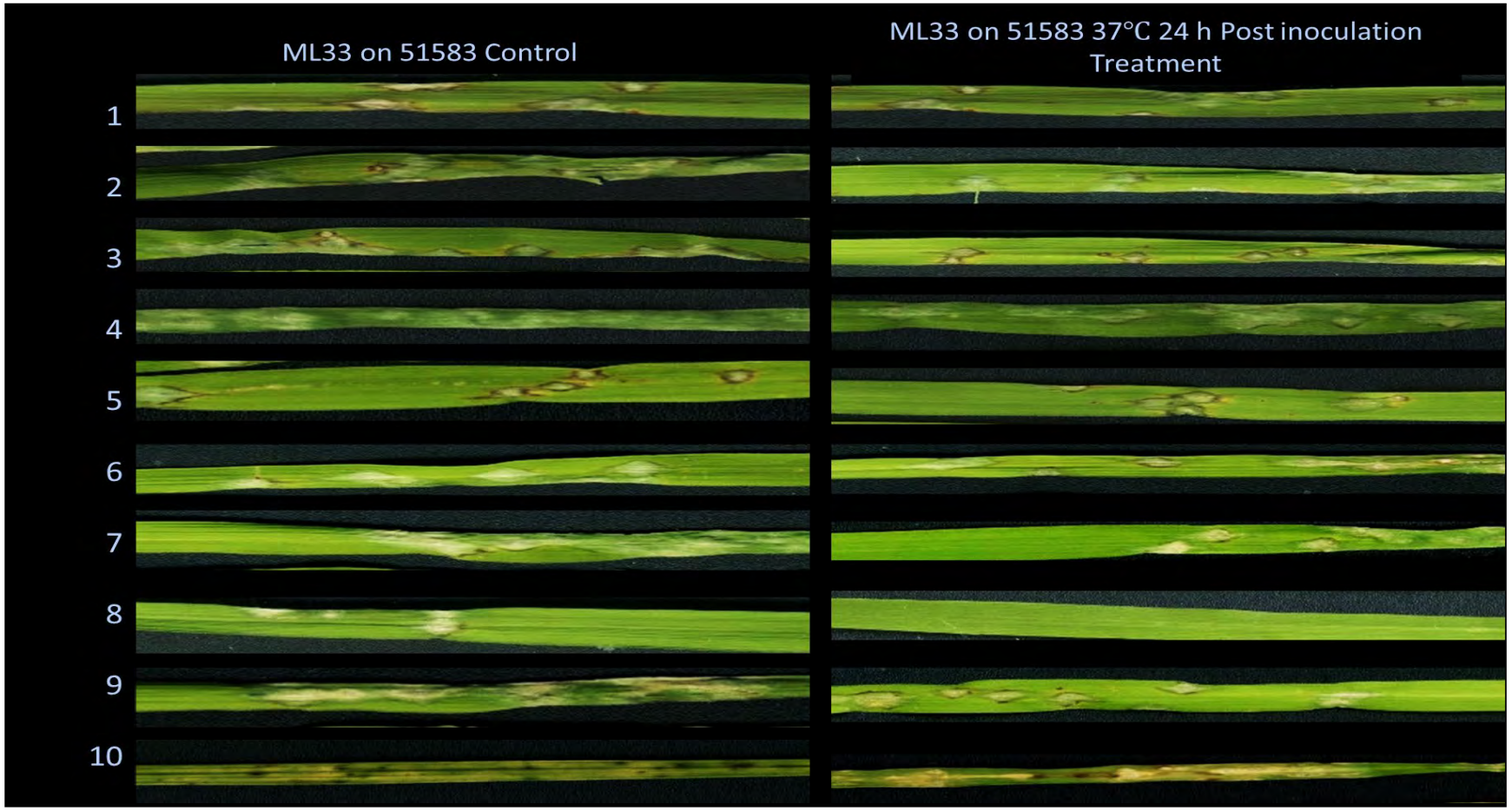
Secondary leaf scans of LTH inoculated with Guy11. Leaves shown are a representative example of the entire pot of plants.



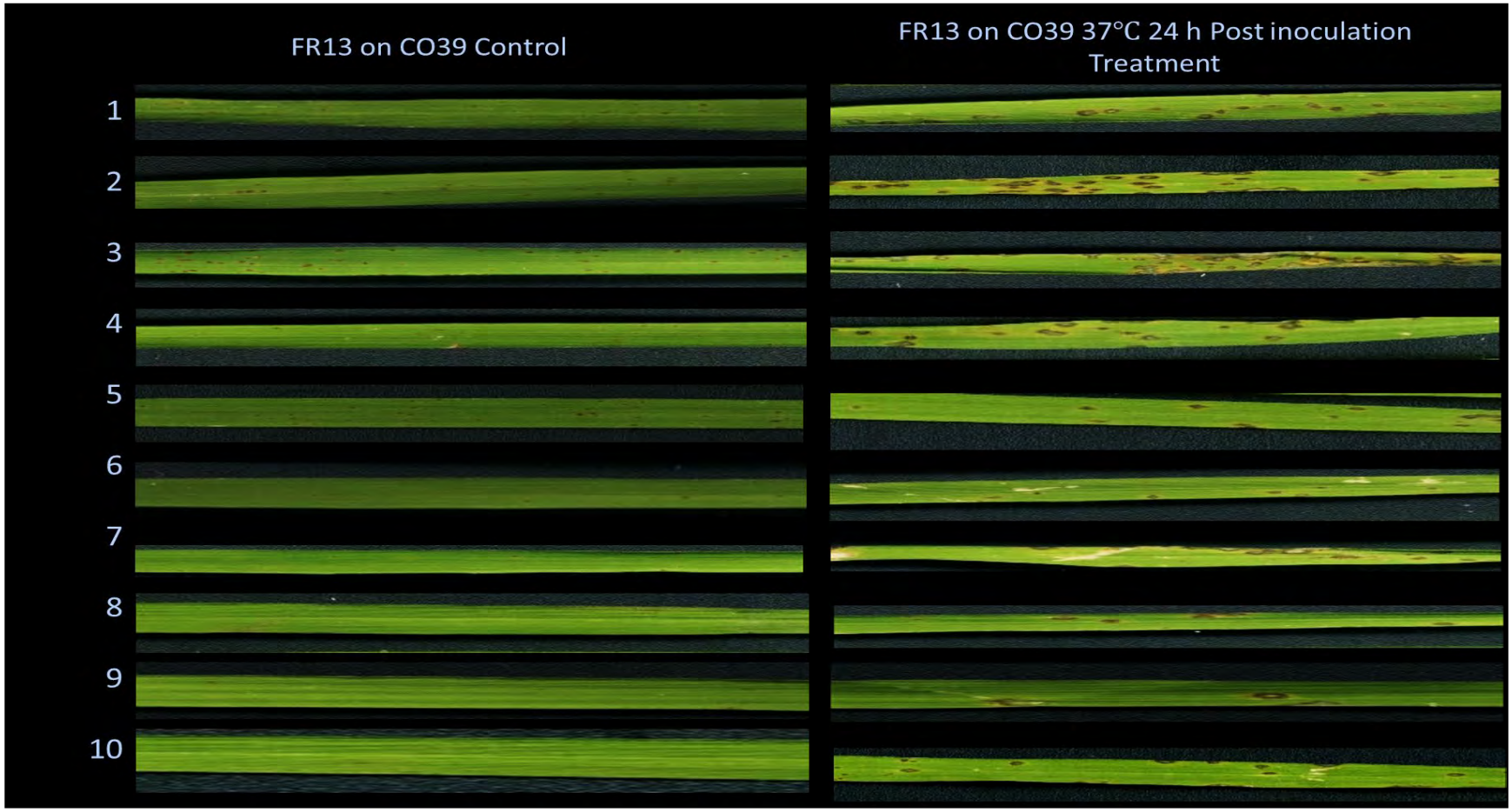
Secondary leaf scans of ToRide inoculated with Guy11. Leaves shown are a representative example of the entire pot of plants.



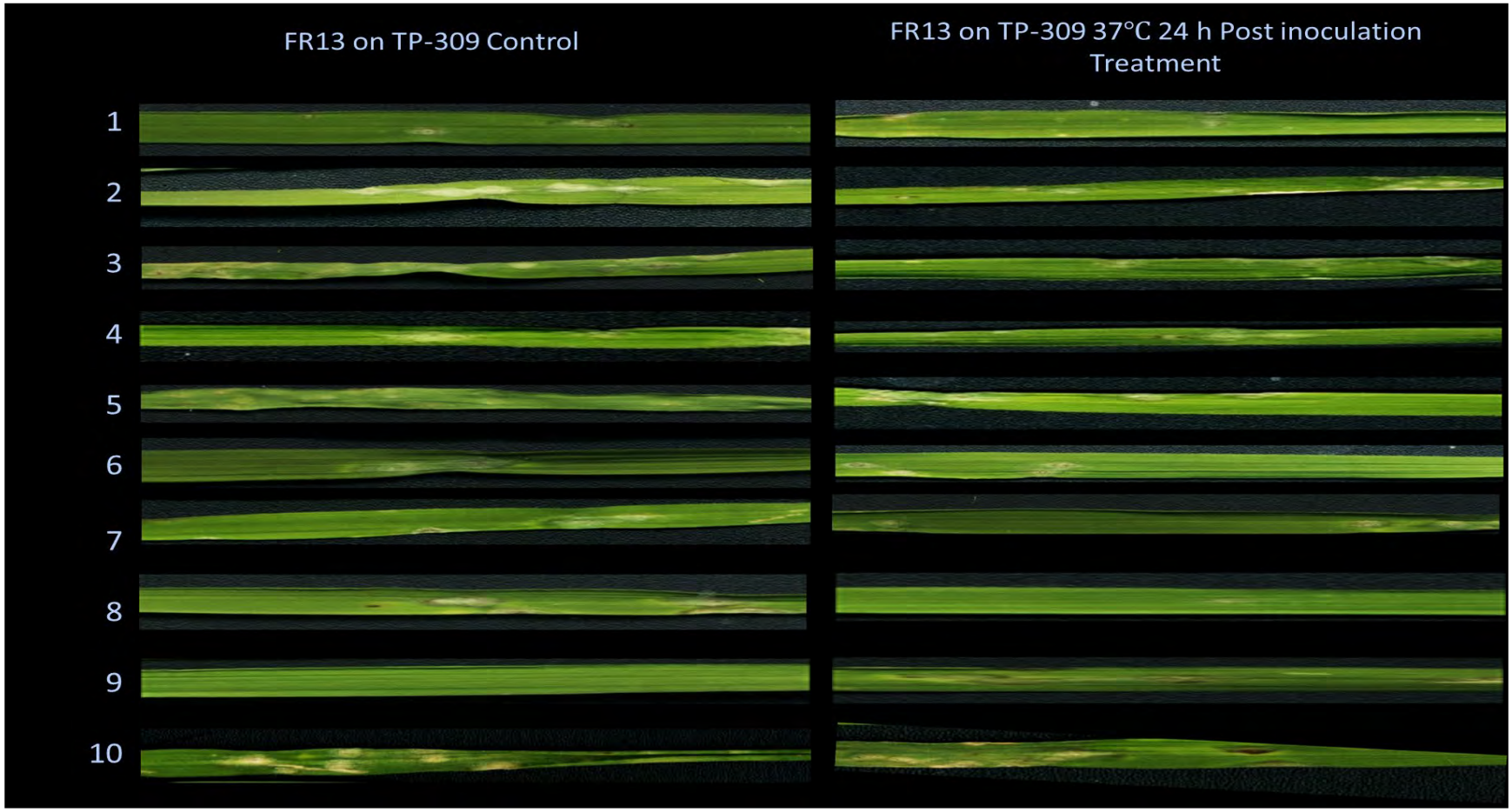
Secondary leaf scans of TP-309 inoculated with ML33. Leaves shown are a representative example of the entire pot of plants.



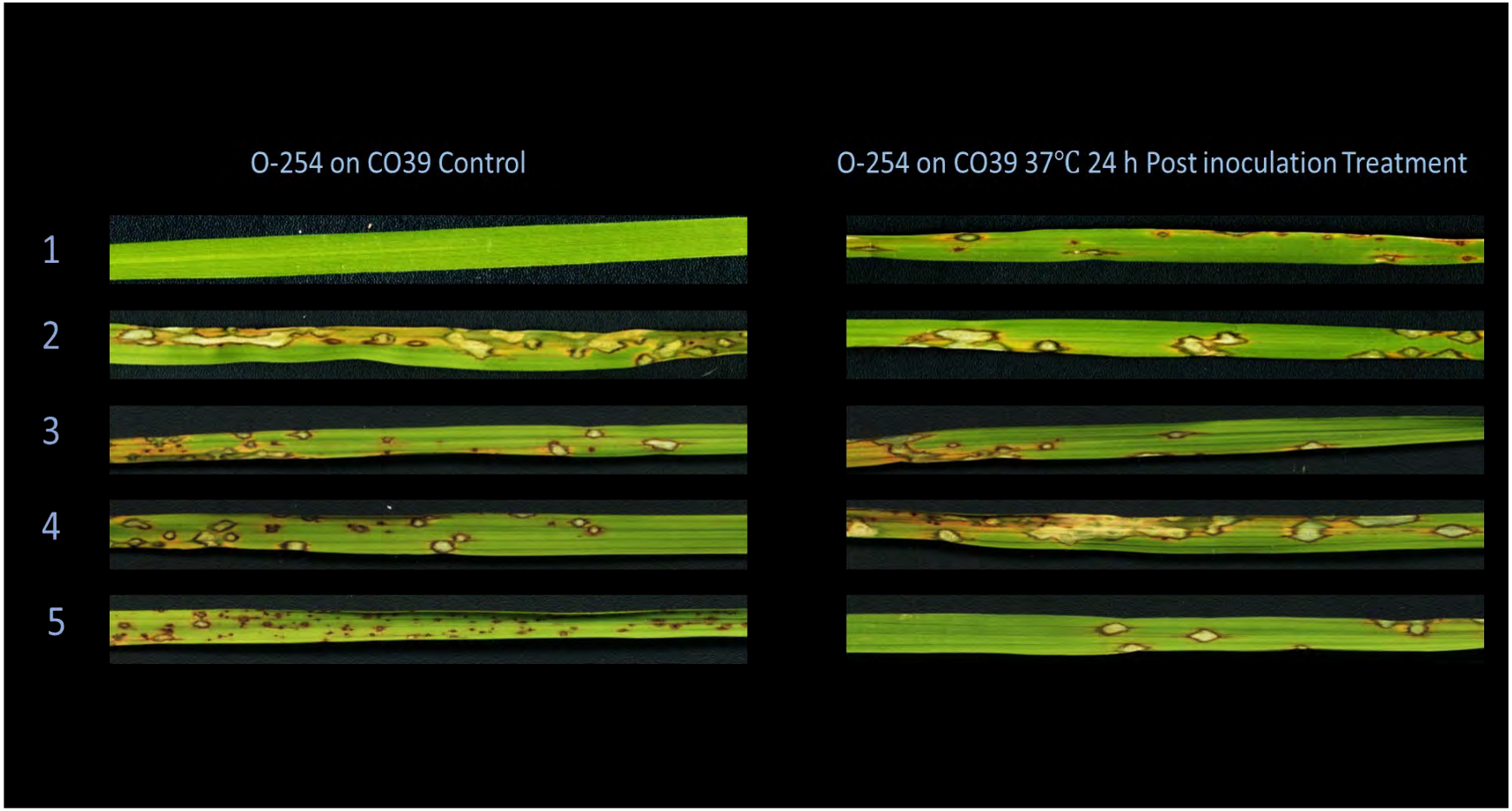
Secondary leaf scans of 51583 inoculated with ML33. Leaves shown are a representative example of the entire pot of plants.



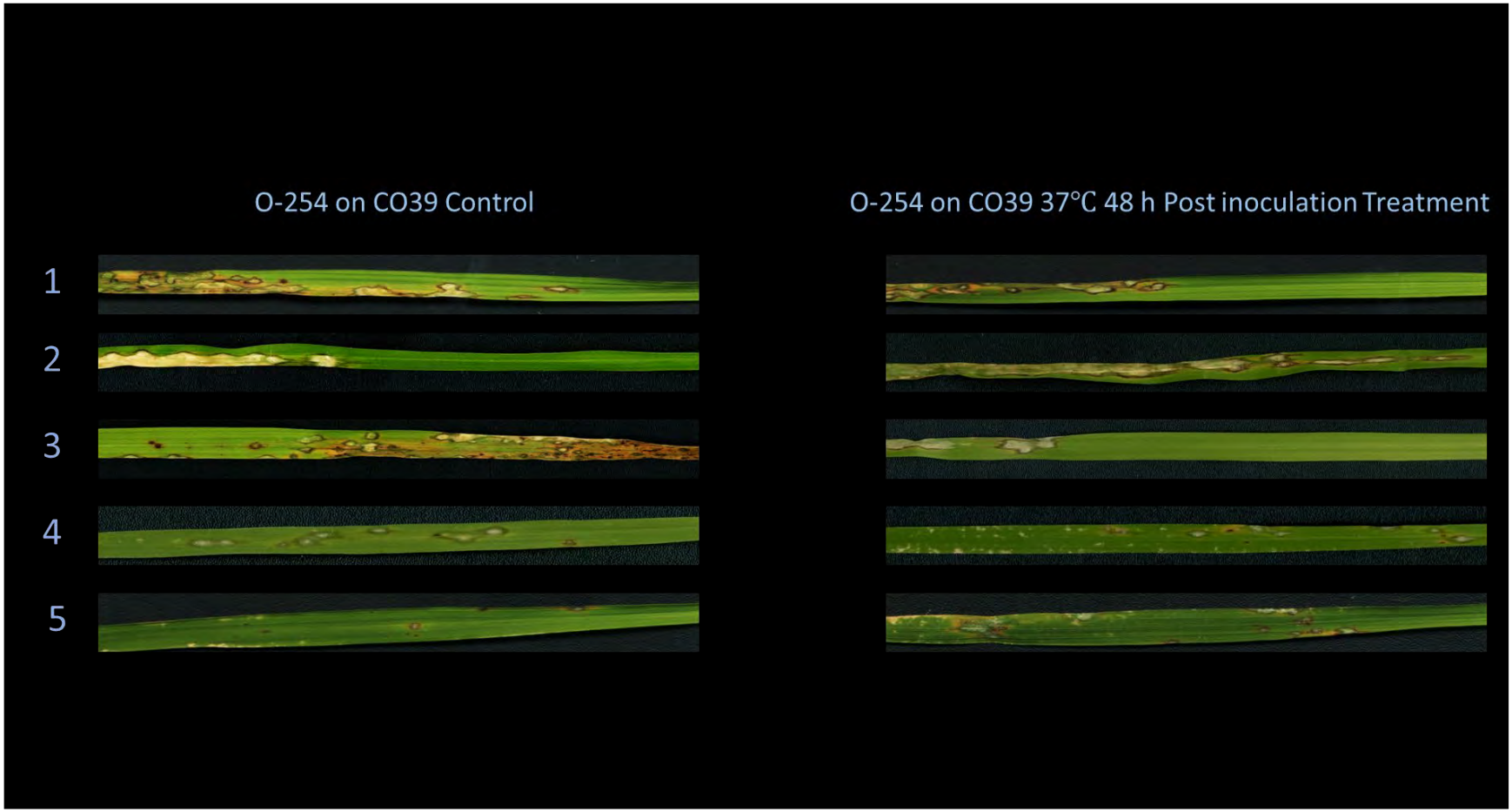
Secondary leaf scans of CO39 inoculated with FR13. Leaves shown are a representative example of the entire pot of plants.



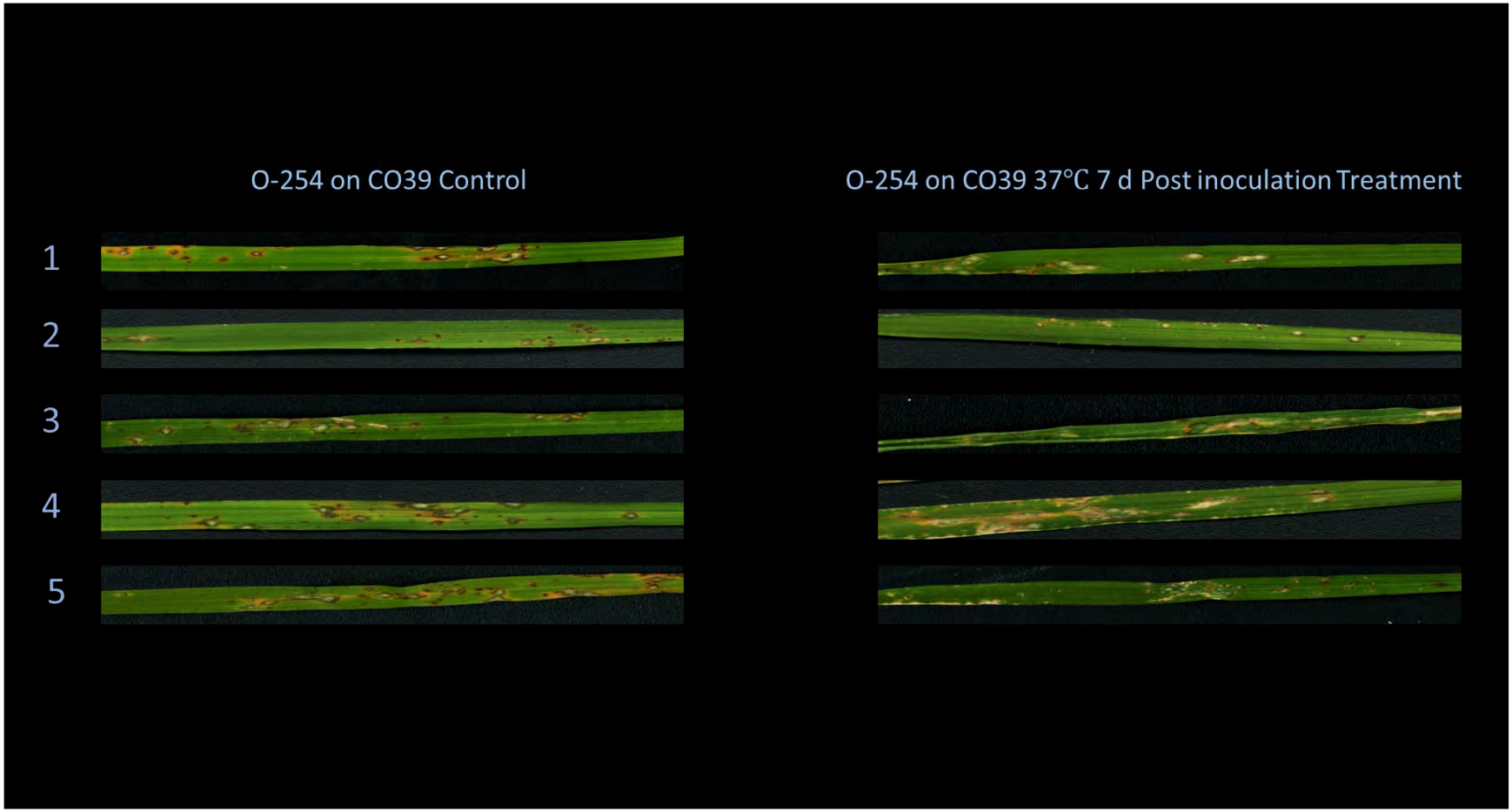
Secondary leaf scans of TP-309 inoculated with FR13. Leaves shown are a representative example of the entire pot of plants.



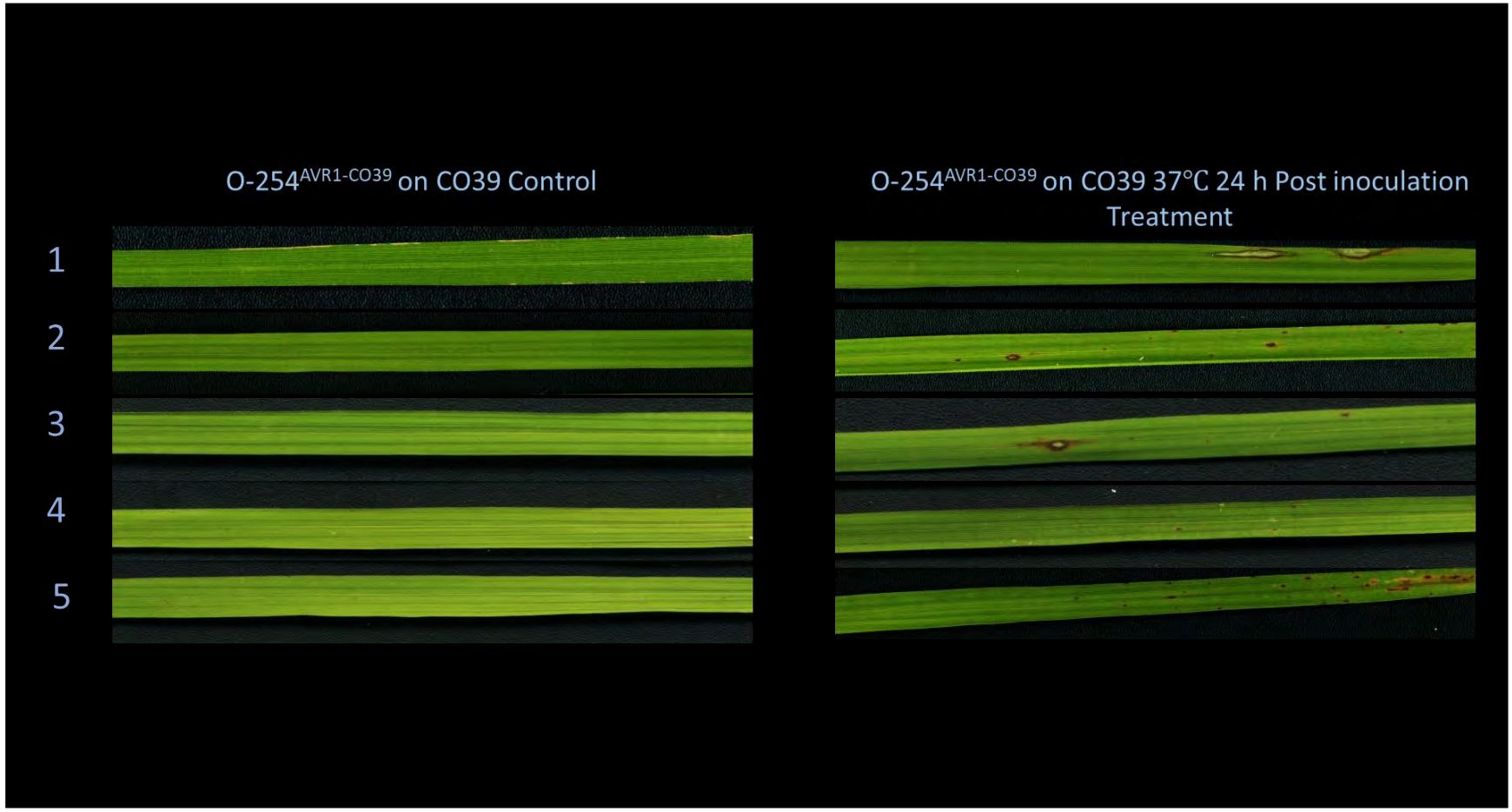
Secondary leaf scans of CO39 inoculated with O-254 with a 24 h post inoculation exposure to 37°C. Leaves shown are a representative example of the entire pot of plants.



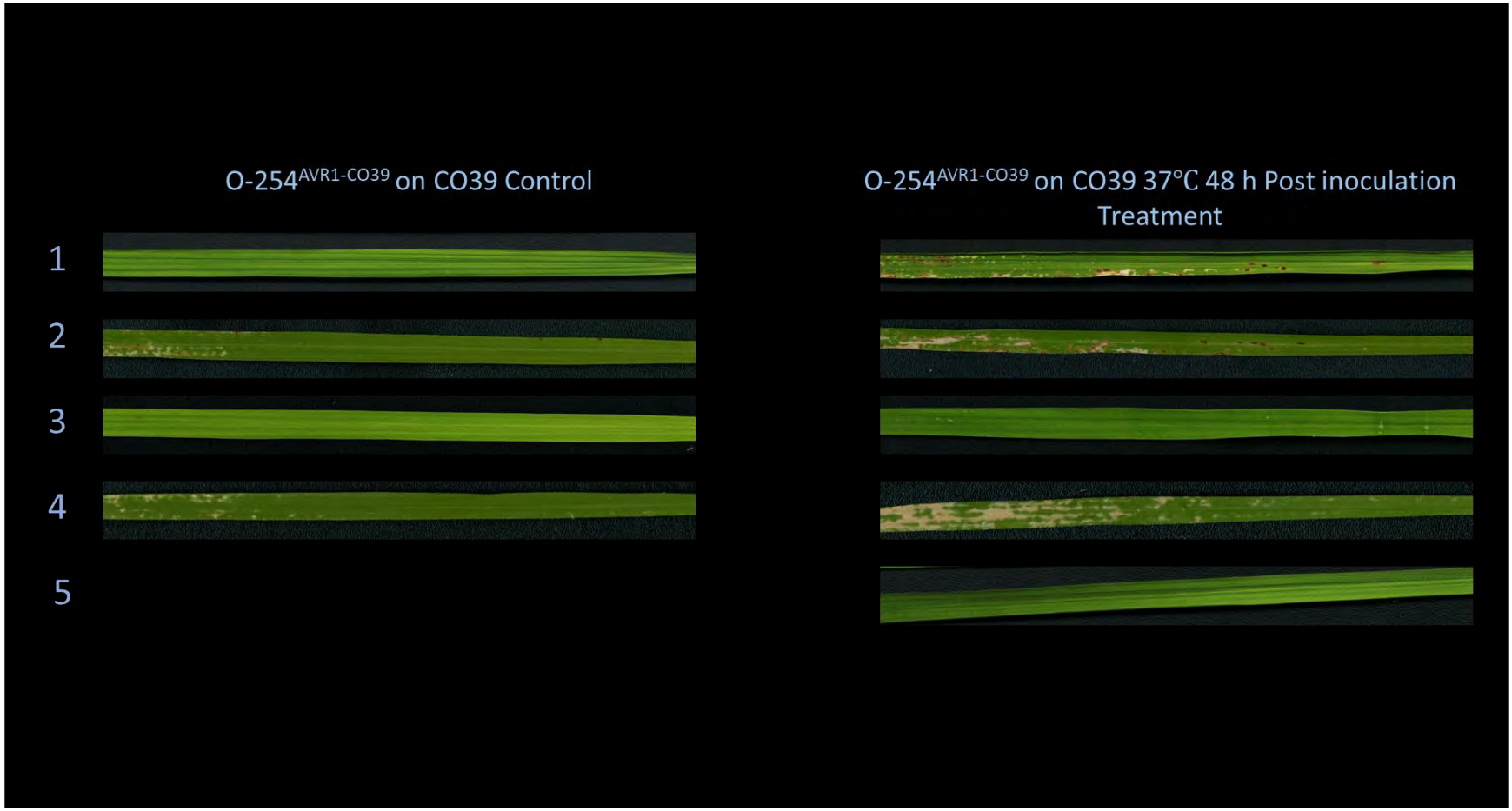
Secondary leaf scans of CO39 inoculated with O-254 with a 48 h post inoculation exposure to 37°C. Leaves shown are a representative example of the entire pot of plants.



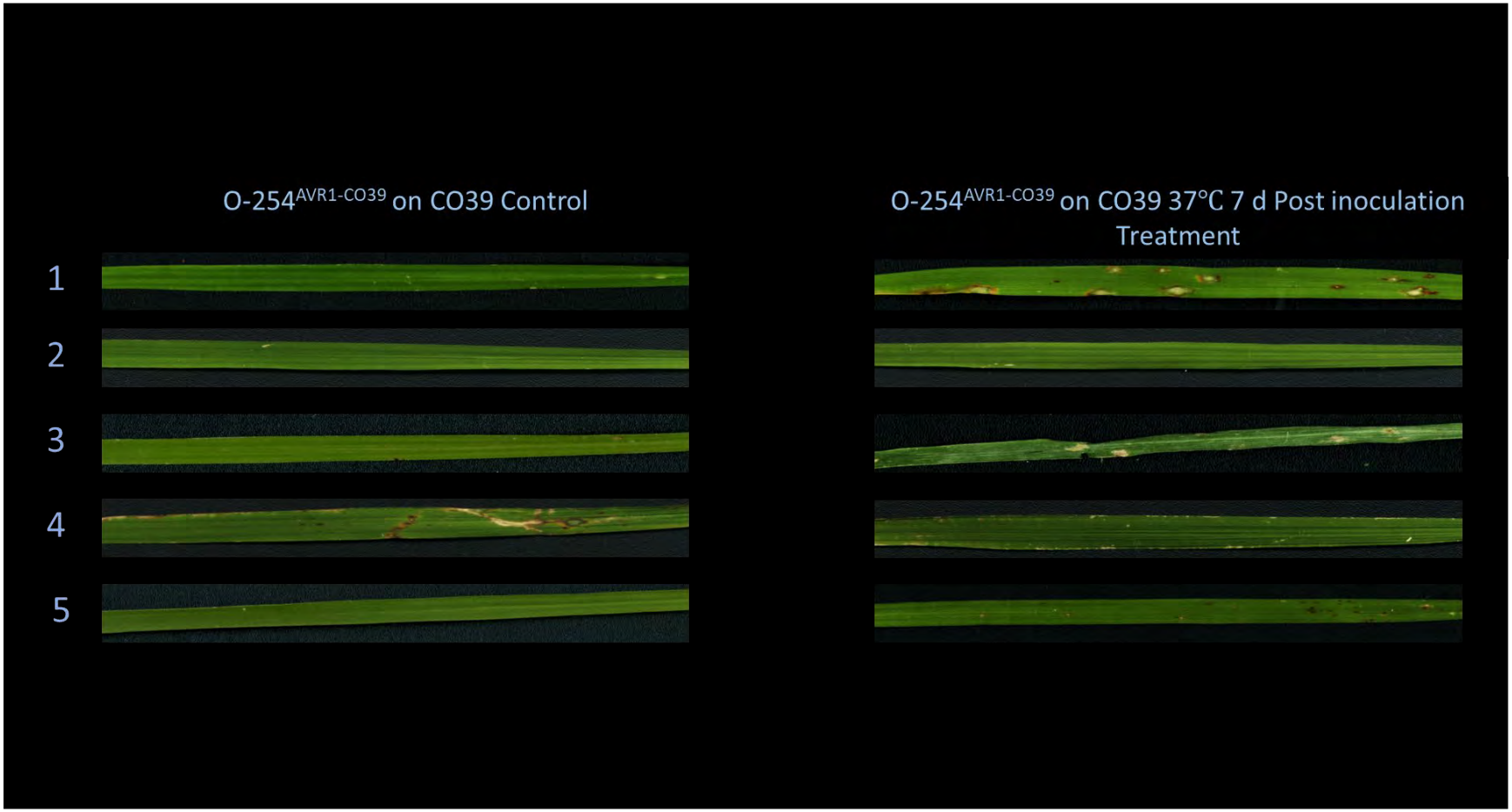
Secondary leaf scans of CO39 inoculated with O-254 with a 7 d post inoculation exposure to 37°C. Leaves shown are a representative example of the entire pot of plants.



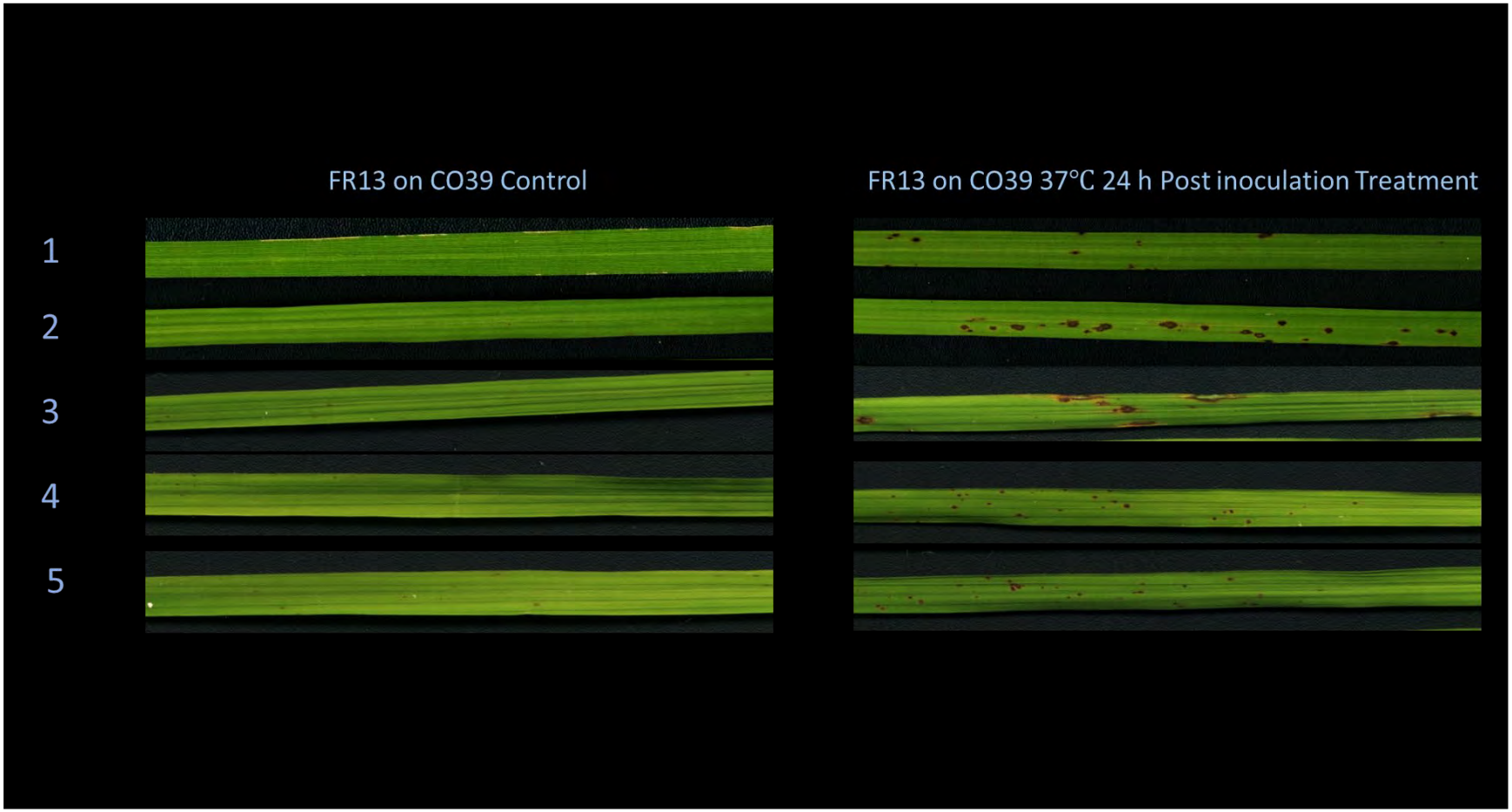
Secondary leaf scans of CO39 inoculated with O-254$^{AVR1\text{-}CO39}$ with a 24 h post inoculation exposure to 37°C. Leaves shown are a representative example of the entire pot of plants.



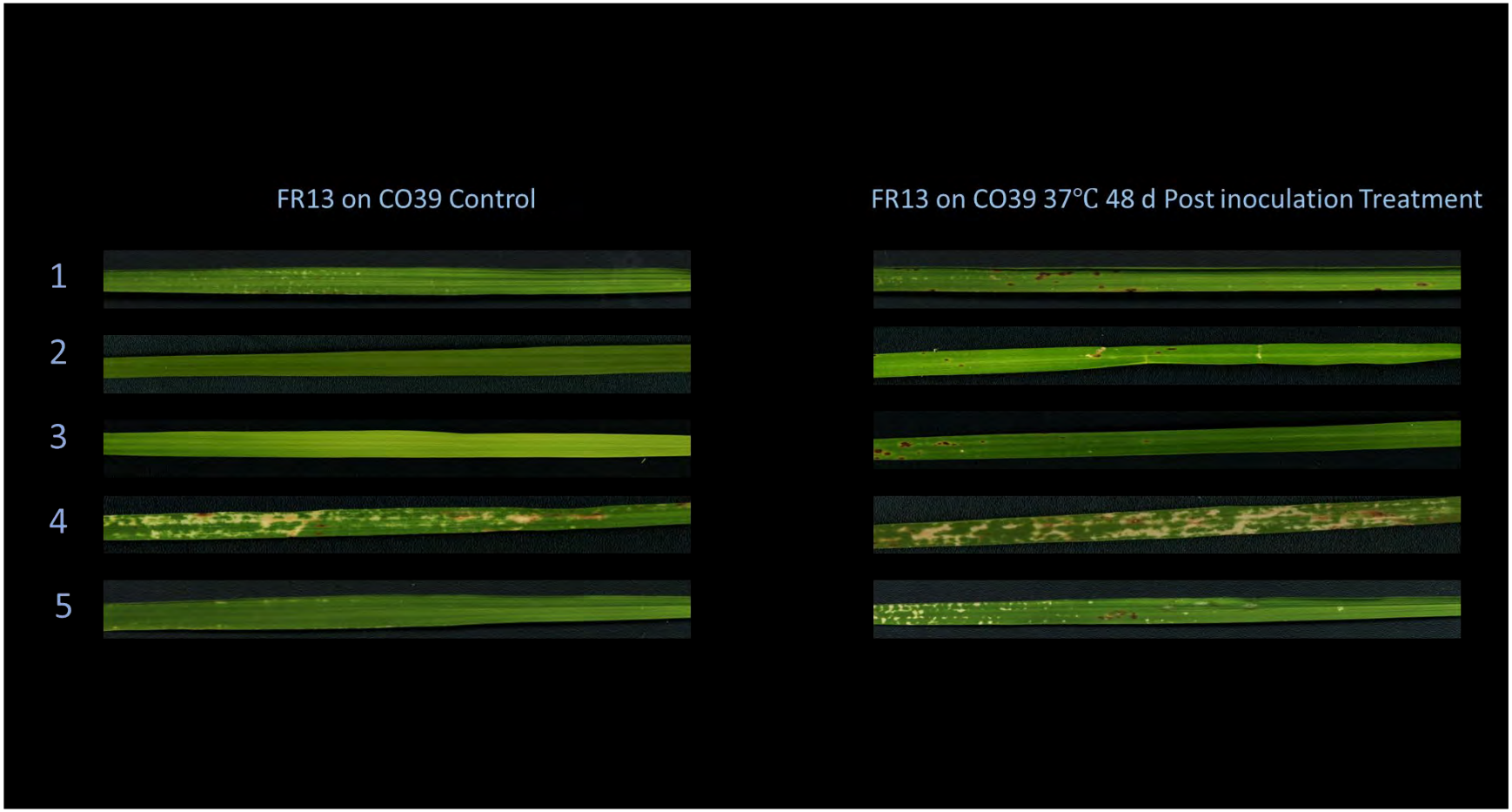
Secondary leaf scans of CO39 inoculated with O-254$^{AVR1\text{-}CO39}$ with a 48 h post inoculation exposure to 37°C. Leaves shown are a representative example of the entire pot of plants.



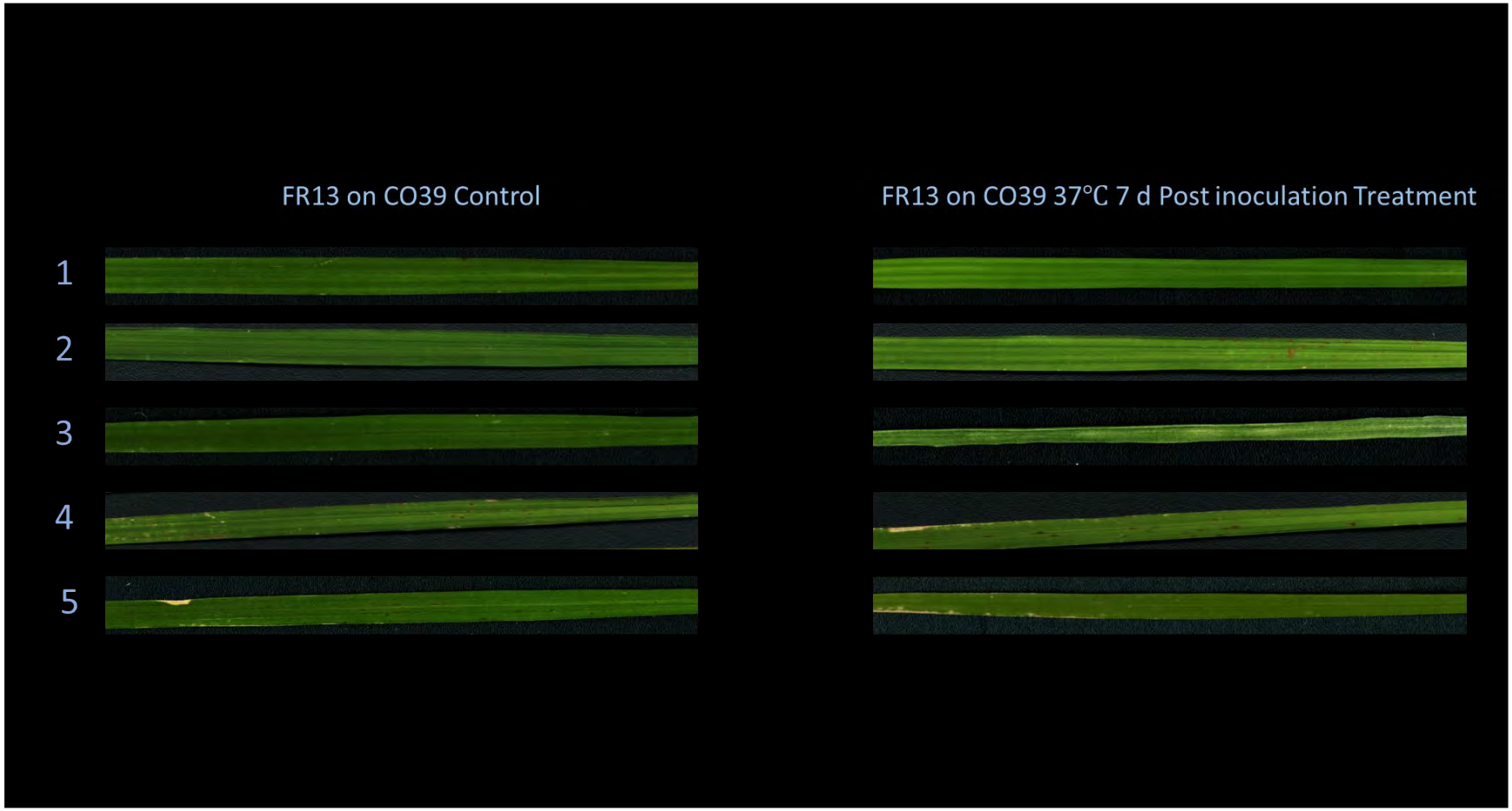
Secondary leaf scans of CO39 inoculated with O-254$^{AVR1\text{-}CO39}$ with a 7 d post inoculation exposure to 37°C. Leaves shown are a representative example of the entire pot of plants.



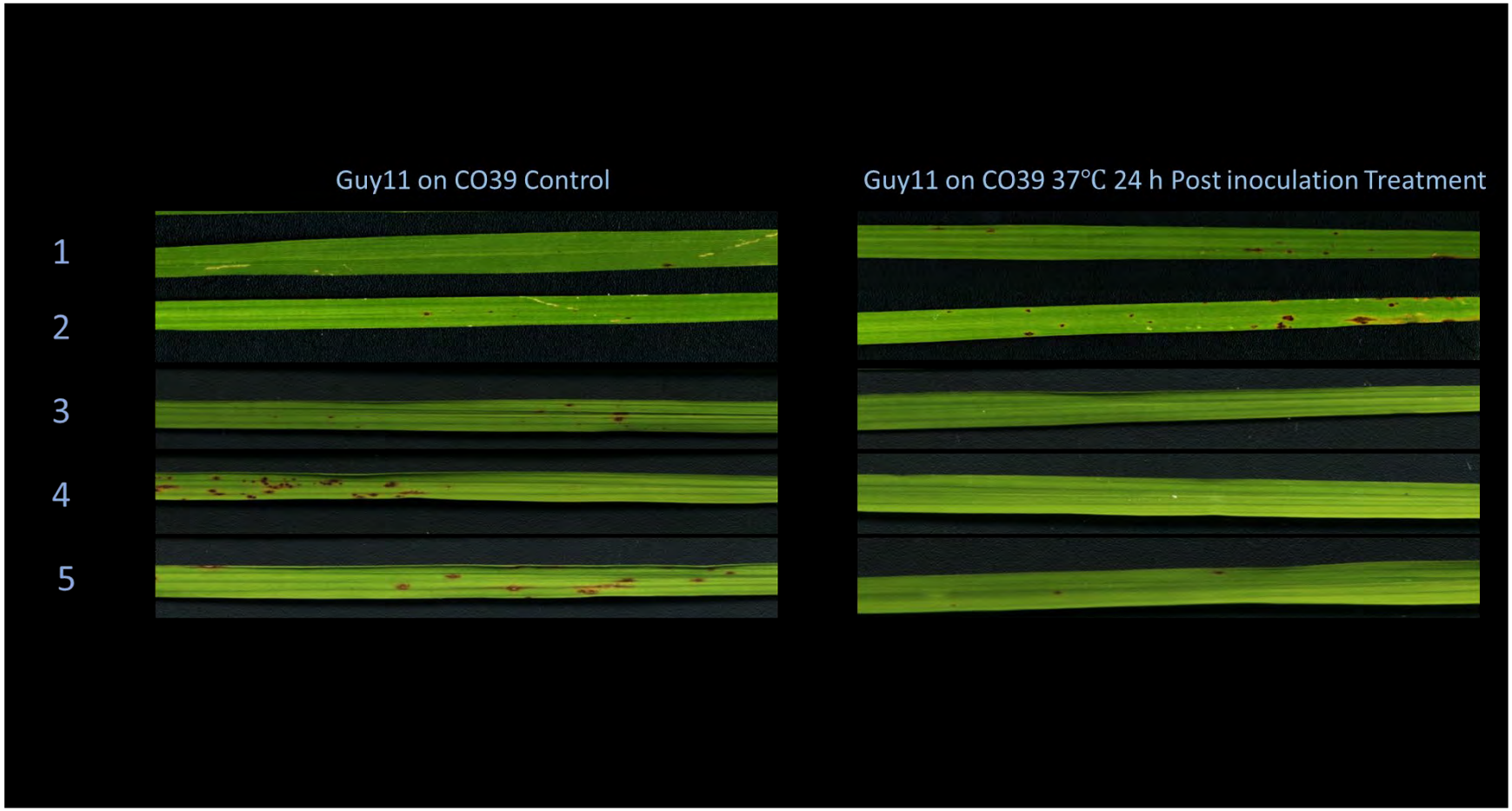
Secondary leaf scans of CO39 inoculated with FR13 with a 24 h post inoculation exposure to 37°C. Leaves shown are a representative example of the entire pot of plants.



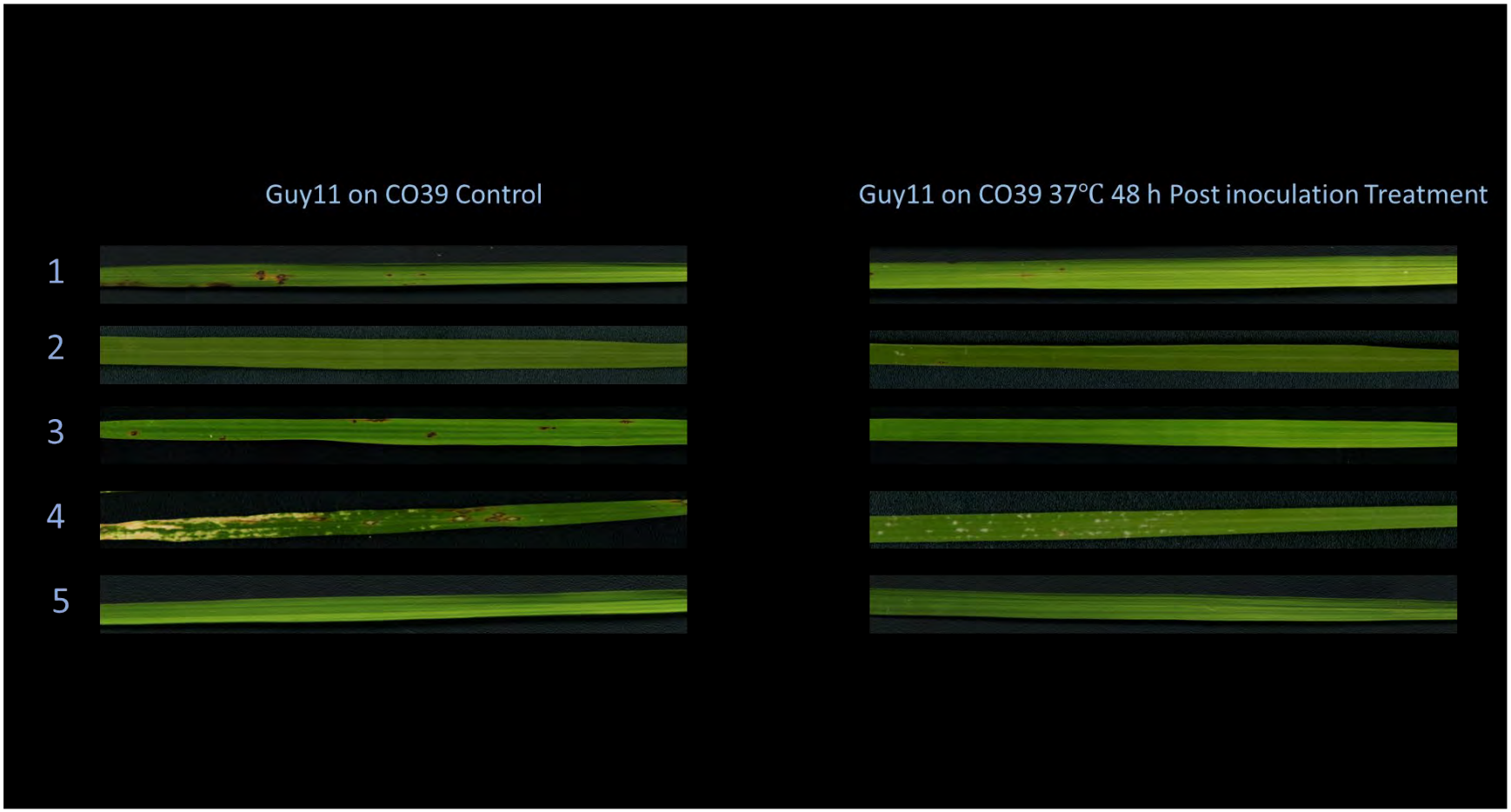
Secondary leaf scans of CO39 inoculated with FR13 with a 48 h post inoculation exposure to 37°C. Leaves shown are a representative example of the entire pot of plants.



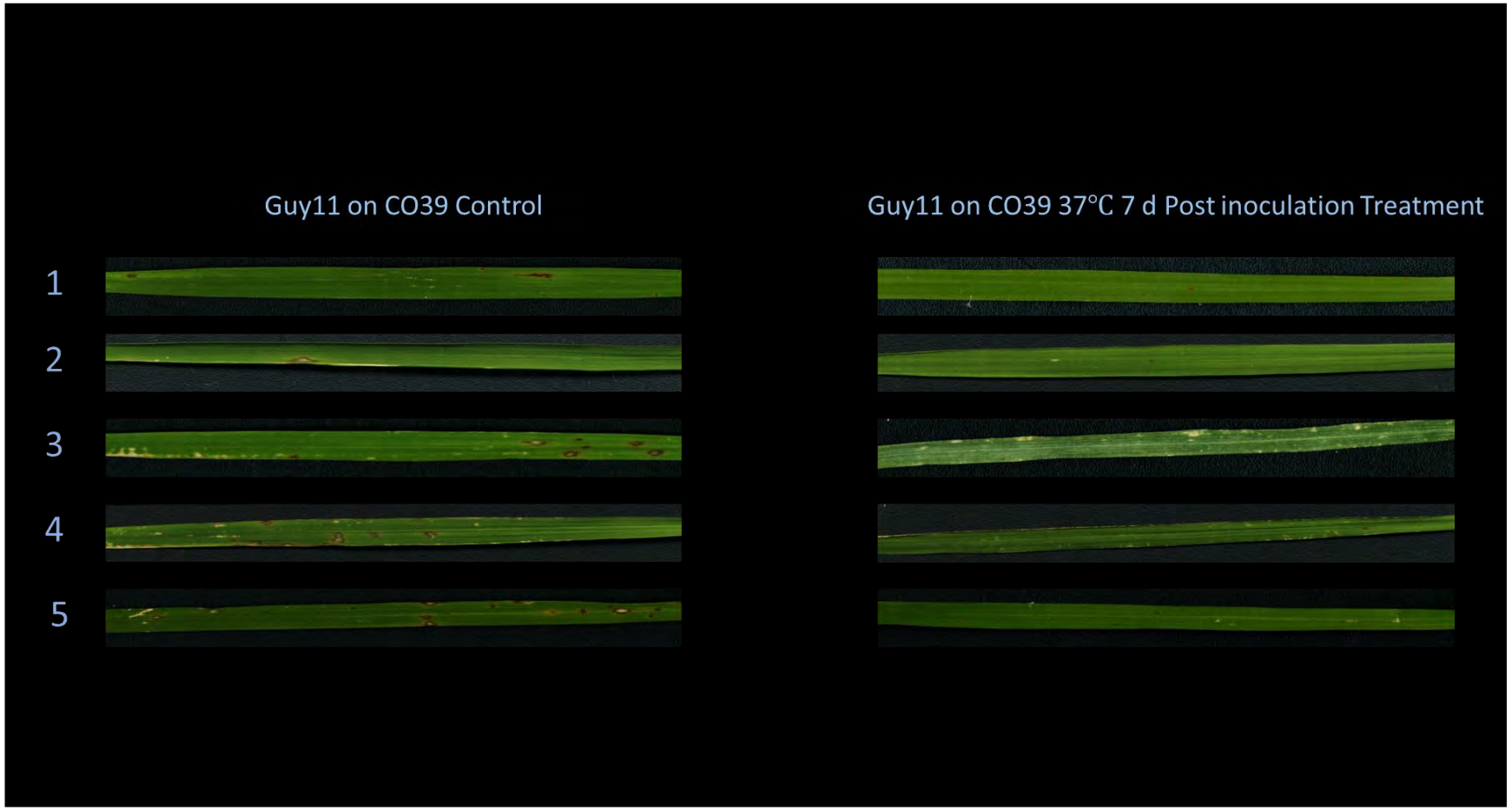
Secondary leaf scans of CO39 inoculated with FR13 with a 7 d post inoculation exposure to 37°C. Leaves shown are a representative example of the entire pot of plants.



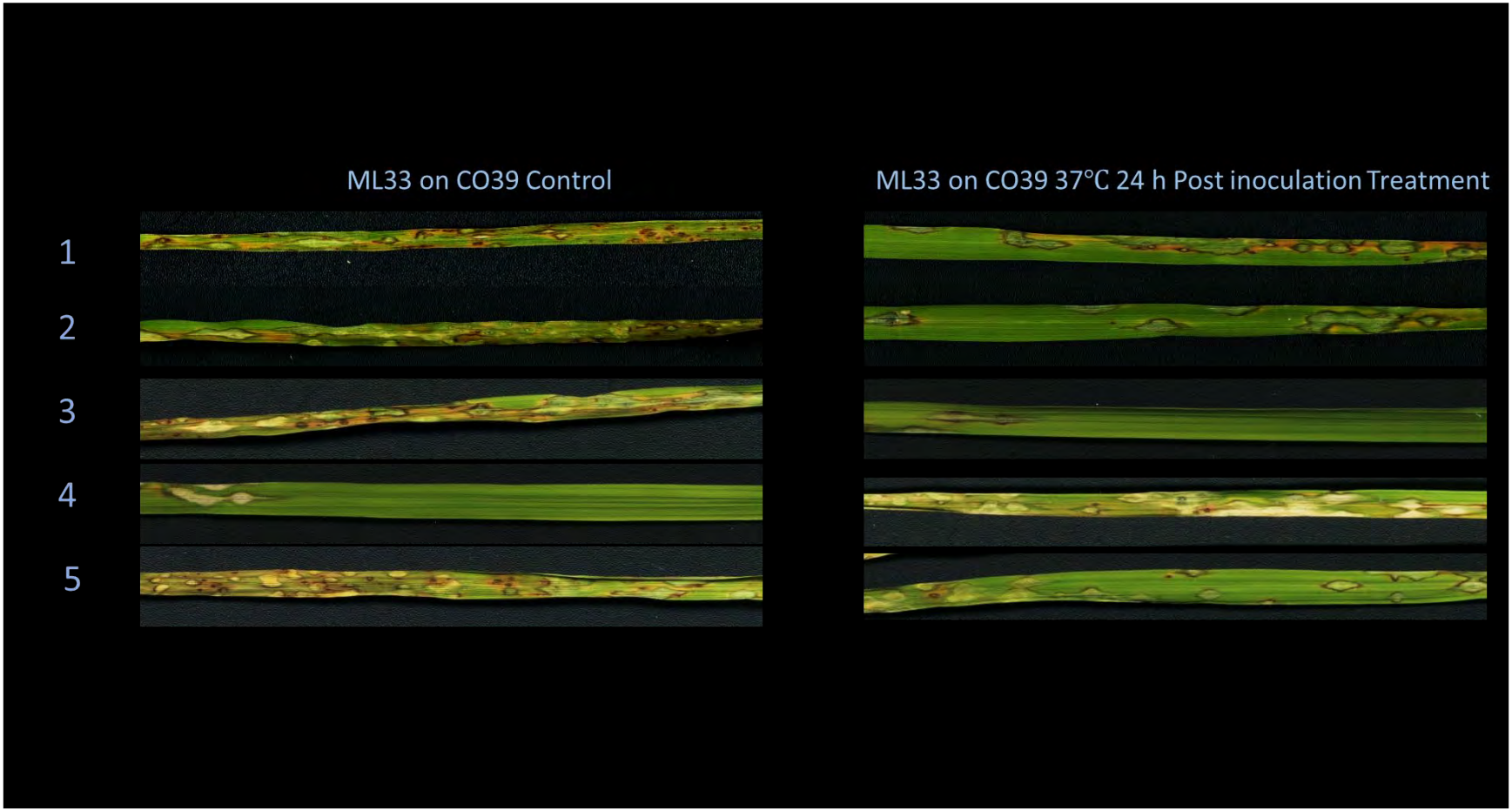
Secondary leaf scans of CO39 inoculated with Guy11 with a 24 h post inoculation exposure to 37°C. Leaves shown are a representative example of the entire pot of plants.



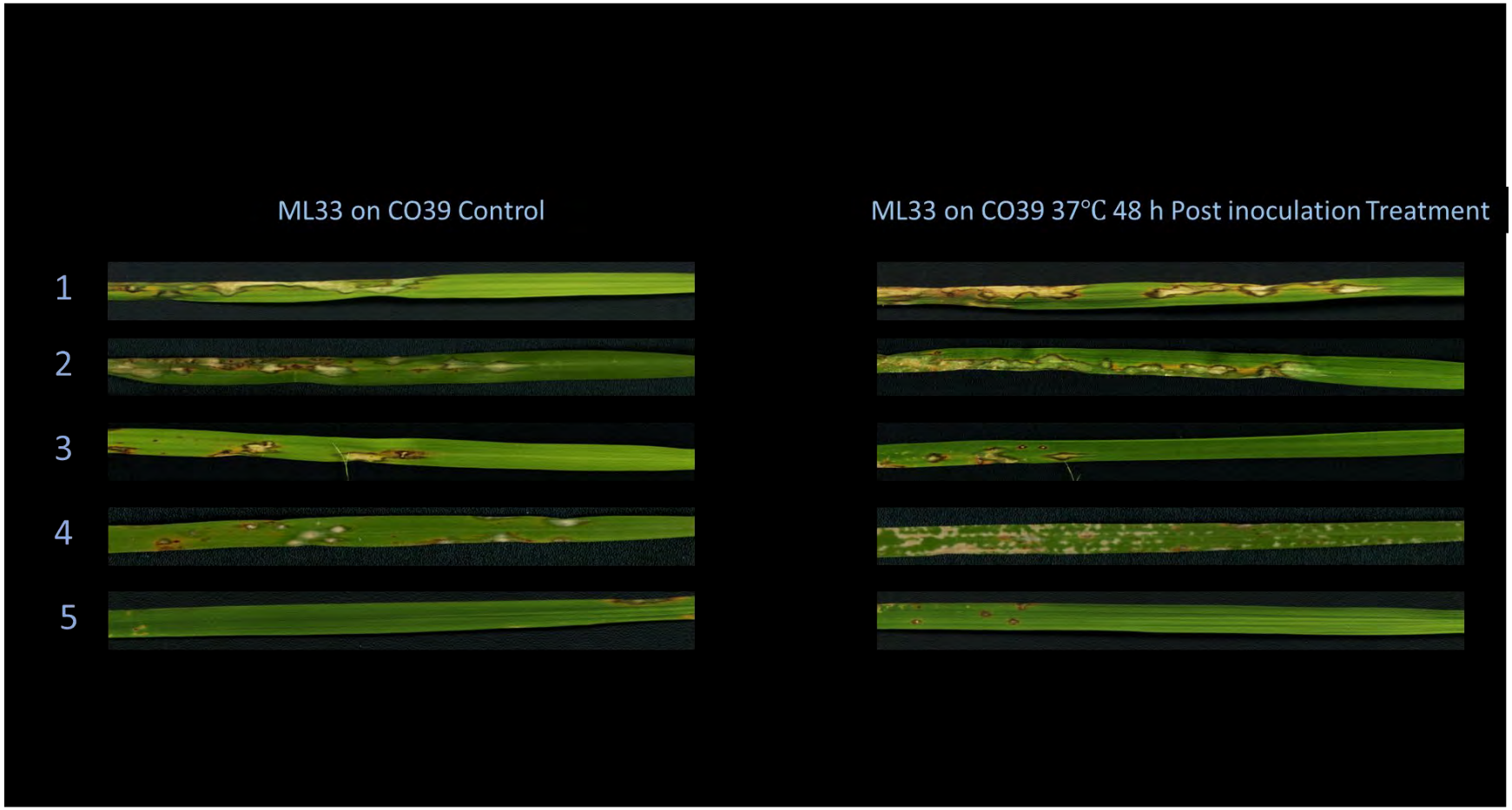
Secondary leaf scans of CO39 inoculated with Guy11 with a 48 h post inoculation exposure to 37°C. Leaves shown are a representative example of the entire pot of plants.



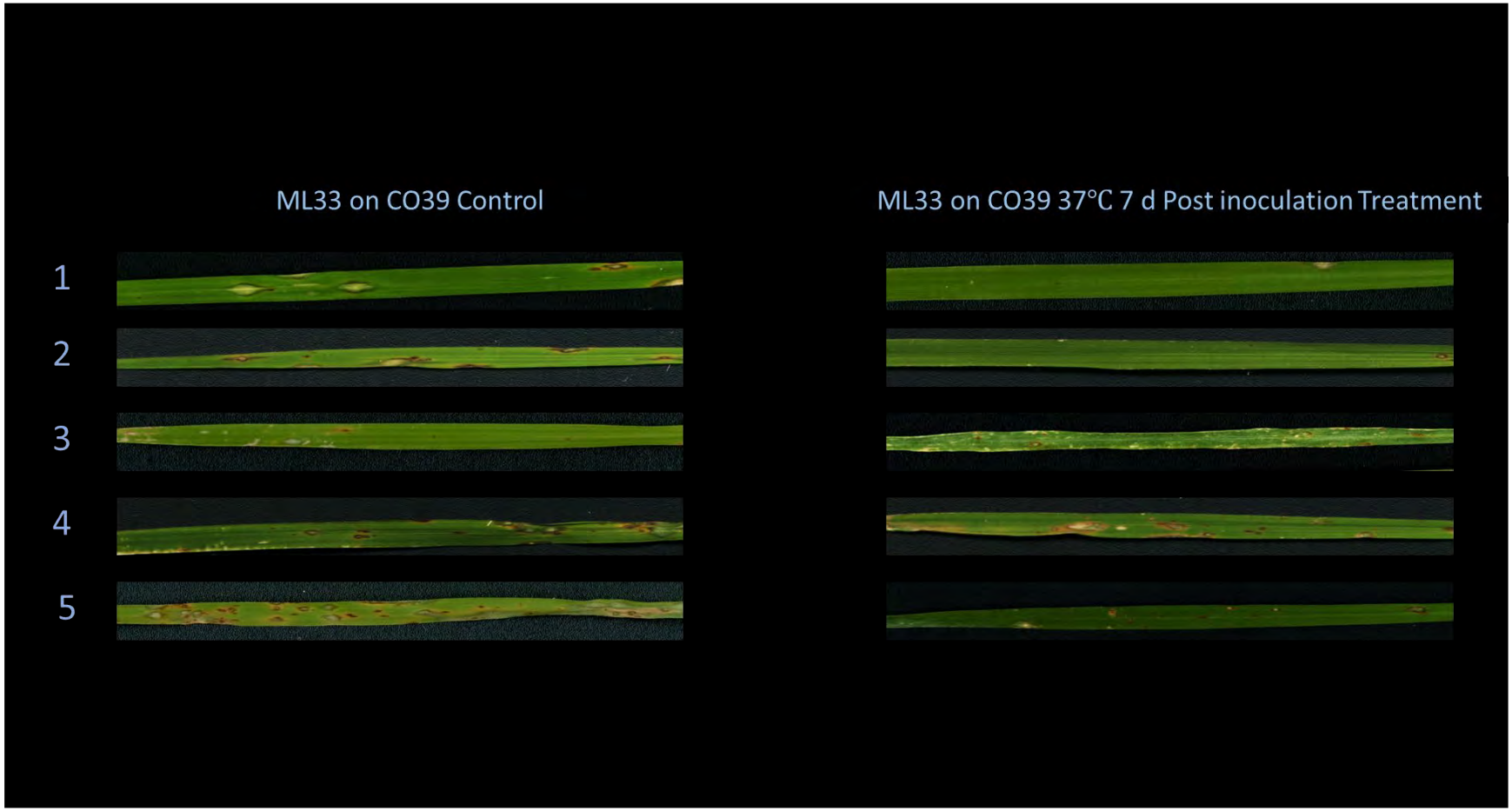
Secondary leaf scans of CO39 inoculated with Guy11 with a 7 d post inoculation exposure to 37°C. Leaves shown are a representative example of the entire pot of plants.



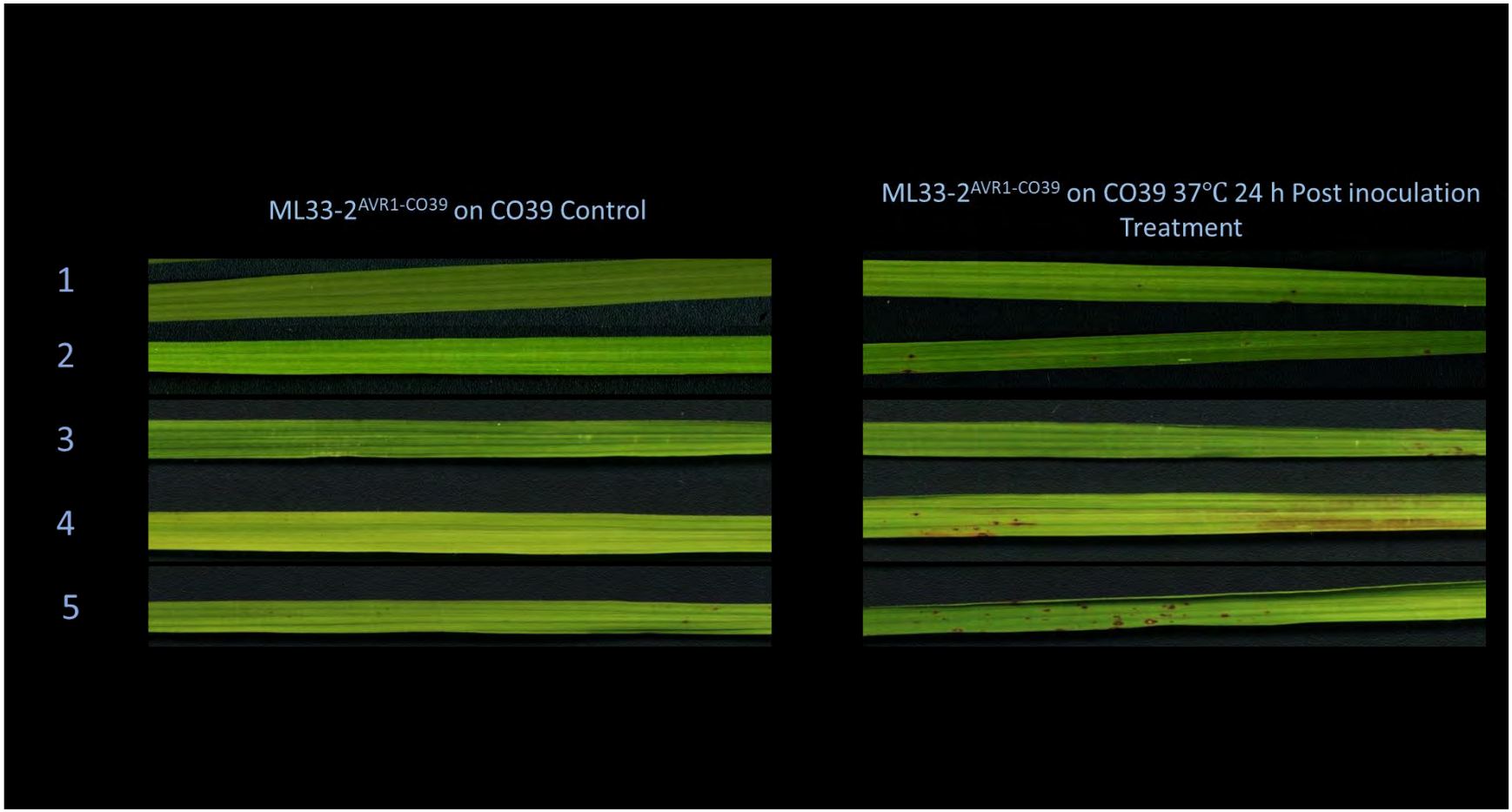
Secondary leaf scans of CO39 inoculated with ML33 with a 24 h post inoculation exposure to 37°C. Leaves shown are a representative example of the entire pot of plants.



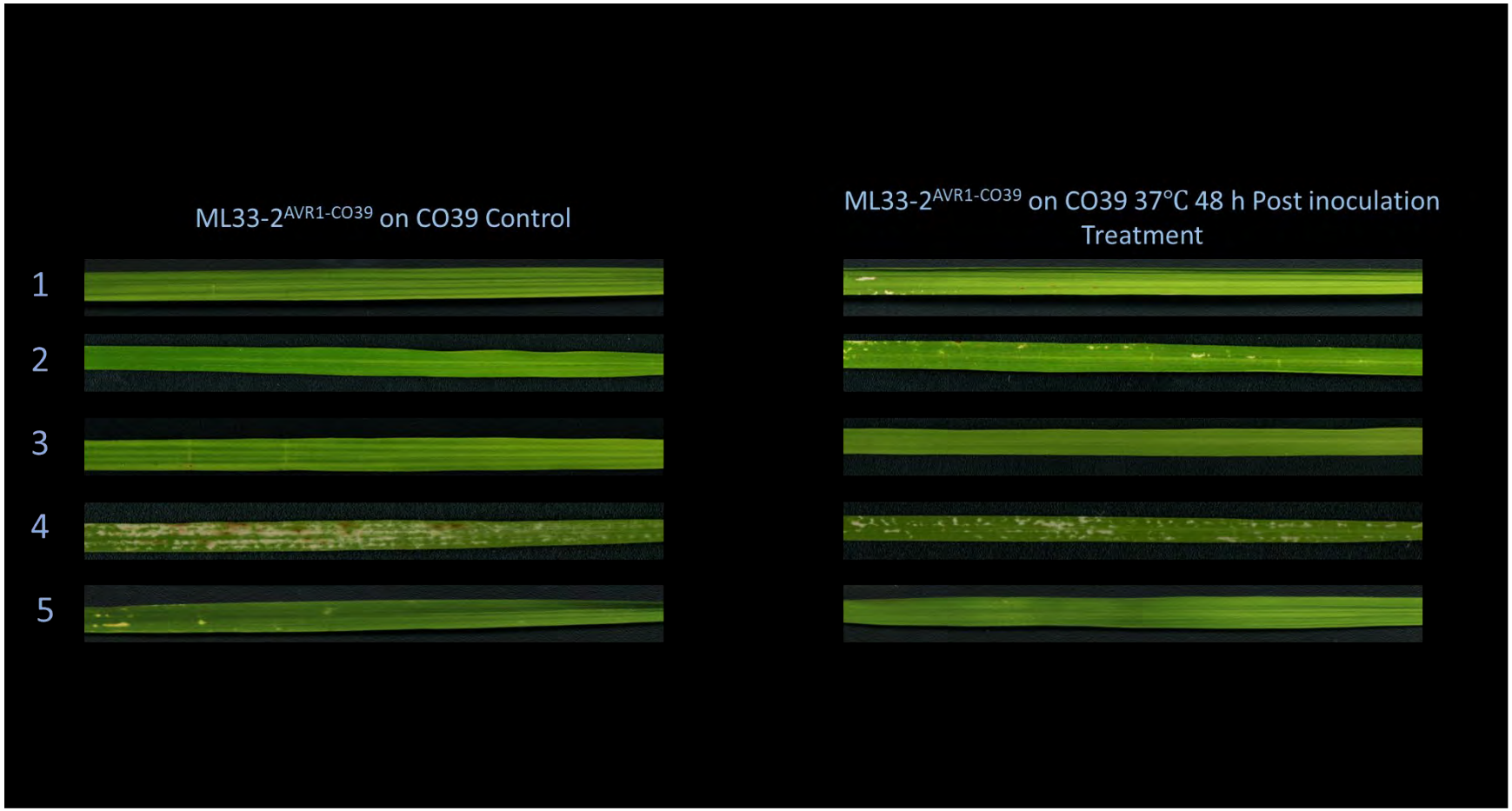
Secondary leaf scans of CO39 inoculated with ML33 with a 48 h post inoculation exposure to 37°C. Leaves shown are a representative example of the entire pot of plants.



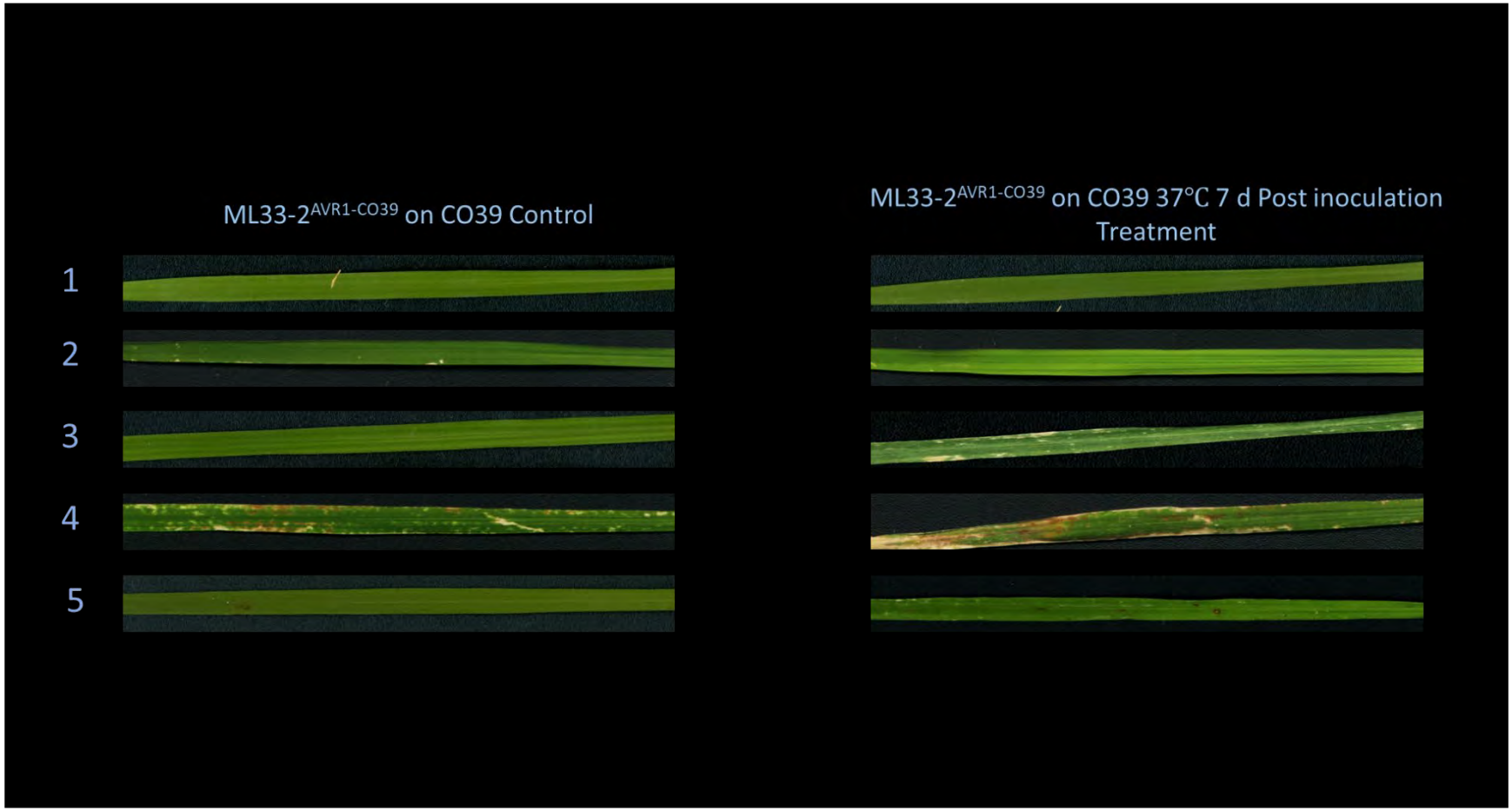
Secondary leaf scans of CO39 inoculated with ML33 with a 7 d post inoculation exposure to 37°C. Leaves shown are a representative example of the entire pot of plants.



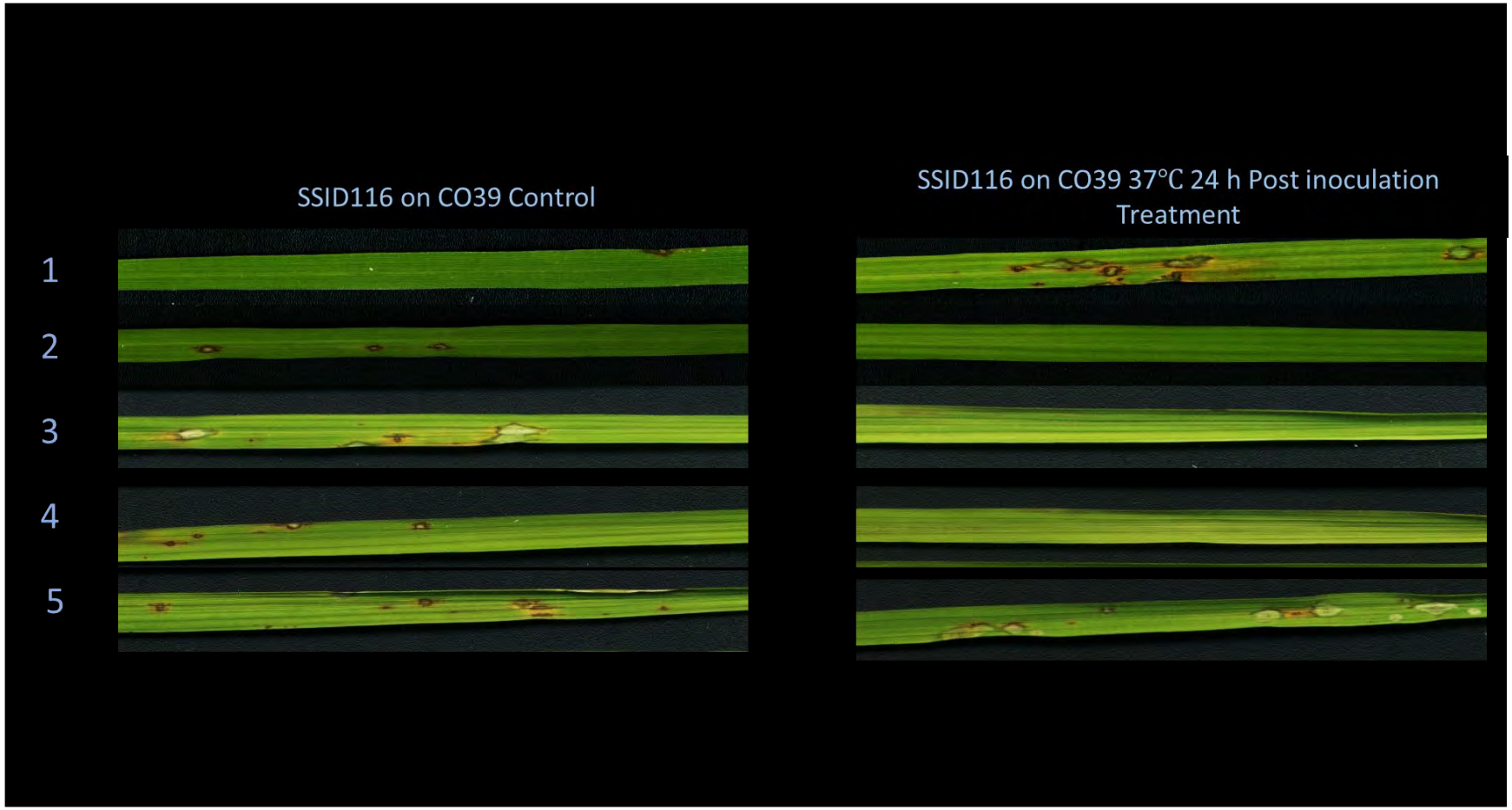
Secondary leaf scans of CO39 inoculated with ML33$^{AVR1\text{-}CO39}$ with a 24 h post inoculation exposure to 37°C. Leaves shown are a representative example of the entire pot of plants.



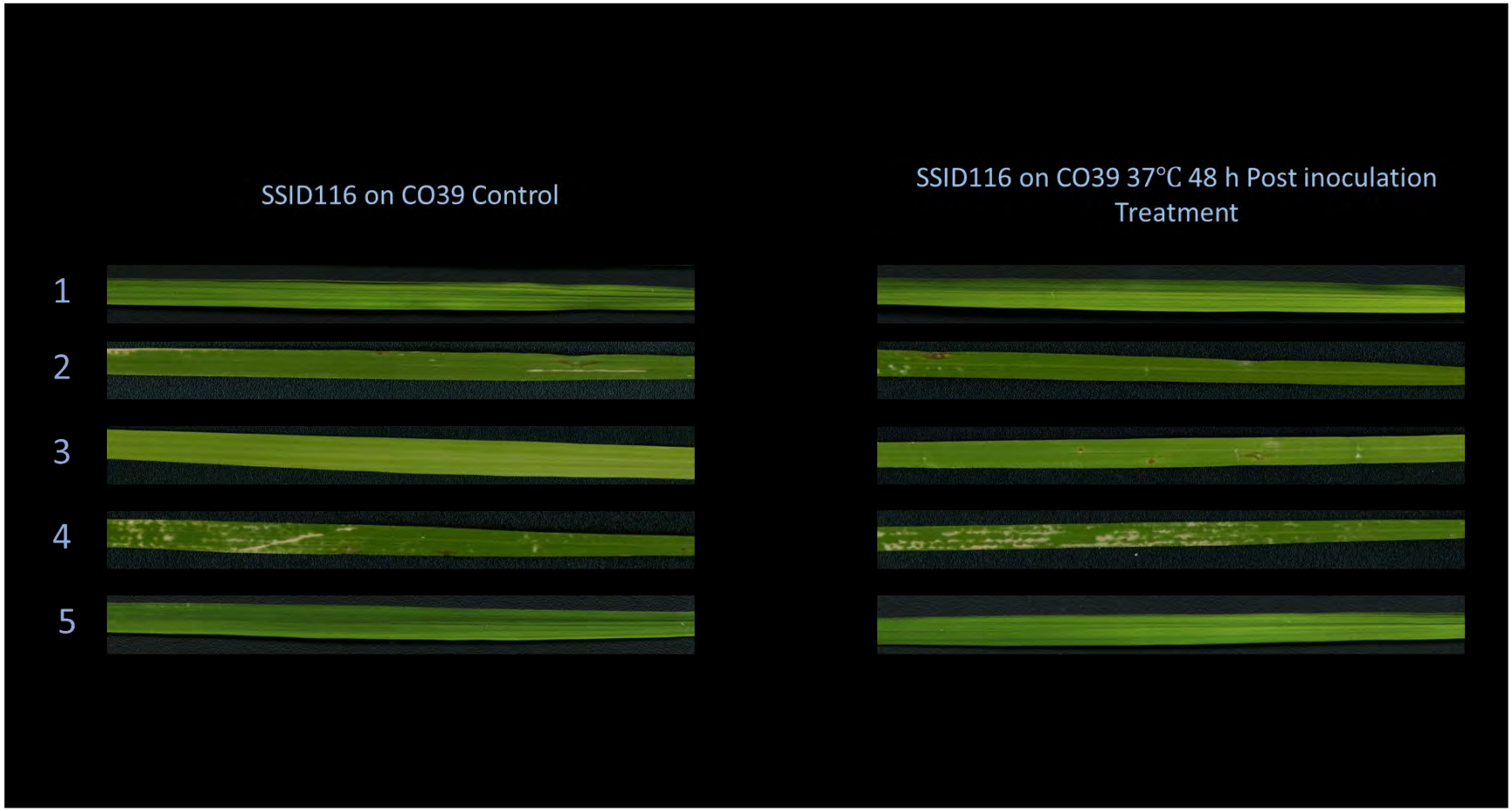
Secondary leaf scans of CO39 inoculated with ML33$^{AVR1\text{-}CO39}$ with a 48 h post inoculation exposure to 37°C. Leaves shown are a representative example of the entire pot of plants.



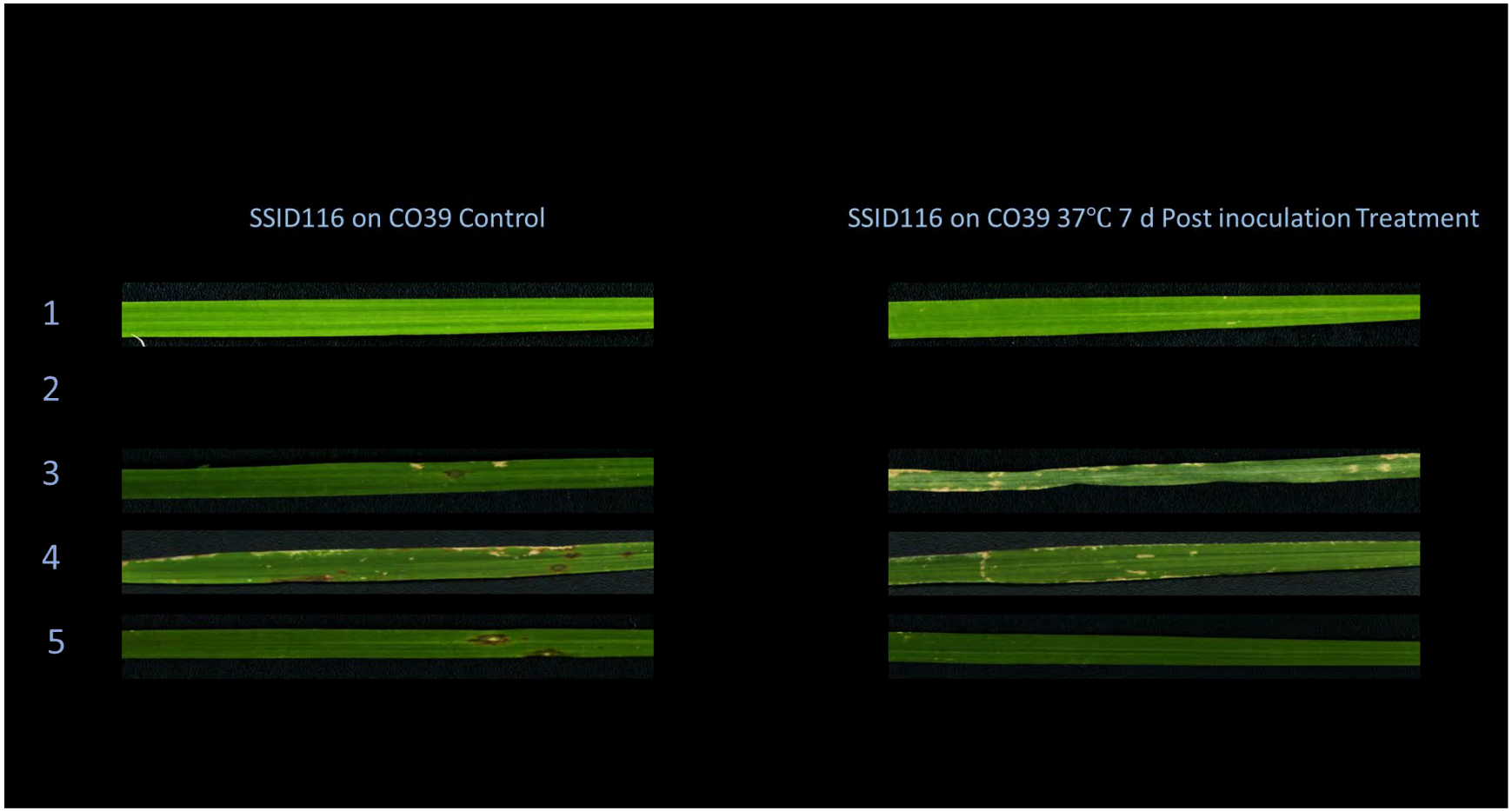
Secondary leaf scans of CO39 inoculated with ML33$^{AVR1\text{-}CO39}$ with a 7 d post inoculation exposure to 37°C. Leaves shown are a representative example of the entire pot of plants.



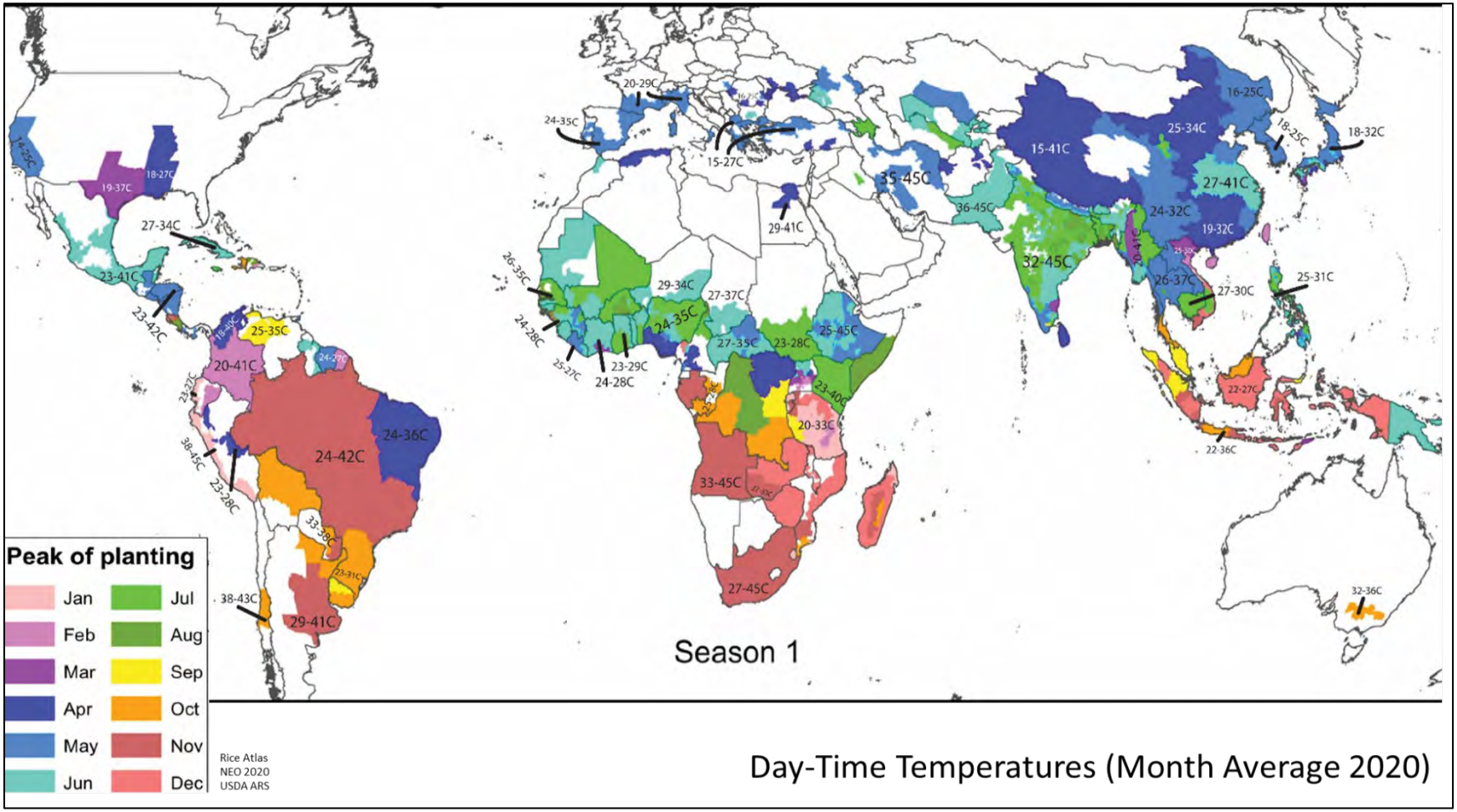
Secondary leaf scans of CO39 inoculated with SSID116 with a 24 h post inoculation exposure to 37°C. Leaves shown are a representative example of the entire pot of plants.



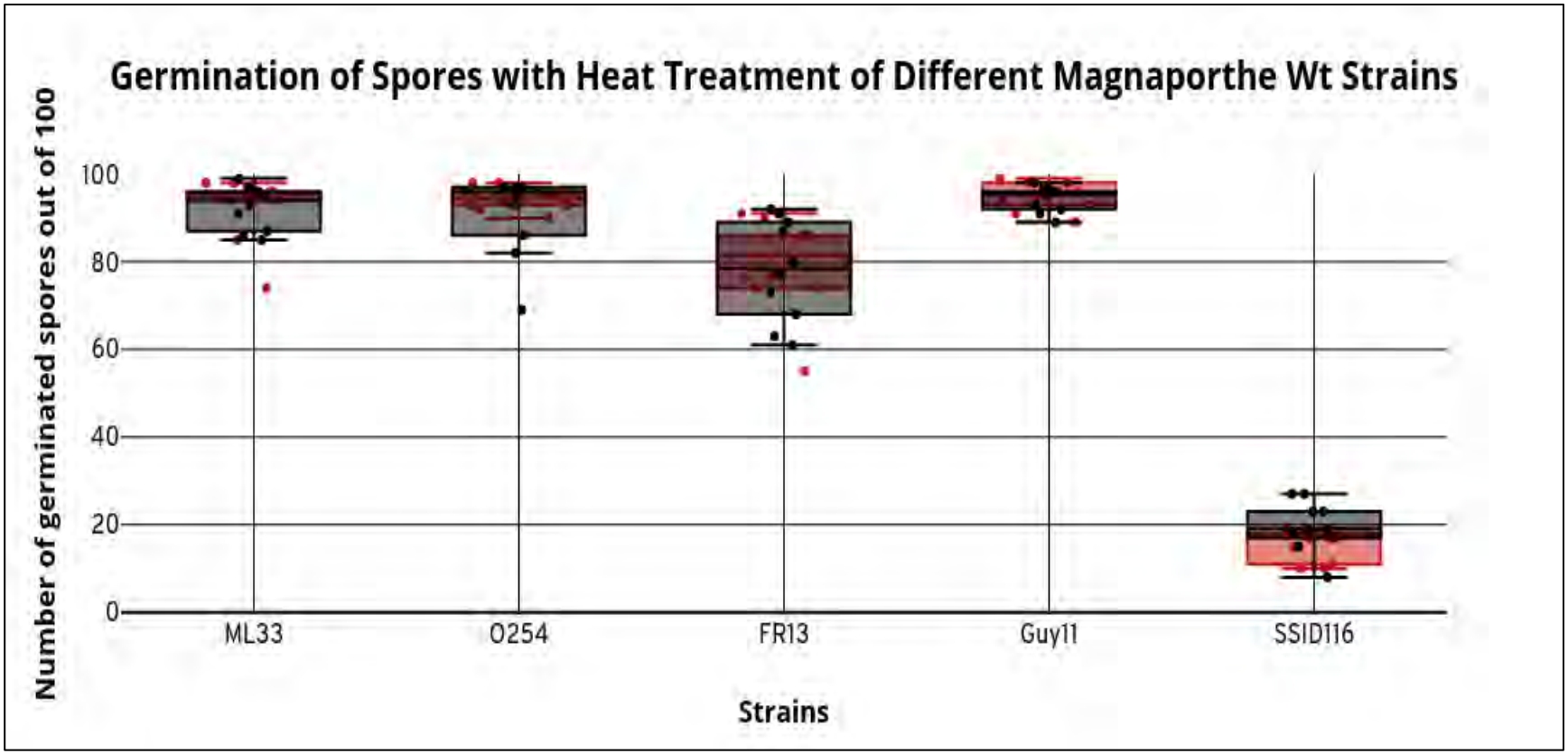
Secondary leaf scans of CO39 inoculated with SSID116 with a 48 h post inoculation exposure to 37°C. Leaves shown are a representative example of the entire pot of plants.



Secondary leaf scans of CO39 inoculated with SSID116 with a 7 d post inoculation exposure to 37°C. Leaves shown are a representative example of the entire pot of plants.

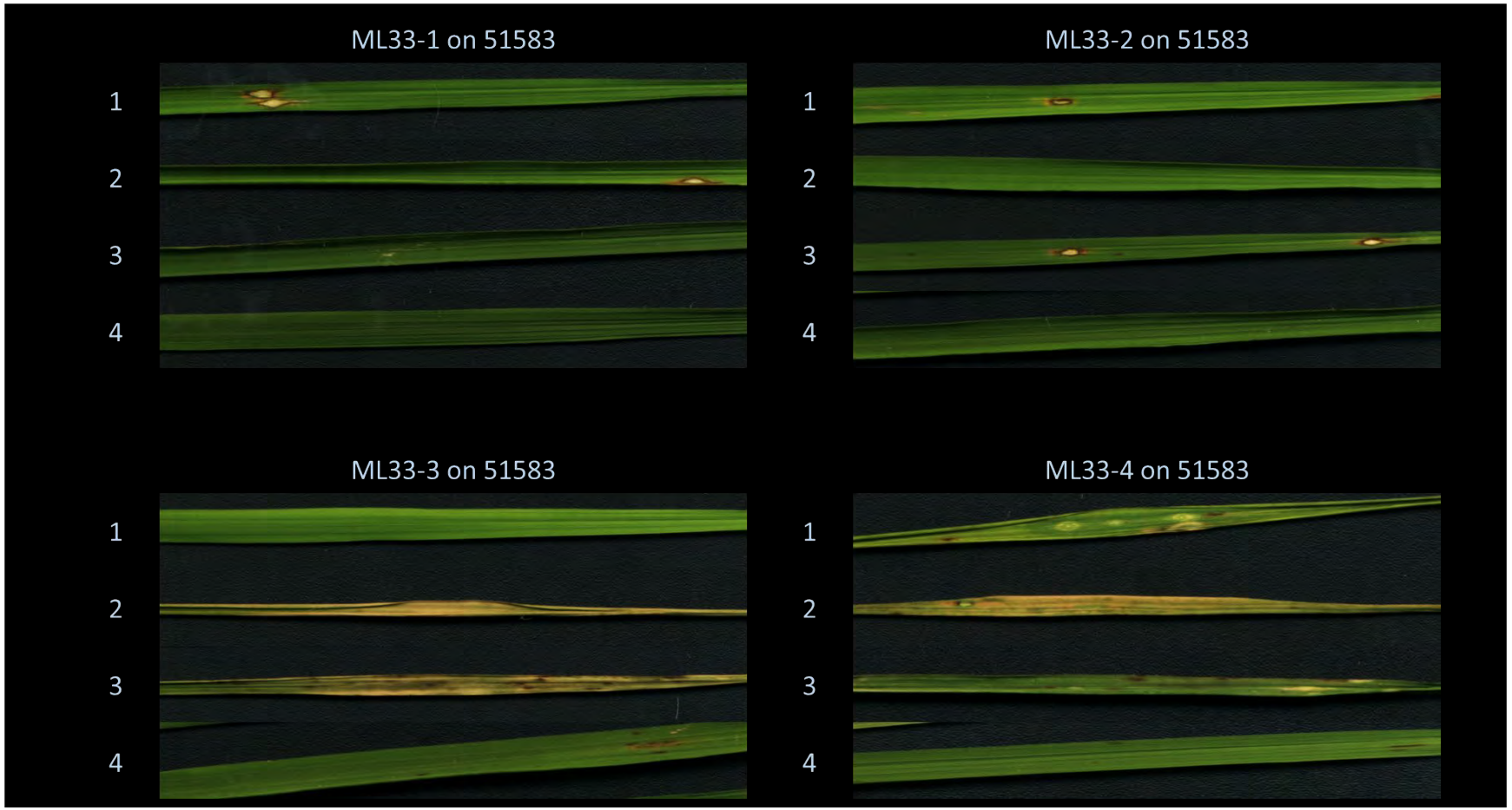


The map displays the range of temperatures for a given country based on the NASA Earth Observations Day-Time Temperatures for 2020. Countries are colored based on the month of peak planting according to the RiceAtlas. Only countries that have reported rice blast have temperature listings.

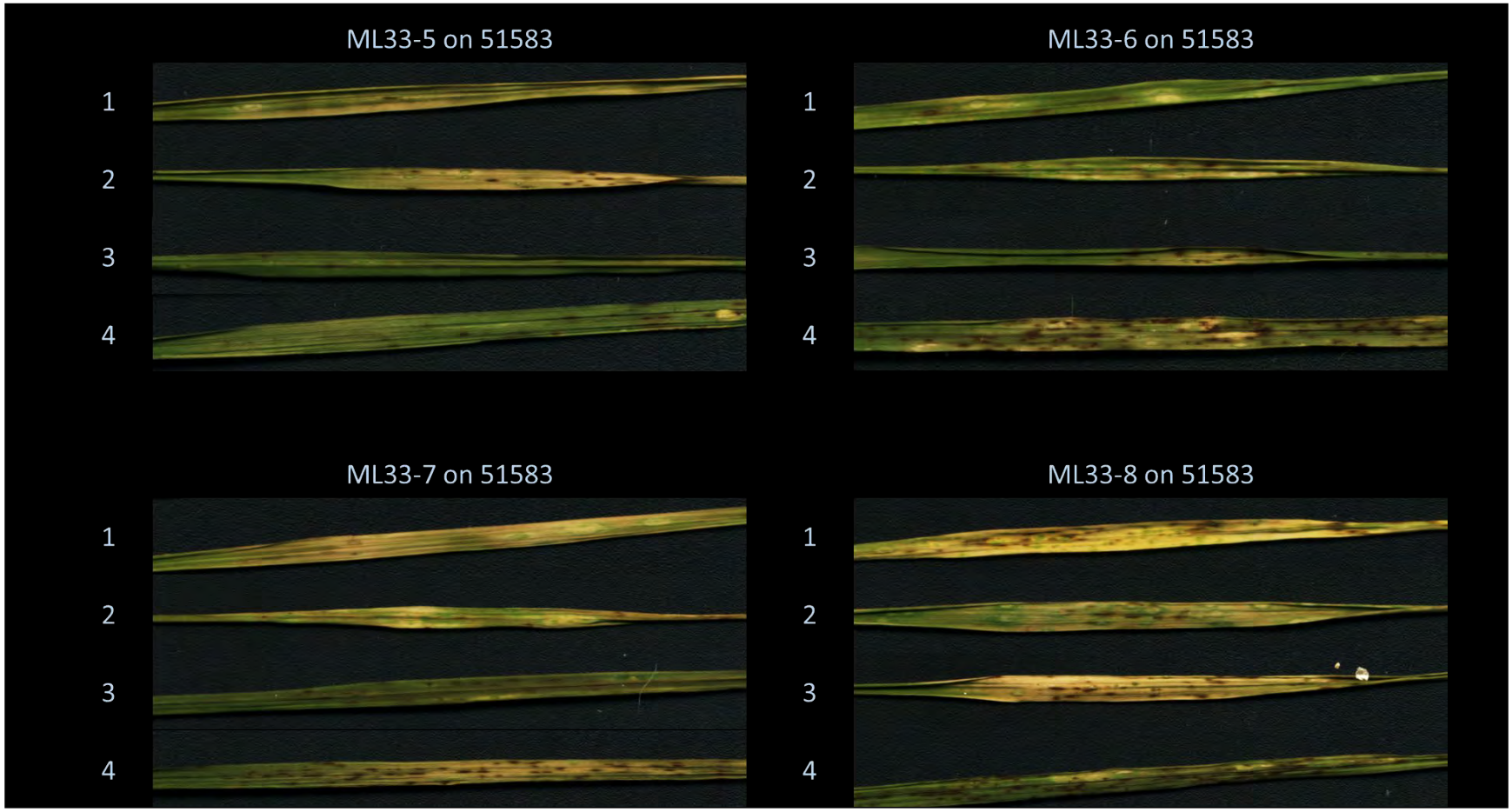


Spore germination of *Magnaporthe oryzae* strains under 37°C heat The ten plates for the treatments and controls are represented by box plots on each strain.

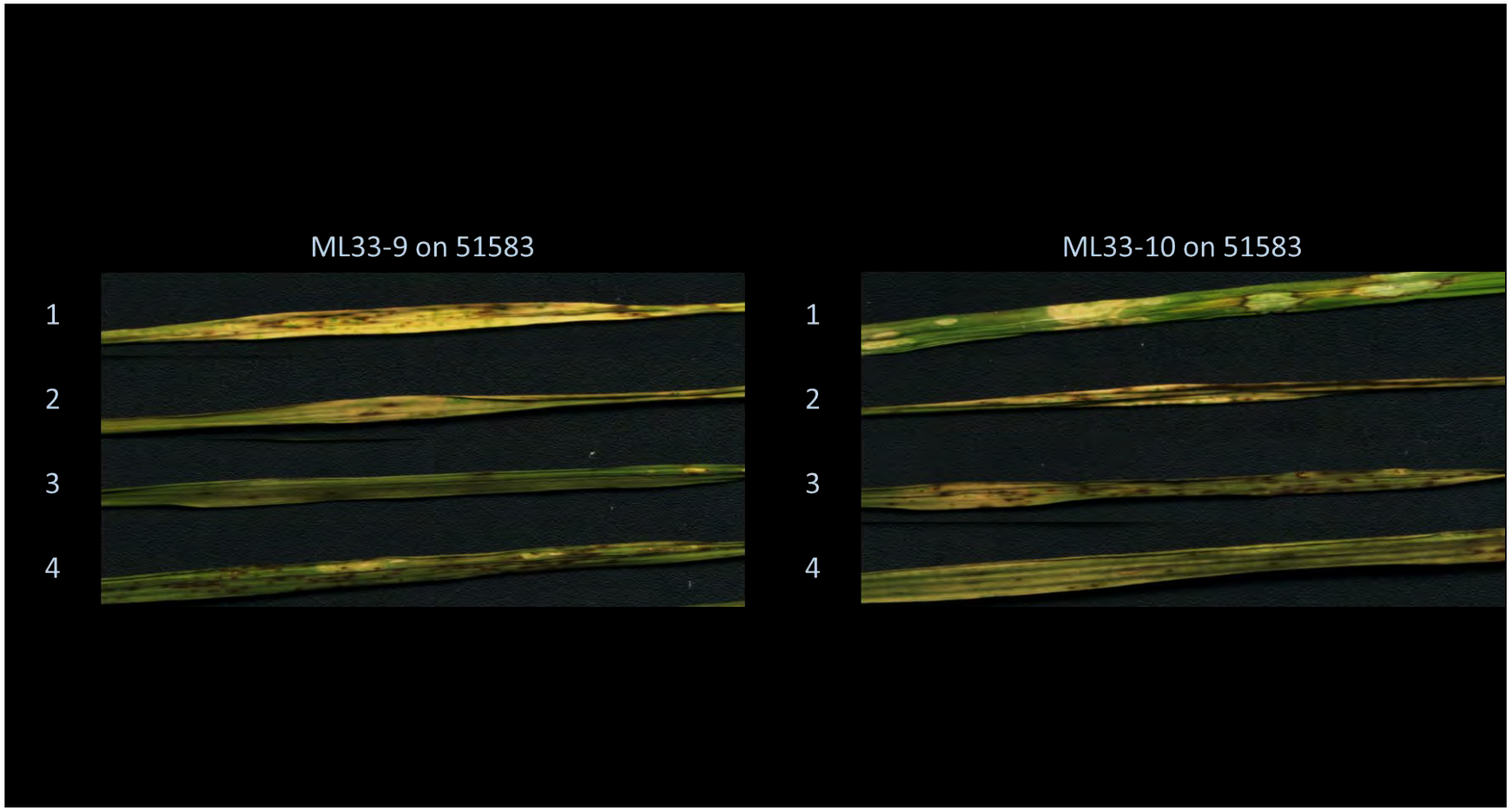
APPENDIX 5: Supplementary Figures for Chapter 7



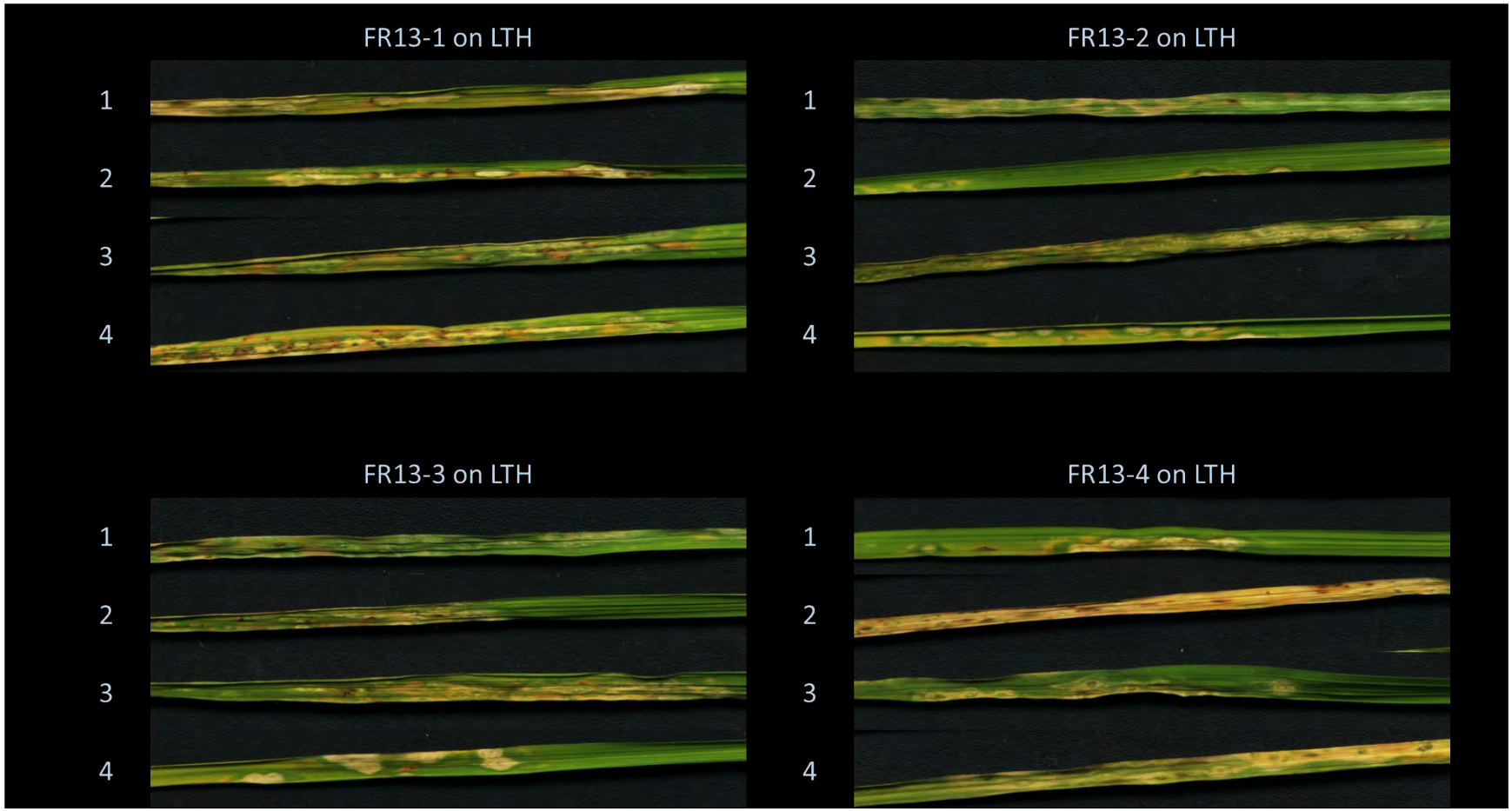
Secondary leaf scans of 51583 inoculated with ML33 stocks for four replications. Leaves shown are a representative example of the entire pot of plants for each inoculation.



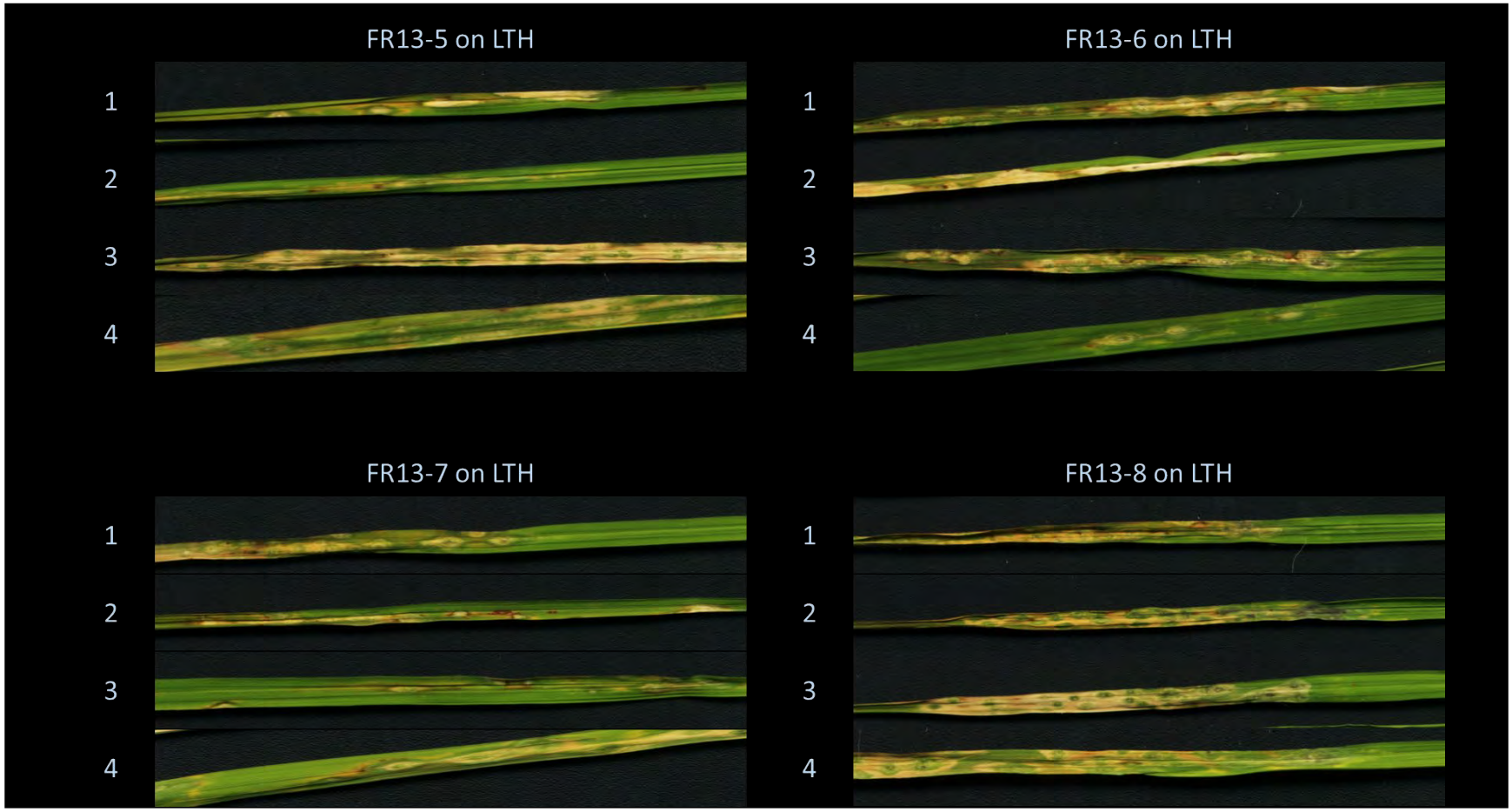
Secondary leaf scans of 51583 inoculated with ML33 stocks for four replications. Leaves shown are a representative example of the entire pot of plants for each inoculation.



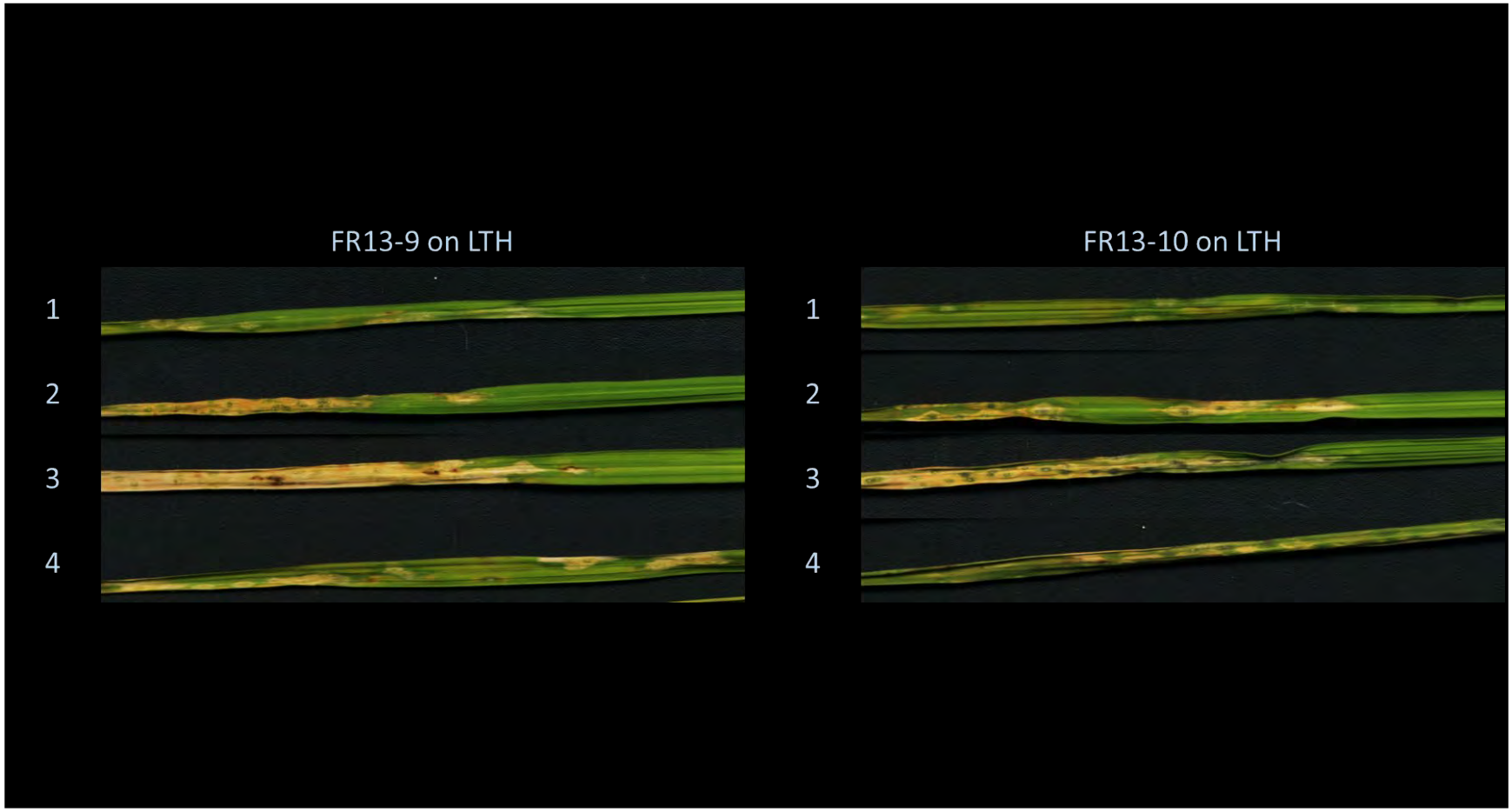
Secondary leaf scans of 51583 inoculated with ML33 stocks for four replications. Leaves shown are a representative example of the entire pot of plants for each inoculation.



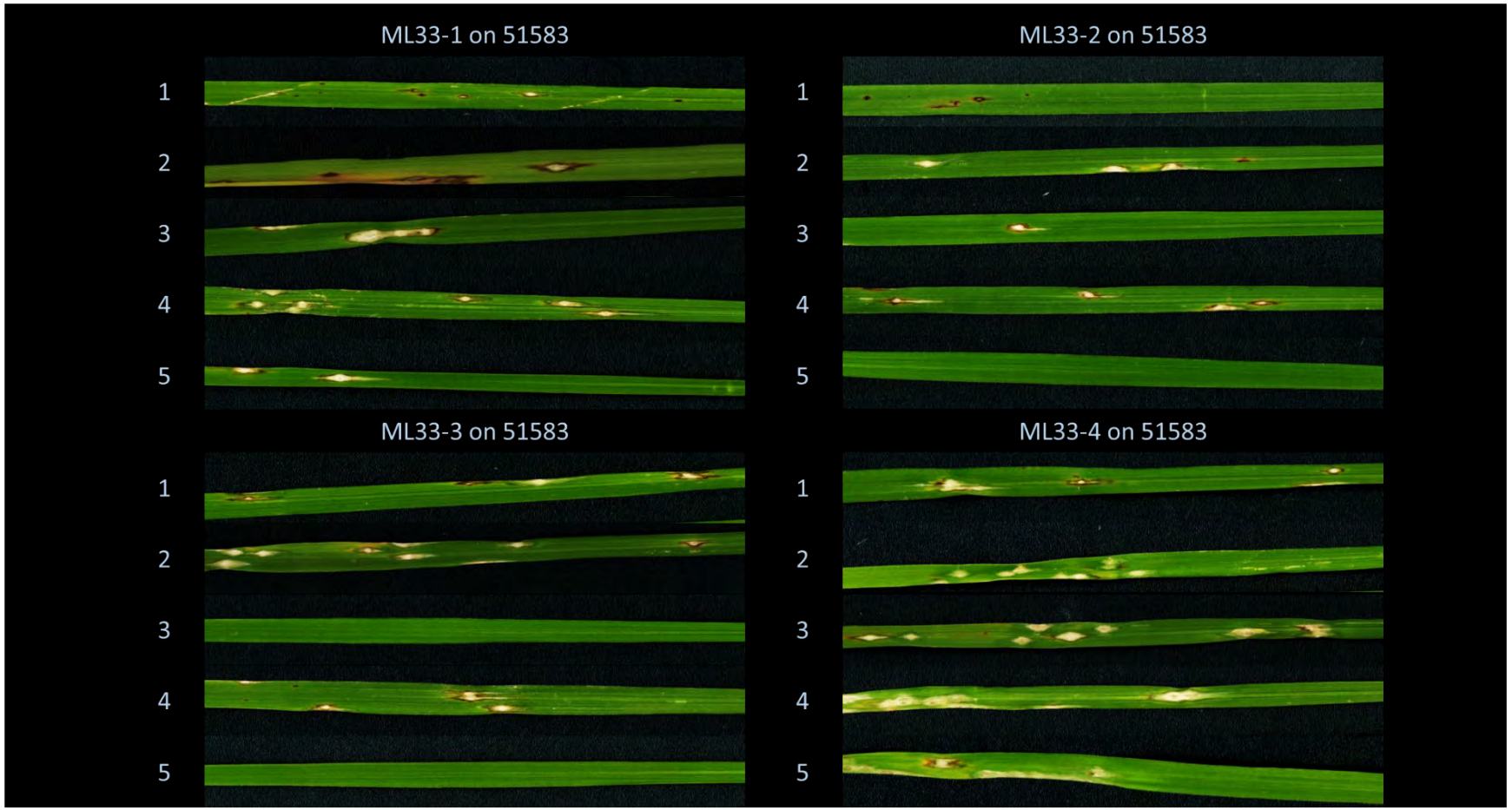
Secondary leaf scans of LTH inoculated with FR13 stocks for four replications. Leaves shown are a representative example of the entire pot of plants for each inoculation.



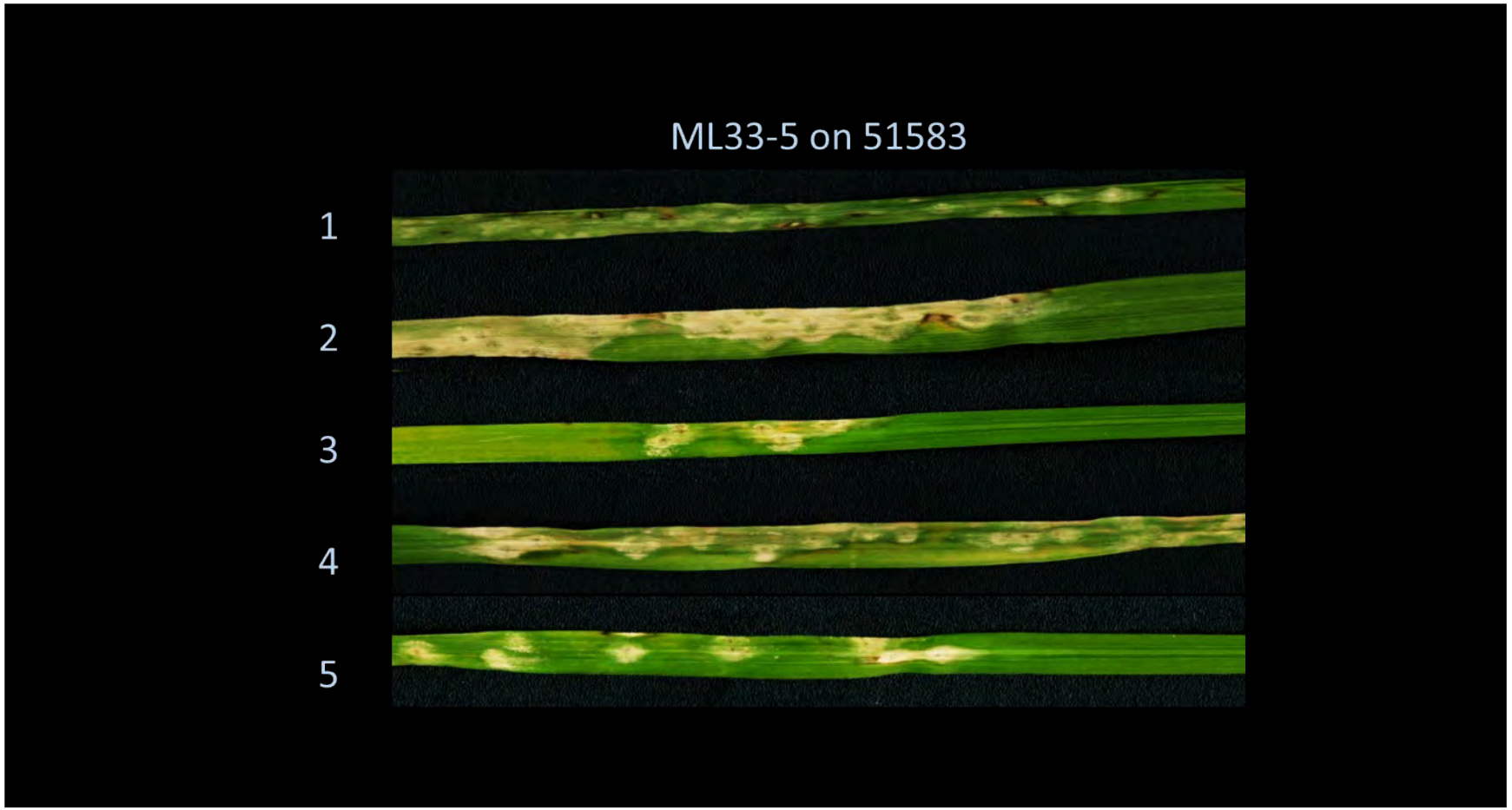
Secondary leaf scans of LTH inoculated with FR13 stocks for four replications. Leaves shown are a representative example of the entire pot of plants for each inoculation.



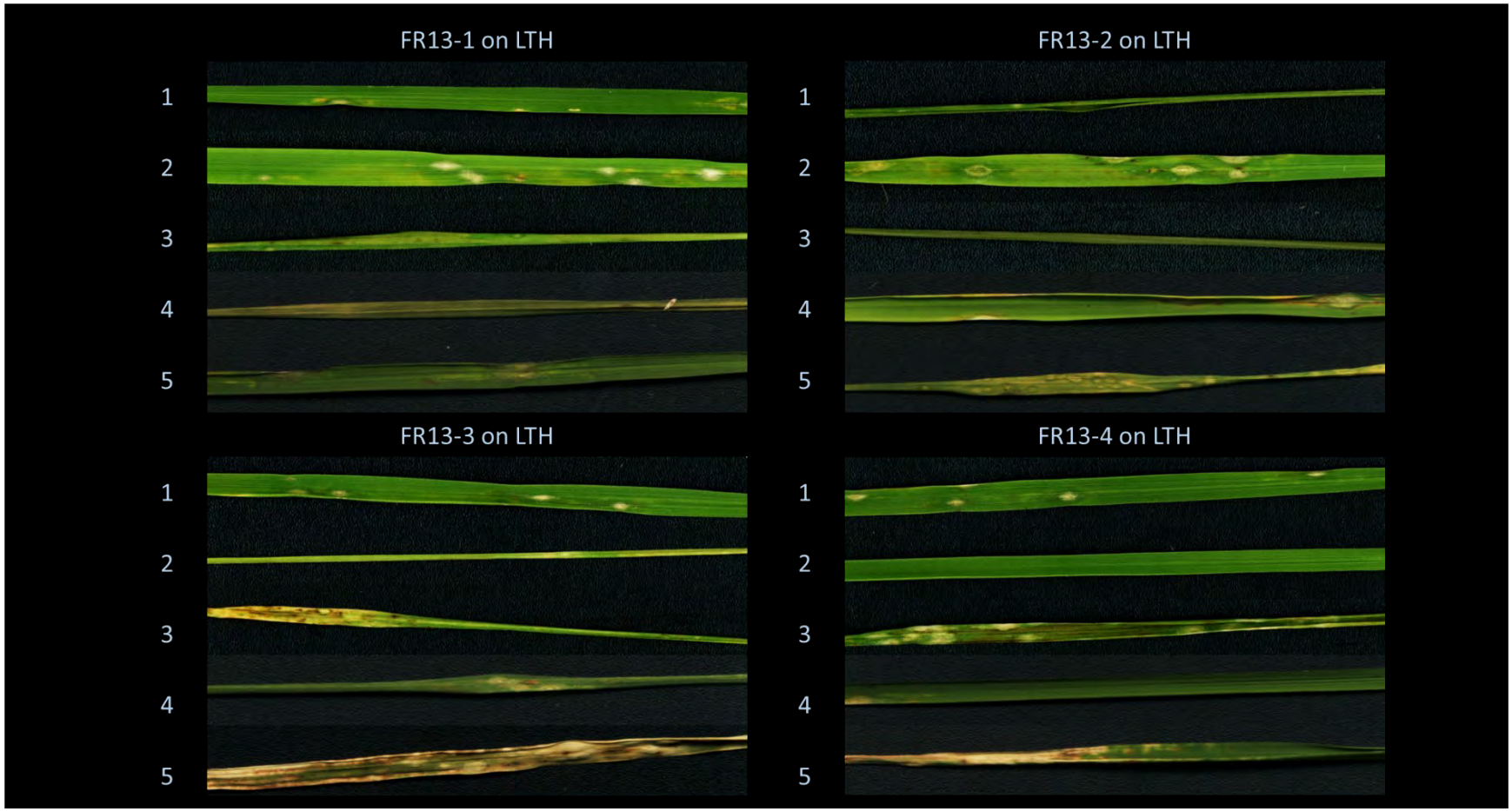
Secondary leaf scans of LTH inoculated with FR13 stocks for four replications. Leaves shown are a representative example of the entire pot of plants for each inoculation.



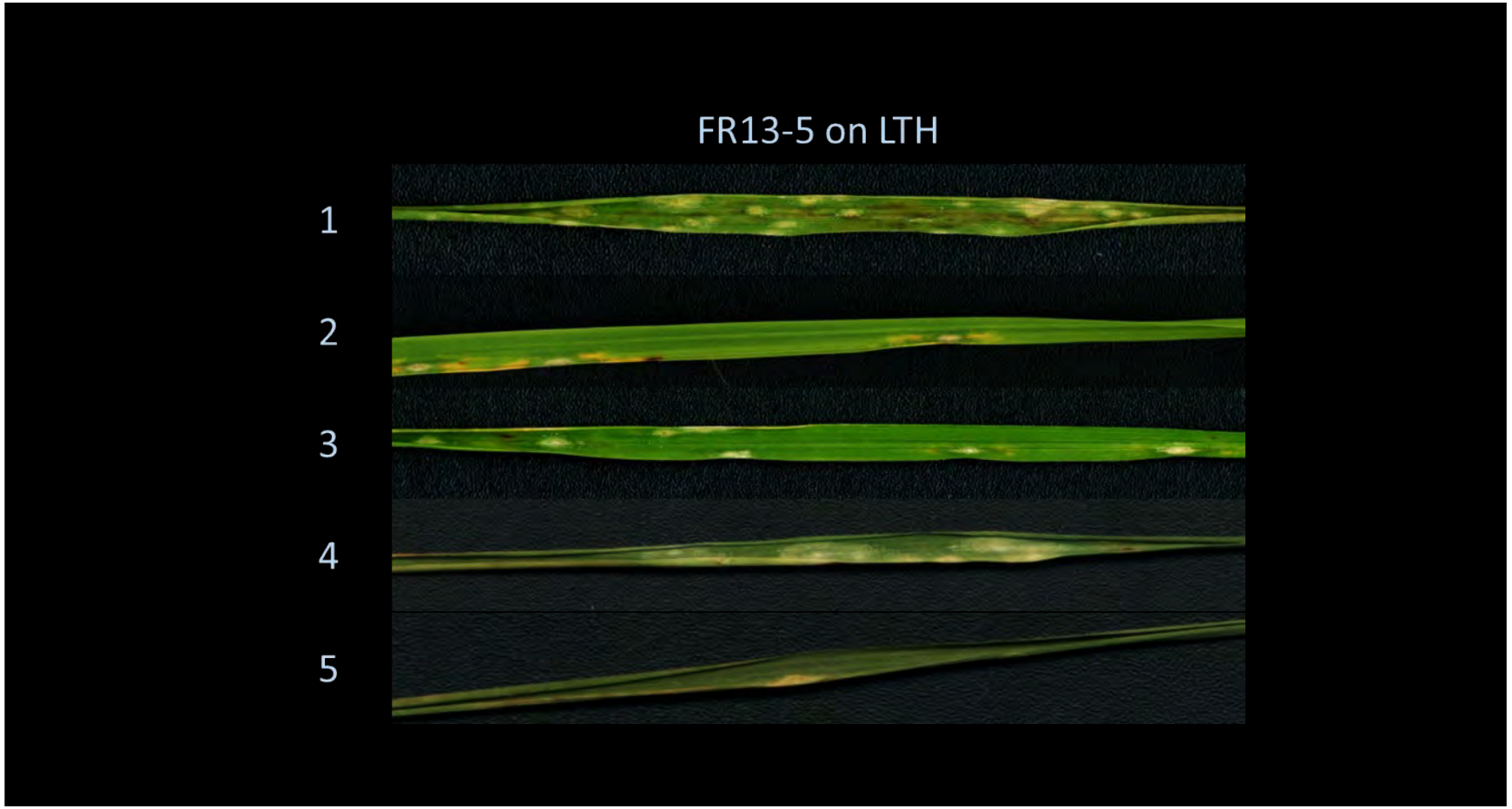
Secondary leaf scans of 51583 in separate trays inoculated with ML33 stocks for five replications. Leaves shown are a representative example of the entire pot of plants for each inoculation.



Secondary leaf scans of 51583 in separate trays inoculated with ML33 stocks for five replications. Leaves shown are a representative example of the entire pot of plants for each inoculation.



Secondary leaf scans of LTH in separate trays inoculated with FR13 stocks for five replications. Leaves shown are a representative example of the entire pot of plants for each inoculation.



Secondary leaf scans of LTH in separate trays inoculated with FR13 stocks for five replications. Leaves shown are a representative example of the entire pot of plants for each inoculation.

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1. Fealko, E., D. Szarka, A. Lamb, B. Amsden, J. Beale, E. Pfeufer. 2019. "First report of black dot root rot, caused by Colletotrichum coccodes, on tomato in Kentucky high tunnels." Plant Disease; Vol. 103, No. 8, 27 May 2019.

Presentations

2. Lamb, A. University of Kentucky Department of Plant Pathology Seminar Series. Lexington, KY Jan. 2023. "Sexist in the Syntax: Grammatical Gender Languages Leading to the Invisibility of Females in Higher Education". (Hybrid Format)

1. Lamb, A. University of Kentucky Department of Plant Pathology Seminar Series. Lexington, KY Sep. 2020. "The Host Microbe Interaction and Epigenetics". (Virtual Format)