Impact of Meteorological Conditions and Maturity of Perithecia on the Release of *Fusarium graminearum* Ascospores

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Academic Abstract

The global food supply is being stressed by climate change, a growing population, and harmful diseases. One risk to vital cereal crops such as wheat and barley is Fusarium head blight (FHB), caused by the fungal plant pathogen *Fusarium graminearum.* Ascospores of the fungus are released from perithecia on the residues of corn and small grains and can be transported long distances (>500 m) through the atmosphere. The overall objective of this work was to assess the influence of meteorological conditions and perithecial maturity on ascospore release. The research focuses on *F. graminearum* because of its damaging impact to staple crops and the global ubiquity of FHB.

The first specific objective was to apply state-of-the-science techniques to identify causal meteorological variables of ascospore release. We analyzed field measurements of airborne ascospores against meteorological conditions at Virginia Tech's Kentland Farm, Blacksburg, Virginia, USA and used convergent cross mapping and multivariate state space reconstruction to identify significant causal agents within this complicated natural and dynamic system. We identified relative humidity, solar radiation, wind speed, and air temperature as predictors of ascospore release.

Our second research objective was to understand the impact of varying meteorological conditions on ascospore release under controlled environmental conditions. We assessed ascospore release in a chamber with controlled temperature (15°C and 25°C) and relative humidity (60%, 75%, and 95%). Ascospores released from ascospore-producing structures (perithecia) were captured on microscope slides placed inside of 3D-printed ascospore discharge devices. Results showed the sensitivity of ascospore release to relative humidity and temperature, with cool temperature and high relative humidity resulting in greater quantities of ascospores released.

Our third research objective was to determine the relationship between the maturity, the number of ascospores, and the hardness of perithecia. A mechanical compression testing instrument was used to investigate the hardness of perithecia at various stages of maturity, producing a mean perithecium compression constant quantifying the uniaxial compression force required to rupture a perithecium. Results indicated that old perithecia contain the greatest amount of ascospores and exhibit increased resiliency, requiring greater forces to rupture, compared to young perithecia.

This research has illustrated the complexities of *F. graminearum* ascospore release by describing the impact of several meteorological conditions and perithecial maturity on the timing and quantity of released ascospores. Collectively, our results may inform wheat growers on the nature and timing of ascospore release, which could help inform FHB management decisions in the future.

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General Audience Abstract

Cereal crops, including wheat and barley, will become increasingly stressed due to growing populations and harmful diseases. One risk to wheat and barley crops is Fusarium head blight, which is caused by the plant pathogen *Fusarium graminearum*. Fusarium head blight diminishes cereal crop quality, reduces cereal crop yields, and produces a toxin harmful to swine and humans. This fungus releases spores capable of long-distance transport through the atmosphere. Improved knowledge about the release of spores would be valuable in predicting disease spread.

Our first research objective was to understand whether certain meteorological conditions caused the release of spores. Field measurements of airborne spores and meteorological conditions obtained at Virginia Tech's Kentland Farm in Blacksburg, Virginia, USA were analyzed using simple linear regression and a more sophisticated technique known as causality analysis. We identified relative humidity, solar radiation, wind speed, and air temperature as predictors of spore release.

The second research objective was to assess spore release in a chamber with controlled temperature and relative humidity. Spore-producing structures were oriented to release spores horizontally onto microscope slides, and the number of spores and distance ejected were measured. Results showed that cooler temperatures (15°C) and high relative humidity (95%) resulted in greater quantities of spores released.

Our final research objective was to determine the relationship between the age of spore-producing structures and the hardness of these structures. A mechanical compression testing instrument was used to investigate their strength and determine the force required to rupture them. Older (mature) structures contained the greatest number of spores and were capable of withstanding the highest forces.

These results may benefit growers as they make Fusarium head blight management decisions. We showed that cool and humid conditions promote spore release and that the age of the spore-producing structures provide useful insight into the quantity of spores that may be released. Additionally, compression testing could be applied in the field to provide growers real-time information on the age of spore-producing structures.

To my parents, for their love and encouragement.

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Chapter 1 – Introduction

Background and Motivation

One question of importance for the global food supply is, how do we protect vital crops from plant pathogens? Bacteria are common causal agents of diseases. For example, *Bacillus pumilus* results in bacterial blot in peaches (Griffin et al. 2001; Kellogg and Griffin 2006) and *Pseudomonas syringae* causes bacterial speck of tomato (Cuppels 1986; Wilson et al. 2002; Buell et al. 2003). Fungi also impact a wide range of crops (Fisher et al. 2012). *Puccinia melancocephala* causes sugarcane rust (Purdy et al. 1985), *Mycosphaerella fijiensis* causes Sigatoka disease in bananas and plantains (Burt et al. 1997; Rutter et al. 1998; Parnell et al. 1998; Burt et al. 1999), *Puccinia graminis* f. sp. *tritici* causes wheat stem rust (Aylor 2003), and *Phakopsora pachyrhizi* causes Asian soybean rust (Schneider et al. 2005). The safety of these staple crops is important in protecting food supplies for a growing global community (Chakraborty and Newton 2011).

Many of these plant pathogens can spread long-distances (> 500 m) from inoculum sources through the atmosphere (Aylor 2003; Brown and Hovmøller 2002; Prussin II et al. 2014a). The framework for modeling the spread of fungal plant pathogens via spores has been proposed (1999, 2003, 1986, 1990; 2001; 1992; 1982; Aylor et al. 2001): the production of spores from a source, the release of the spores from the canopy, transport and reactions within the atmosphere, preservation of pathogenicity during transport, and infection of a new crop via deposition. While the processes involved are well understood (Aylor 1986), there remain several unresolved factors that can determine successful transmission of disease within each of these phases (Rotem and Aust 1991; Sung and Cook 1981). The ability of plant pathogens to reach high altitudes in the

troposphere is an important factor as it prevents spore deposition within the canopy and provides access to horizontal wind currents preferential for long-distance transport (Nagarajan and Singh 1990). The timing of a release event may influence a plant pathogen's ability to remain viable, as exposure to greater than 6 h of UV radiation reduces viability of *Mycosphaerella fijiensis* ascospores (Parnell et al. 1998). Rainfall events can play a large role in wet deposition events (Chen and Yuan 1984; Reis 1990; Gilbert and Tekauz 2000). These factors, along with many other meteorological parameters, can affect the transport and virulence of harmful plant pathogens.

An important fungus that is responsible for spreading Fusarium head blight (FHB) to wheat and barley crops is *Fusarium graminearum* (Goswami and Kistler 2004). Fungal pathologists rank *Fusarium graminearum* as the fourth most important fungus based on its scientific and economic significance (Dean et al. 2012). Fusarium head blight has resulted in more than $3 billion in crop loses in the United States (McMullen et al. 1997; Paulitz 1999; Schmale III and Bergstrom 2003) and $220 million in Quebec and Ontario (Windels 2000) over a 10-year period. Beyond economic losses, the fungus produces the mycotoxin deoxynivalenol (Snijders 1990; Desjardins et al. 1993), which causes vomiting and nausea in swine and humans (Sutton 1982) when ingested in feed or finished food product, respectively. The life cycle of *F. graminearum* is well understood. The fungus resides in the debris of corn and small grains (Sutton 1982). *Fusarium graminearum* overwinters as saprophytic mycelium (Sutton 1982) and produces perithecia (ascospore-producing structures) during the spring season (Trail and Common 2000). The perithecia forcibly discharge ascospores (Trail and Common 2000; Parry et al. 1995; Schmale III et al. 2005a) at an acceleration of 870,000 g (Trail et al. 2005) and are capable of traveling more

than 500 m in the atmosphere (Sutton 1982; Prussin II et al. 2014a). The ascospores infect hosts between anthesis and the soft stage of kernel development (Schroeder and Christensen 1963; Jenczmionka et al. 2003) and produce conidia to infect neighboring crops via rain splash (Sutton 1982; Doohan et al. 2003; Paul et al. 2004). While the disease pathway is understood, the number of ascospores potentially released from a field remains an unresolved parameter in many models of spore release (Aylor 1986, 1999).

The scientific literature on the relationship between *F. graminearum* ascospore release and meteorological conditions contains both laboratory (Gilbert et al. 2008; Tschanz et al. 1975; Trail et al. 2002) and field (Inch et al. 2005; Paulitz 1996; Fernando et al. 2000; Ayers et al. 1975) studies. While these studies generally report a relationship between spore release and relative humidity (RH) (Paulitz 1996; Tschanz et al. 1975; Inch et al. 2005; Reis 1990), questions remain regarding what levels of RH are required (Trail et al. 2002; Ayers et al. 1975), whether other meteorological variables also affect ascospore release (Del Ponte et al. 2009; Sutton 1982; Prussin II et al. 2014b; Paulitz 1996), and if *F. graminearum* maturity influences these release events (Trail and Common 2000). Previous field studies (Inch et al. 2005; Paulitz 1996; Fernando et al. 2000; Ayers et al. 1975) focused on identifying trends in the data and correlations, but not causal relationships, between meteorological conditions and ascospore release.

In many investigations of the atmospheric transport of *F. graminearum*, RH (Paulitz 1996; Tschanz et al. 1975; Inch et al. 2005; Reis 1990) and air temperature (Del Ponte et al. 2009; Sutton 1982; Paulitz 1996) have been associated with ascospore release. Previous studies

performed in a wind tunnel identified the greatest numbers of ascospores released at RH values greater than 92% (Trail et al. 2002). A field study conducted in Quebec, Canada (Paulitz 1996) found that ascospore release events occurred at 60% to 95% RH. In a field study performed in Pennsylvania (Ayers et al. 1975), the greatest numbers of ascospores collected from wheat and corn fields coincided with RH between 95% and 100%, respectively. Ascospore release has been found to occur over a range of temperatures (11 to 30° C) in field experiments (Paulitz 1996). In some of these experiments, there was a daily periodicity of ascospore release; the greatest number of ascospores was recovered during the cooler, nighttime periods (Fernando et al. 2000; Prussin II et al. 2014a; Prussin II et al. 2014b).

Along with RH and air temperature, several other meteorological factors including certain light levels (Trail et al. 2002), rainfall (Chen and Yuan 1984; Reis 1990; Gilbert and Tekauz 2000), and wind speed (Prussin II et al. 2014b) have also been associated with ascospore release events or FHB epidemics. These investigations have focused on identifying correlations between meteorological variables and ascospore release events, whereas the nonlinear dynamics and confounding variables of natural systems (Holling 2001) indicate the need for a more powerful tool to identify causal relationships within these systems. Causality analyses have been applied within the natural (Sugihara et al. 2012; Deyle et al. 2013) and social sciences (Stern and Enflo 2013), and their application to plant pathology has the potential to produce new insight into the complex relationships underlying the epidemiology of plant diseases.

The complex, connected dynamic relationships between variables (Sugihara et al. 2012; Deyle et al. 2013) makes investigating how varying meteorological factors influence ascospore release a

challenge. While researchers have associated RH (Trail et al. 2002; Paulitz 1996) and temperature (Paulitz 1996; Tschanz et al. 1975; Manstretta and Rossi 2015; Gilbert et al. 2008) with ascospore release, it is difficult to separate the effects of RH from solar radiation, air temperature, and other meteorological factors because many follow distinct diurnal cycles. Since these two metrological parameters have been correlated with ascospore release by several researchers (Paulitz 1996; Sutton 1982), controlled laboratory studies that allow for the assessment of each parameter's impact on ascospore release number and discharge distance would more mechanistic insight into the process.

While the timing of meteorological parameters appears to be important in predicting ascospore release events (Schmale III et al. 2006; Schmale III et al. 2005b; Trail et al. 2002; Paul et al. 2007), the role of perithecial maturity on the ability for and the amount of ascospore release warrants investigation. A model for ascospore release includes four essential steps: a signal indicating the presence of mature ascospores within a perithecium, the sufficient accumulation of turgor pressure to discharge the ascospores from the perithecium, the extension of the ascospore-containing ascus through which ascospore discharge occurs, and the opening of the ascus (Trail and Seminara 2014). Investigating the relationship between perithecial maturity and the amount of mature ascospores capable of participating in release events is a necessary step towards assessing the potential ascospore source strength in the field.

Objectives

The main objective of this work was to understand the influence of meteorological conditions and perithecial maturity on ascospore release. As motivated above, we identified two research

foci with respect to *F. graminearum* ascospore release: (1) the impact of meteorological conditions on the timing and quantity of ascospore discharge; and (2) the role of perithecial maturity on ascospore availability during release events. This research revolves around the following specific research hypotheses and objectives:

Hypothesis 1: Relative humidity, solar radiation, and wind speed are causal agents for ascospore release.

Objective 1: Determine which meteorological variable(s) influence ascospore release through causality analysis of field measurements.

Hypothesis 2: Ascospores are discharged at greater numbers and farther distances at higher RHs and at cooler temperatures.

Objective 2: Determine the effects of RH and temperature on the number and distance of discharged ascospores under controlled conditions.

Hypothesis 3: Mature perithecia are more resilient to compressive forces and contain greater numbers of mature ascospores.

Objective 3: Mechanically compress perithecia at various stages of maturity to understand the force required to rupture the perithecia.

Organization of the Dissertation

Chapter 2, *Identification of meteorological predictors of* Fusarium graminearum *ascospore release using correlation and causality analyses,* addresses Objective 1. In this study, we analyzed ascospore concentrations obtained during a 2011 and 2012 field campaign in Blacksburg, VA against several meteorological variables. We used two types of causality analyses, convergent cross mapping and multivariate state space forecasting, to identify meteorological causal agents of ascospore release. Convergent cross mapping identified RH, solar radiation, wind speed, and air temperature as predictors of ascospore release. Multivariate state space forecasting identified solar radiation and RH as effective predictors of ascospore release. Due to its applicability when investigating complex natural systems, the use of causality analyses may be useful in examining the spread of other plant pathogens and bioaerosols.

Chapter 3, *Ascospore release and discharge distances of* Fusarium graminearum *under controlled temperature and relative humidity,* addresses Objective 2. We describe the design of a chamber with controlled temperature and RH used in measuring the numbers of ascospores discharged from perithecia and the distance discharged. We investigated ascospore release at two temperatures (15°C and 25°C) and at three RHs (60%, 75%, and 95%). Greater numbers of ascospores were released at 15°C and increased with increasing levels of RH. Ascospores traveled farther at the highest RH (95%) and at 25°C. The results indicate ascospore quantity and discharge distance are affected by temperature and RH. This knowledge may be applicable to FHB modelers and researchers in the field of plant disease epidemiology.

Chapter 4, *Associations between the maturity of perithecia, quantity of ascospores, and strength of the perithecial wall in the plant pathogen,* Fusarium graminearum, addresses Objective 3 and describes the relationship between a perithecium's maturity and its force-deformation characteristics. We determined this relationship by uni-axially compressing perithecium using a mechanical testing instrument. The results indicated that as perithecia mature, a greater amount of compressive force is required to rupture the perithecial structure and that numbers of ascospores within the perithecia increase with maturity. Our results suggest that as the internal structures of perithecia mature, the entire structure becomes more resilient to outside compressive forces. Compression testing may provide a novel method of determining perithecial maturity in the field.

Chapter 5 concludes this dissertation by describing the main outcomes of the work and making recommendations for future work.

Attributions

Dr. Linsey C. Marr served as a major co-advisor for this work. She provided guidance and suggestions regarding project design, execution, and writing for the work in Chapters 2, 3, and 4. She is listed as a co-author in Chapters 2, 3, and 4. She has also offered editing advice for other portions of this dissertation. Dr. Marr also provided financial support for this work (NSF EF-0830093; IGERT: MultiScale Transport in Environmental and Physiological Systems (MultiSTEPS) NSF DGE-0966125)

Dr. David G. Schmale, III served as a major co-advisor for this work. He provided guidance and suggestions regarding project design, execution, and writing for the work in Chapters 2, 3, and 4. He is listed as a co-author in Chapters 2, 3, and 4. He has also offered editing advice for other portions of this dissertation. Dr. Schmale also provided financial support for this work (Virginia Small Grains Board 449281, Improving the Management of FHB through an Increased Understanding of how the Pathogen Releases its Spores; Virginia Small Grains Board 449428, Advancing FHB Management Strategies by Increasing Knowledge of how the Pathogen Develops its Spore-release Structures; IGERT: MultiScale Transport in Environmental and Physiological Systems (MultiSTEPS) NSF DGE-0966125).

Dr. Shane D. Ross served as a committee member and was listed as a co-author in Chapter 2. Dr. Ross provided guidance through the completion of this dissertation. Dr. Ross provided guidance and suggestions regarding data interpretation, writing, and editing for the work presented in Chapter 2.

Dr. Amy J. Pruden-Bagchi served as a committee member and provided guidance through the completion of this dissertation.

Dr. Korine N. Kolivras served as a committee member and provided guidance through the completion of this dissertation.

Dr. Amir E. BozorgMagham provided expertise on causality analyses. Dr. BozorgMagham is listed as a co-author in Chapter 2. Dr. BozorgMagham provided guidance and suggestions regarding data interpretation, writing, and editing for the manuscript resulting from that work.

Dr. Frances Trail provided expertise on the *Fusarium graminearum* perithecial structure. Dr. Trail is listed as a co-author in Chapter 4. Dr. Trail provided the parent strain (PH-1) and the mutant strain (4417) that were analyzed and provided suggestions regarding writing and editing for the manuscript resulting from that work.

Michael Reinisch provided experimental design advice, assistance with compression experiments, and data interpretation for the work presented in Chapter 4. Mr. Reinisch is listed as a co-author in the manuscript resulting from that work.

Dr. Aaron Prussin, II designed and conducted the field experiments in 2011 and 2012 that were analyzed in Chapter 2.

Dr. Steve McCartney and Jay Tuggle provided assistance in the collection of the compression

data presented in Chapter 4.

Craig Powers assisted with designing the spore discharge devices described in Chapter 3.

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Chapter 2 – Identification of meteorological predictors of *Fusarium graminearum* ascospore release using correlation and causality analyses

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Abstract

Fusarium head blight (FHB), caused by the plant pathogen *Fusarium graminearum*, is a significant threat to small grains production worldwide. Additional knowledge is required to clarify the influence of meteorological conditions on the release of ascospores of *F. graminearum*. Here, a new application of causality analysis is used to determine how meteorological conditions cause ascospore release. Two types of causality analyses, convergent cross mapping and multivariate state space forecasting, were applied to field measurements of airborne ascospores of *F. graminearum* over two years. Convergent cross mapping identified relative humidity, solar radiation, wind speed, and air temperature as predictors of ascospore release. Multivariate state space forecasting identified solar radiation and relative humidity as effective predictors of ascospore release. Increased concentration of ascospores in the atmosphere primarily occurred during periods of high relative humidity, low solar radiation, and low wind speed. Results from this study may assist producers in managing FHB in small grains

by narrowing the timing and application of fungicides around major ascospore release intervals predicted by meteorological conditions.

Keywords: Fungus; Fusarium head blight; Bioaerosol; Causality analysis; Convergent cross mapping; Disease management; *Fusarium graminearum*; Multivariate forecasting

Introduction

Fusarium head blight (FHB), produced by the fungus *Fusarium graminearum* Schwabe, caused damage to small grains yields in the UK (Jennings and Turner 1996) and $3 billion of losses to wheat production in the US alone between 1990 and 2000 (Windels 2000). Grain infected with *F. graminearum* may contain mycotoxins (Schollenberger et al. 2002) that threaten the health of domestic animals and humans (McMullen and Stack 1983). The fungus forcibly discharges ascospores from perithecia produced from overwintered residues of corn and small grains (Gilbert and Fernando 2004; Paulitz 1999), and these ascospores may be transported through the atmosphere over long distances to susceptible crops (Prussin II et al. 2015; Schmale III et al. 2012). Prior studies have suggested that the release of ascospores and FHB epidemics are associated with light intensity and spectral energy distribution (Trail et al. 2002), rainfall (Chen and Yuan 1984; Reis 1990; Gilbert and Tekauz 2000), air temperature (Del Ponte et al. 2009; Sutton 1982), wind speed (Prussin II et al. 2014b), and relative humidity (Paulitz 1996). A complete understanding of the causal relationship, in which one event is responsible for a second event, between meteorological conditions and the release of ascospores is needed to accurately predict the spread of disease. Detecting causal relationships in natural systems, however, is

extremely challenging because of large variability, nonlinear dynamics, and confounding variables (Holling 2001).

A series of innovative and promising methods have been developed to identify causation in weakly connected dynamic systems, such as ecological time series (Sugihara et al. 2012; Deyle et al. 2013). These methods, known collectively as causality analyses, have been applied within the natural (Sugihara et al. 2012; Deyle et al. 2013) and social sciences (Stern and Enflo 2013), but have not yet been applied to the field of plant pathology. The application of causality analysis to plant pathology has the potential to produce new insight into the complex relationships underlying the epidemiology of plant diseases. In contrast to regression analysis, which can identify correlation but not causation, causality analysis is able to determine whether one variable causes an effect on another variable and the direction of the relationship.

The objective of this study was to determine which meteorological variable(s) influence ascospore release through causality analysis. This objective was based on the hypothesis that meteorological variables (relative humidity, solar radiation, and wind speed) are causal agents for ascospore release. This hypothesis was tested using convergent cross mapping analyses (Sugihara et al. 2012; BozorgMagham et al. 2015) and multivariate state space forecasting (Deyle et al. 2013). Results explicitly identified cause-and-effect agents and will allow for more accurate representations of ascospore release in disease forecasting models. Ultimately, results will enable growers of small grains to make informed FHB management decisions based on meteorological predictors of ascospore release.

Materials and Methods

Experimental data collection

Field-scale experiments with *F. graminearum* took place in 2011 and 2012 at the Kentland Farm in Blacksburg, Virginia and have been described previously by Prussin et al. (2014a; 2014b). Briefly, corn stalks were inoculated with the strain Fg_Va_GPS13N4_3ADON and stored at room temperature for approximately 10 weeks to permit colonization of the stalks. After the fungus colonization period, a 3716 m^2 wheat field was artificially inoculated with the infected stalks.

The timeline of measurements of airborne ascospore concentration was designed to capture background conditions and the release of ascospores from the infected field. In 2011, the field was inoculated on 2 May, and measurements began 17 days later on 19 May and continued until 3 June (15 days). In 2012, the field was inoculated on 16 April, and measurements started 10 days later on 26 April and were completed on 14 May (18 days). The background concentration of ascospores was established in 2012 via hourly measurements gathered for 7 days prior to artificial inoculation of the field (measurements began on 9 April).

A volumetric, active ascospore sampler (Quest Developments, Brits, South Africa) with a flow rate of 0.22 $m^3\ h^{-1}$ was used to measure airborne ascospore concentration as a function of time. The sampler was installed at the center of the field and was outfitted with a circular rotating disk coated with silicone grease to collect ascospores released from the inoculum source. The ascospores on the sample disks were stained using Calberla's Stain (Multidata Inc., Saint Louis Park, Minnesota), and were counted under a microscope at 400x magnification. The concentration of ascospores per cubic meter of air was calculated as the ascospore collection rate

divided by the ascospore sampler's volumetric flow rate. The concentration was assumed to be a proxy for ascospore release, as the measurement height of 50 cm was well within the canopy, approximately half its height (Prussin II et al. 2014b), where the effects of dilution in the atmospheric boundary layer were considered to be minimal. A direct relationship between ascospore release and capture was assumed, as the effects of residence time within the canopy were presumed to be negligible.

Meteorological data

A weather station was situated approximately 250 m northwest and 350 m northwest from the center of the inoculum source (i.e., the location of the Quest sampler) in 2011 and 2012, respectively. It recorded air temperature (^{o}C), soil temperature (^{o}C), relative humidity (%), rainfall (mm), wind speed (m s^{-1}), and solar radiation (W m^{-2}). Absolute humidity was calculated using the ideal gas law and the Magnus-Tetens approximation (Bolton 1980). The 15-min records were averaged over 1-h intervals to match the temporal resolution of the ascospore measurements.

Causality analyses

Convergent cross mapping

The convergent cross mapping (CCM) method (Sugihara et al. 2012; BozorgMagham et al. 2015) was used to investigate the relationship between ascospore concentration and meteorological conditions. This method is based loosely on a parent-child relationship. Information associated with a driver (i.e., the parent) is passed on to a response (i.e., the child). It is anticipated that in examining characteristics of the response (i.e., the child), attributes about a specific driver would be apparent. For our application, the driver was the meteorological variable of interest and the response was ascospore concentration.

Time-lagged components (Abarbanel 1996; Takens 1981; Sauer et al. 1991) of ascospore concentration, the response signal, were used to estimate the dynamics of each meteorological variable, the candidate driver. An improvement in the recovery of a meteorological variable shows that the variable has an influential causal impact on ascospore concentration. The meteorological observations were compared to the CCM-estimated meteorological values obtained using the time-lagged ascospore concentration, resulting in the calculation of a Pearson correlation coefficient. The Pearson correlation coefficient quantifies the strength between a dynamically-connected set of variables. An average of the Pearson correlation coefficients, over subsets of length L of the historical data sets, was obtained and termed the convergent cross mapping coefficient, ρ. Causality is indicated by a ρ value that often increases with an increasing length of subset library length, L. A thorough description of the creation of the reconstructed phase spaces and determination of the CCM coefficient can be found in **Online Resource 1**.

Multivariate state space forecasting

The meteorological variables determined as influential causal agents by the CCM method were further analyzed using the multivariate state space forecasting method. The multivariate state space forecasting method (Deyle et al. 2013) is inspired by a conceptual combination of the Granger causality method (Granger 1969) and the simplex method (Farmer and Sidorowich 1987). The multivariate state space forecasting method aims to improve the prediction of the ascospore concentration by leveraging the historical information of the ascospore concentrations augmented by the information of the causal meteorological variables. Predicted ascospore concentration was obtained using single-variable (by exploiting only the past information of ascospore concentration and without using the causal meteorological data) and multivariable

forecasting methods. The root mean square (RMS) error, defined as the difference between the experimentally-obtained ascospore concentration and the forecasted ascospore concentration, was determined for single variable and multivariate forecasting methods. The improvement resulting from the use of augmented information of the causal meteorological data was quantified by calculating the difference between the two RMS error values. Statistical significance of the improvement was studied for each augmented meteorological variable (for example, temperature and solar radiation). A thorough description of the methodology used to create the reconstructed phase spaces and the calculation of the RMS of forecasting error is available in **Online Resource 1**.

Statistical analyses

Exploratory statistical analyses were conducted using the JMP Pro 10.0.2 (SAS Institute Inc., Cary, NC). Statistical significance was defined at a level of 0.05 for all analyses, unless specified otherwise. A two-sample test for independent groups was conducted on ascospore concentration in 2011 and 2012 to determine whether the concentration differed between the two years. This necessitated the use of a Welch's t-test because the standard deviations between the populations could not be assumed to be approximately equal. Due to expected differences in ascospore release during daytime v. nighttime, analysis of variance (ANOVA) was used to compare the means of daytime (0700 to 1900 local time, EDT) and nighttime (1900 to 0700) ascospore concentration. The one-way ANOVA used periods of the day as the treatment (categorical variables) with treatment levels of daytime or nighttime.

A bivariate analysis of ascospore concentration and individual meteorological variables was performed to determine if there were any significant relationships. All meteorological variables

analyzed were continuous unless otherwise stated as nominal. In all correlation analyses, all data points were included unless otherwise noted. A logarithmic base-10 transformation of the ascospore data was applied because of the skewed nature of the distribution. Simple linear regression was employed to model the relationship between the continuous response variable (ascospore concentration) and the continuous predictor variable (meteorological variables).

Results

Causality analysis

The CCM coefficient, ρ, between the candidate driver (meteorological variables) and the response signal (ascospore concentration) is shown in Fig. 2-1 (2011) and Fig. S1 (2012) (**Online Resource 2**); results are similar between the two years. The parameter ρ denotes the strength of the causal relationship with a value of one indicating the strongest causal relationship and lower values signifying a weaker relationship. The convergence of ρ as L increased for air temperature, relative humidity, wind speed, and solar radiation indicated that these four variables were the driving signals. The clustering of ρ for large L for these variables suggested that no single one was dominant. In addition, soil temperature and absolute humidity had the smallest influence on ascospore concentration.

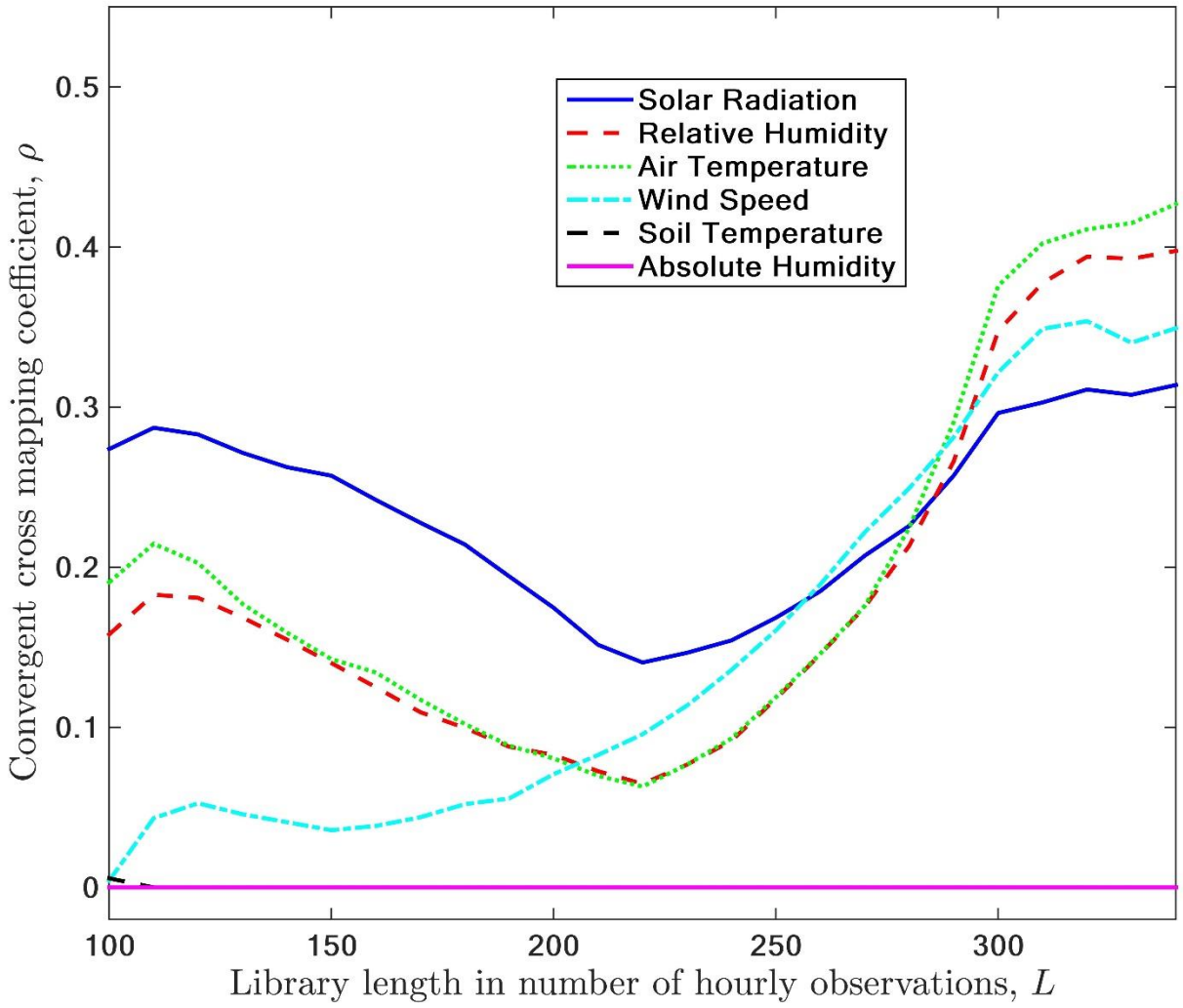

Figure 2-1: The CCM coefficient, ρ, between driver signals (meteorological variables) and response signals (ascospore concentration) for the 2011 monitoring period. Solar radiation, relative humidity, air temperature, and wind speed show CCM coefficients ρ that increase significantly with increasing library length and are significantly greater than zero for the large library length. Thus, they are the most important drivers influencing ascospore release.

The multivariate state space forecasting analysis was performed considering a time difference of 0 to 8 h between the meteorological and ascospore concentration time series, such that the

meteorological variable preceded the ascospore concentration signal. At each time step, ascospore concentration was predicted for a 12 h lead time. Forecasting began on 11 May 2012 at 0700 and was performed for hourly intervals. Figure S2 (2012) (**Online Resource 2**) shows one sample of the RMS of single variable (solid line) and multivariate state space forecasting errors (dashed line). In this example the solar radiation signal, leading the ascospore concentration signal by 4 h, was considered as the augmented variable.

This study investigated whether the augmentation of the meteorological variables significantly improved the forecasting of ascospore concentration. A t-test was applied between the RMS errors for each case. The null hypothesis was that the RMS of errors of the multivariate and single variable forecasting results were identical and the differences were random. Table S1 shows the results of this analysis, where the cases that rejected the null hypothesis are denoted by +1 if the forecast was improved and -1 if the forecast was degenerated. Solar radiation and relative humidity were among the most influential external driving forces in this system. Absolute humidity, as the augmented variable, significantly changed the results, but degenerated the forecast.

Correlation analysis

Atmospheric ascospore concentration was significantly higher after the introduction of inoculated corn stalks to the field ($P < 0.0001$). In 2012, the average ascospore concentration was 176 ascospores m^{-3} pre-inoculation and 3066 ascospores m^{-3} post-inoculation, indicating that a great majority of the ascospores collected originated from the inoculated field and not from background sources.

Atmospheric ascospore concentration varied by period of the day in both 2011 and 2012. Analysis of variance (ANOVA) was employed to determine whether the mean daytime concentration (between 0700 to 1900 h) was significantly different from the nighttime concentration (between 1900 to 0700 h). In 2011 (n = 352), the daytime ascospore concentration (mean ± standard error= 278 ± 2810 ascospores m^{-3}) was significantly different ($P < 0.0001$) from the nighttime ascospore concentration (15,509 ± 2747 ascospores m^{-3}). In 2012 (n=424), the mean concentration during the nighttime (5542 ± 523) was significantly higher than that during the daytime (524 ± 533).

Elevated ascospore concentrations occurred episodically; most of the time, concentrations were zero or close to zero. Each ascospore event documented in the field was categorized as either zero (0 ascospores), low (between 1 and 4999 ascospores m^{-3}), minor (between 5000 and 49,999 ascospores m^{-3}), or major (≥ 50,000 ascospores m^{-3}) (Table 2-1). In 2011, 90% (37/41) of all "minor" events and 100% (12/12) of all "major" events occurred during the nighttime hours. The results for 2012 were similar, with 98% (64/65) of "minor" events and 100% (2/2) of "major" events occurring during the nighttime hours.

Table 2-1: Classification of ascospore release events in 2011 and 2012

	Zero Event[1]		Low Event[2]		Minor Event[3]		Major Event[4]	
Year	No. of Events	Percentage	No. of Events	Percentage	No. of Events	Percentage	No. of Events	Percentage
2011*	133	37.78%	166	47.16%	41	11.65%	12	3.41%
2012†	98	23.11%	259	61.08%	65	15.33%	2	0.47%

* There were 352 ascospore release events recorded in 2011
† There were 424 ascospore release events recorded in 2012
[1] A zero event was associated with an ascospore concentration of zero
[2] A low event was associated with an ascospore concentration between 1 and 4,999 ascospores m^{-3}
[3] A minor event was associated with an ascospore concentration between 5000 and 49,999 ascospores m^{-3}
[4] A major event was associated with an ascospore concentration ≥ 50,000 ascospores m^{-3}

Correlation analysis identified significant relationships between ascospore concentration and the following: time of day, wind speed, solar radiation, air temperature, relative humidity, and soil temperature in both years. Results for absolute humidity, rainfall, and soil temperature were not consistent between the two years. However, due to pronounced issues with heteroscedasticity following the log transformation, the correlation results were indeterminate. There was no rainfall during the field campaign in 2011, so analysis of the relationship between rainfall and ascospore release was not possible.

There was a clear relationship between relative humidity and ascospore concentration (Fig. 2-2 (2011) and Fig. S3 (2012)). The box plots for each nominal relative humidity value in Fig. 2-3 (2011) and Fig. S4 (2012) depict the spread of ascospore concentration. A prominent wedge pattern depicts higher ascospore concentration at elevated levels of relative humidity, shown by the higher median values and wider intervals between the first and third quartile of the box plots. The equation of a line defining the upper boundary of the wedge was created by binning relative humidity in 1% increments and fitting a line to the maximum ascospore concentration in each

bin. The linear relationships determined for both years were significant (P <0.0001), with calculated slopes of 0.049 and 0.018 in 2011 and 2012, respectively.

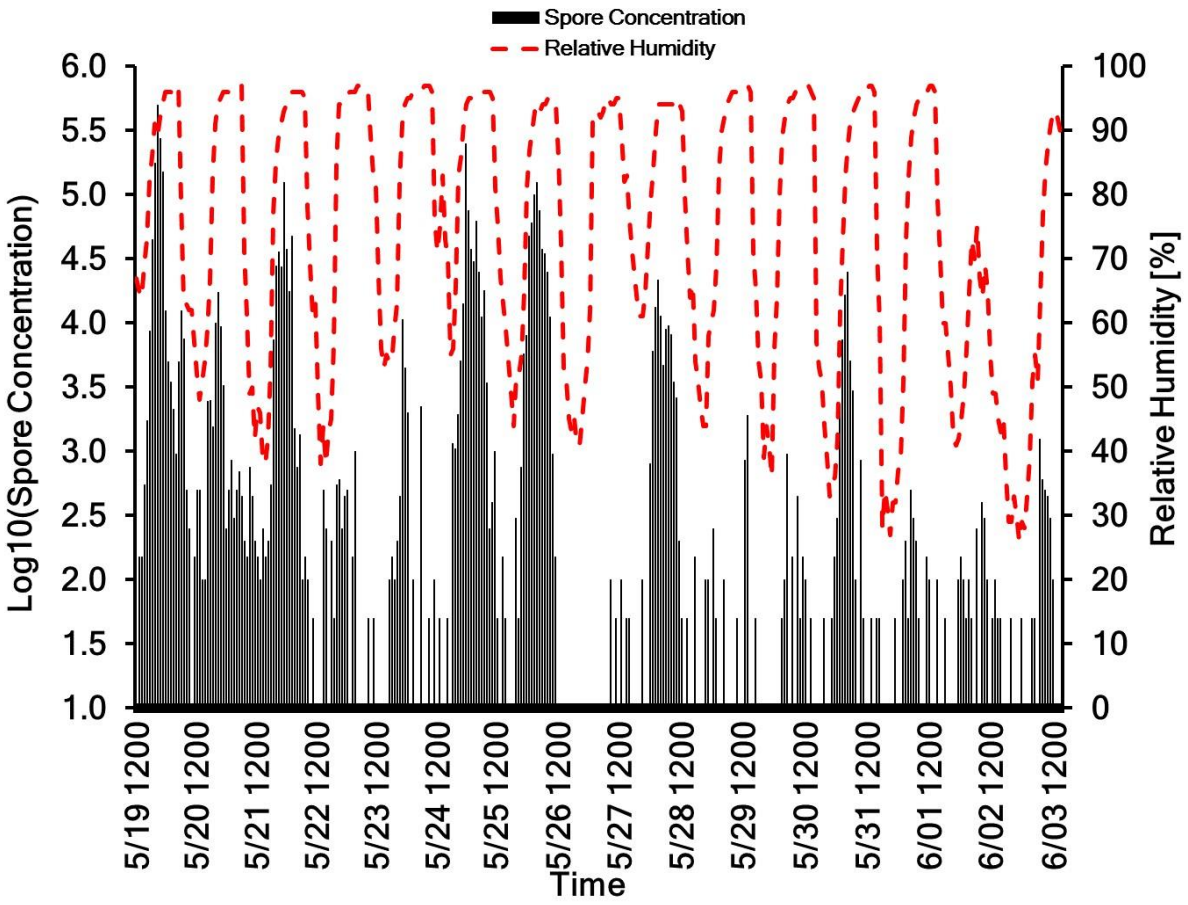

Figure 2-2: Hourly ascospore concentration (black bar graph) and relative humidity (RH, dashed red line) for a field source of F. graminearum between 1700 h 19 May 2011 to 800 h 3 June 2011. A 1-acre wheat field plot was inoculated with a strain of F. graminearum (FGVA4) and subsequently monitored for airborne ascospore concentration using a Quest volumetric ascospore sampler

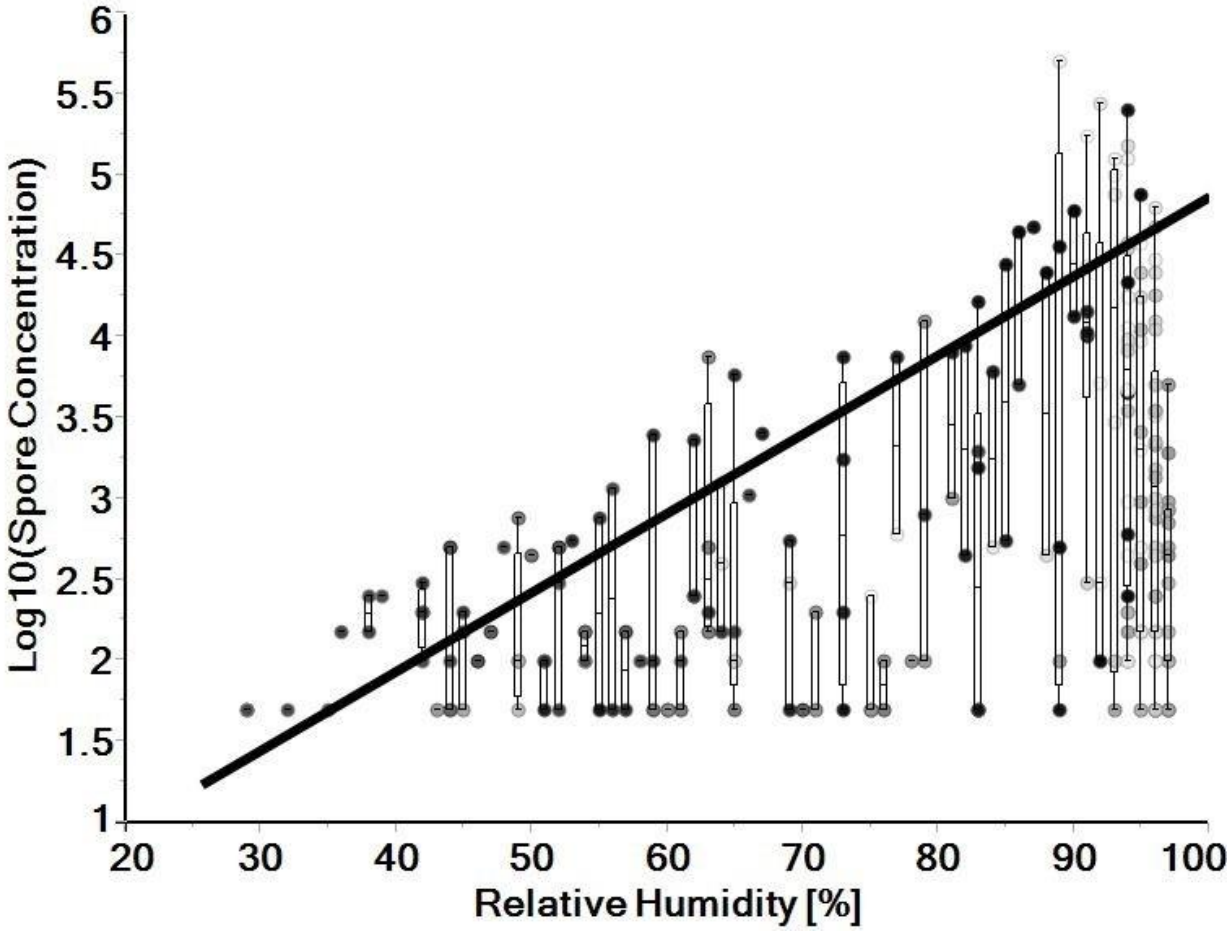

Figure 2-3: F. graminearum ascospore concentration versus relative humidity for a field-scale source of F. graminearum during the monitoring period in 2011. The shading distinguishes nighttime (black and white) from daytime (grey variants) events. The black line fits the highest concentration of ascospores for each 1% range in relative humidity to illustrate where an apparent threshold exists

The relationship between ascospore concentration and air temperature is shown in Fig. 2-4 and Fig. S5 (**Online Resource 2**). The black and white markers in the figures illustrate ascospore concentration during the nighttime period (1900 to 0700), and the grey markers indicate daytime concentration (0700 to 1900). A wedge pattern was evident in 2011, when ascospore

concentrations were higher at the lower temperatures. In 2012, there was more scatter in the data, and no clear trend was evident. Temperature was binned in 1^{o}C increments, and the resulting linear fit to the maximum concentration in each bin is shown in **Table S2**. The resulting linear fit was significantly different from the null hypothesis in 2011 ($P < 0.0001$) but not in 2012 ($P = 0.70$).

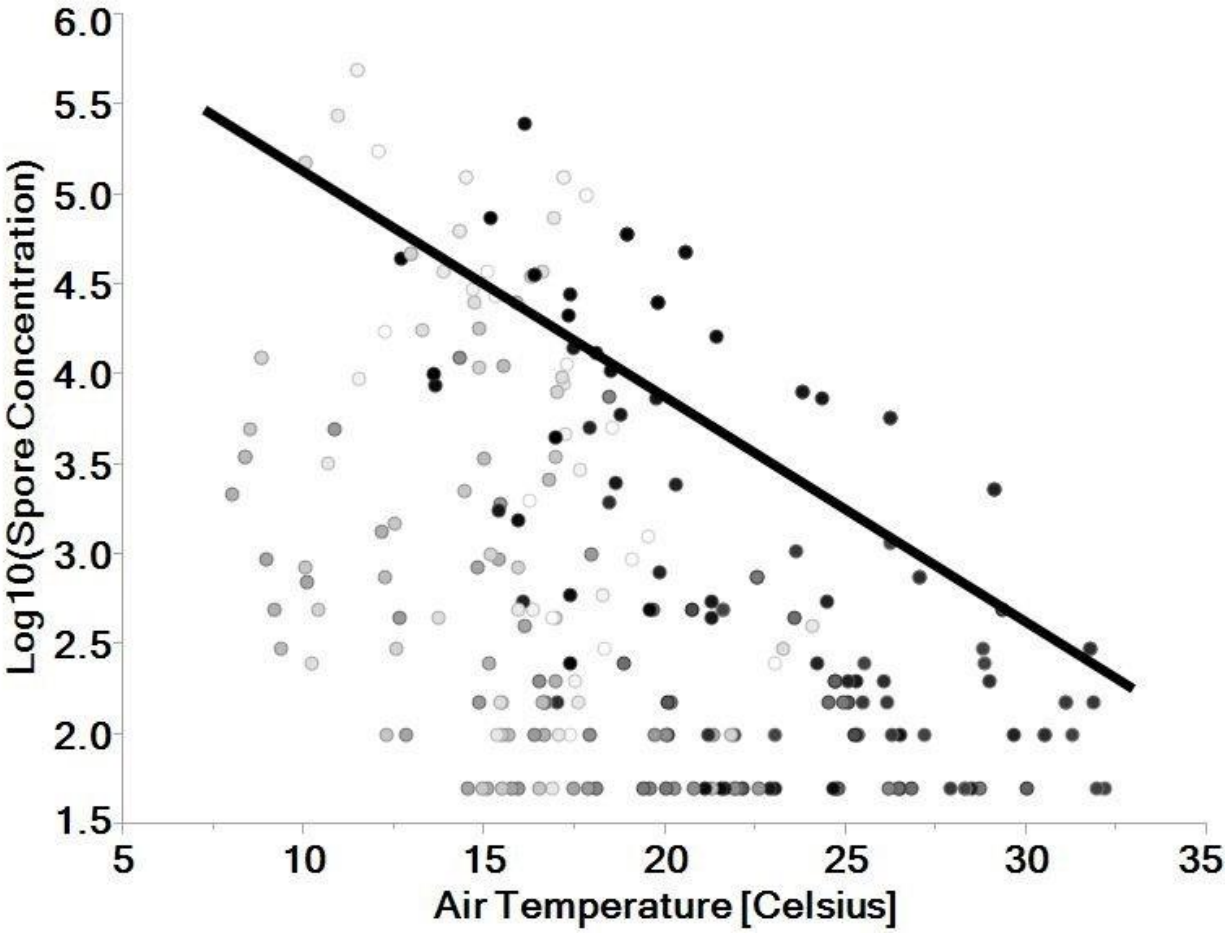

Figure 2-4: F. graminearum hourly ascospore concentration versus air temperature in 2011. The shading distinguishes nighttime (black and white) from daytime (greys variants) events. The black line fits the highest concentration of ascospores within each 1° C range in temperature to illustrate where an apparent threshold exists

Discussion

The impact of meteorological conditions on the release of ascospores of *F. graminearum* into the atmosphere is understudied. This study aimed to extend previous field (Inch et al. 2005; Paulitz 1996; Fernando et al. 2000; Ayers et al. 1975) and laboratory experiments (Gilbert et al. 2008; Tschanz et al. 1975; Trail et al. 2002) by examining two seasons of ascospore concentration data from the same farmland, unique for the large size (3716 m^2) of inoculum generated under controlled conditions (Prussin II et al. 2014a; Prussin II et al. 2014b; Prussin II et al. 2015). Associations between meteorological conditions and ascospore concentration were investigated through causality analyses developed to provide information about systems using small ecological data sets (Sugihara et al. 2012; Deyle et al. 2013; Clark et al. 2015).

To the authors' knowledge, this is the first study to employ causality analyses to predict the release of fungal ascospores into the atmosphere (convergent cross mapping and multivariate state space forecasting). Figure 2-1 and Fig. S1 (**Online Resource 2**) show that solar radiation, air temperature, wind speed, and relative humidity are causally linked to release of *F. graminearum* ascospores. Two causal analysis methods found that: (1) solar radiation and relative humidity were the most influential causal factors; (2) soil temperature and absolute humidity were not causal; and (3) wind speed and air temperature, which may be indicators of atmospheric stability, were causal by one method but not the other. The results further indicated that the identified relationships provide information beyond correlation and actually show that certain meteorological conditions lead to ascospore release. Because of the coupled nonlinear dynamics present within ecological systems, apparent relationships can swing by varying degrees due to minor changes in parameters (Sugihara et al. 2012); thus knowledge of the direction and strength of the relationships is valuable when predicting future conditions.

The results of the 2011 and 2012 field trials illustrated a strong tendency for ascospore concentration to be higher at nighttime (1900 to 0700), agreeing with previous research (Schmale III and Bergstrom 2004; Schmale III et al. 2005b; Schmale III et al. 2006; Inch et al. 2005; Fernando et al. 2000; Ayers et al. 1975; Paulitz 1996). *Mycosphaerella fijiensis* exhibits similar afternoon or nighttime-dominated release events (Meredith et al. 1973; Burt et al. 1997), while other fungal pathogens, such as *Bremia lactucae* (Su et al. 2000), *Venturia inaequalis* (Gadoury et al. 1998), and *Venturia pirina* (Spotts and Cervantes 1994) tend to release ascospores during the daytime or exhibit no apparent temporal preference, such as *Sclerotinia sclerotiorum* (Clarkson et al. 2003).

The largest ascospore concentrations occurred at the highest relative humidity levels. Both types of causality analyses (convergent cross mapping and multivariate state space forecasting) identified relative humidity as an important controlling signal as opposed to factors such as soil temperature and absolute humidity. This study adds to previous research that has identified a relationship between relative humidity and ascospore release (Paulitz 1996; Tschanz et al. 1975; Inch et al. 2005; Reis 1990). Researchers have hypothesized that relative humidity may be the trigger responsible for ascospore release (Paulitz 1996; Tschanz et al. 1975). Turgor pressure within the ascus may be the mechanism for forcibly discharging ascospores (Trail et al. 2002; Trail et al. 2005; Trail 2007), and the balance between water inside the ascus and water in the vapor phase in the atmosphere may help to determine ascospore release.

Convergent cross mapping identified air temperature to be a causal agent of ascospore concentration and a negative correlate per linear regression (Fig. 2-4 and Fig. S5 (**Online Resource 2**)). The results agree with a field trial that found a negative correlation between ascospore release and temperature (Paulitz 1996). Similarly, controlled laboratory studies found that lower temperatures favored ascospore discharge events (Tschanz et al. 1975). The apparent preference for low temperature aligns with studies of *F. graminearum* ascospores showing that germination rates increased with decreasing temperatures (Gilbert et al. 2008), highlighting that low temperatures may favor both release and ascospore survival.

Wind speed was identified as a causal agent of ascospore release with the highest concentration observed during periods with the lowest wind speeds. (Fig. 2-1 and Fig. S1 (**Online Resource 2**)). Researchers have encountered similar daily periodicity in other field data and have hypothesized that the higher number of ascospores could be associated with ascospore release events during the afternoon coupled with the transition to more stable overnight conditions (Fernando et al. 2000). Higher nighttime ascospore concentration may be at least partially explained by more stable atmospheric conditions. We speculate that turbulent mixing associated with an unstable atmosphere during the daytime results in fewer ascospores settling to the ground, whereas stable nighttime conditions produce less mixing in the atmosphere and are more favorable for ascospore deposition and capture (Maldonado-Ramirez et al. 2005; Schmale III and Bergstrom 2004; Schmale III et al. 2005a). Additional research is needed to determine the nature of the relationship between wind speed, ascospore release, and ascospore deposition.

The causality analyses identified a relationship between solar radiation and ascospore concentration. A chamber study showed that light quality and intensity similar to that encountered during mid-day rain events was associated with the largest ascospore release events (Trail et al. 2002). In a field study in New York, the highest ascospore deposition events within a corn canopy were encountered during the nighttime (Schmale III and Bergstrom 2004), while another field study with a 60-m sampling height found no significant difference between daytime and nighttime ascospore counts (Maldonado-Ramirez et al. 2005). The release of ascospores during low levels of solar radiation may not be an advantageous strategy to achieve long distance transport (Schmale III et al. 2006; Oke 1987), but may prolong ascospore survival (Rotem and Aust 1991; Sung and Cook 1981). Research on the effect of UV radiation on *Mycosphaerella fijiensis* ascospores showed that exposure to UV radiation beyond 6 h reduced spore viability, so the timing of a release event could influence the potential for long-distance transport (Parnell et al. 1998). The strong correlation of solar radiation with other meteorological variables makes it challenging to isolate its effect. Additional laboratory experiments are required to further determine how solar radiation may induce ascospore release or if the relationship may be a byproduct of other conditions.

Future research within the area of ascospore release and meteorological conditions would benefit from an understanding of the limitations of this study. An atmospheric transport model of ascospores that accounts for the large difference in atmospheric stability between the day and night would help define the vertical concentration gradient of ascospores and resolve some of these questions, although reliable information about ascospore emission rate as a function of conditions is also needed for such a model to be useful. The number of ascospores measured

during the field experiments was always less than the number released because the sampling method captured only those ascospores that reached the instrument's inlet height; many ascospores were not sampled and deposited back to the ground (Buttner and Stetzenbach 1993; Hart et al. 1994). This disparity between released and captured ascospores has been identified in *M. fijiensis* field investigations as well (Burt et al. 1999; Rutter et al. 1998).

These results highlight meteorological variables that are drivers of increased ascospore concentration. By enabling refinements to models of ascospore transport and dissemination, these findings could help improve predictions of the relative risk of infection (Prussin II et al. 2015; Schmale III and Ross 2015). While the parameters describing ecological, biological, and meteorological conditions are continuously changing and are coupled in complex ways, the ability to describe their interactions can inform decision-making with respect to species management, plant and animal protection, and human health.

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Electronic Supplementary Material

Additional Supplementary Material may be found in the online version of this article:

Online Resource 1:

Causality Methods

Online Resource 2:

Fig. S1 The CCM coefficient, ρ, between candidate driver signals (meteorological variables) and the response signal (ascospore concentration) for the 2012 monitoring period.

Fig. S2 Forecast root mean square (RMS) errors in cases of multivariate forecasting (dashed red line) and single variable forecasting (solid black line) with solar radiation as the augmented information.

Fig. S3 Hourly ascospore concentration (black bar graph) and relative humidity (RH, dashed black line) for a field source of *F. graminearum* between 1800 hours 26 April 2012 to 1100 hours 14 May 2012.

Fig. S4 Ascospore concentration and the relationship to relative humidity (greyscale markers) for a field-scale source in 2012.

Fig. S5 Ascospore concentration versus air temperature for a field-scale source of *F. graminearum* during the monitoring period in 2012.

Fig. S6 Schematic of two time series indicating concept of library length**.**

Fig. S7 Schematic of the reconstructed phase spaces of two variables and the process for calculation of ρ.

Table S1 Results of t-test between RMS of errors corresponding to the cases with and without augmented meteorological signals.

Table S2 Results of bivariate analysis of ascospore concentration and meteorological variables.

Chapter 3 – Ascospore release and discharge distances of *Fusarium graminearum* under controlled temperature and relative humidity

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Abstract

Understanding the influence of meteorological conditions on the release of pathogen spores is necessary for crop management decisions and development of spore transport models. This study investigated the release of ascospores of *Fusarium graminearum* in a controlled chamber at two temperatures (15 °C and 25 °C) and at three relative humidities (60%, 75%, and 95%). Filter paper pieces containing perithecia from a single isolate of *F. graminearum* were placed inside custom 3D-printed spore discharge devices, and the number of ascospores released and distance the ascospores were discharged were measured. The number of ascospores released was higher at 15 °C, and increased with increasing levels of relative humidity. Ascospores were discharged 0.5 mm to over 12 mm from perithecia and traveled farther from the perithecia at 25 °C and at the highest levels of relative humidity. Even small differences in discharge distances may be important for the escape of ascospores from the laminar boundary layer and into the turbulent layer. Spore transport models need to consider the impact of environmental conditions on spore release and transport.

Keywords: Fungus; *Fusarium graminearum*; Fusarium head blight; Bioaerosol; Relative humidity; Spore discharge; Perithecia; Chamber investigation; Disease management; Aerobiology

Introduction

An increased understanding of each of the various stages of atmospheric transport is important for controlling the spread of disease and managing crops (Aylor 1999). *Fusarium graminearum*, causal agent of Fusarium head blight (FHB) of wheat and barley (Windels 2000; McMullen et al. 1997; Goswami and Kistler 2004), may be transported over long distances in the atmosphere (Prussin II et al. 2014a; Prussin II et al. 2015). Symptoms of FHB include premature bleaching and shriveled kernels, which have contributed to economic losses totaling $2.7 billion (US dollars) from 1998 to 2000 in the Northern Great Plains and Central United States (Nganje et al. 2002; Goswami and Kistler 2004). Grain may be contaminated with trichothecene mycotoxins such as deoxynivalenol, threatening the health of monogastric animals and humans if consumed (Okubara et al. 2002; Schollenberger et al. 2002; McMullen and Stack 1983).

Some plant pathogens are commonly transported through the atmosphere, and their spread is initiated by the release of a pathogen from a source (Schmale III and Ross 2015). Some examples include the oomycete *Phytophthora infestans*, causal agent of late blight of potato and tomato (Fry et al. 1993; Brown and Hovmøller 2002), the fungus *Puccinia melancocephala*, causal agent of sugarcane rust (Purdy et al. 1985), and *Bacillus pumilus*, causal agent of bacterial blot in peaches (Kellogg and Griffin 2006; Griffin et al. 2001). Some plant pathogenic ascomycetes, such as *Fusarium graminearum*, discharge sexual ascospores into the atmosphere from infested crop debris and become the primary inoculum for local or regional epidemics

(Elbert et al. 2007; Sutton 1982). Environmental conditions, such as relative humidity (RH) and atmospheric stability, can influence the aerobiological processes of spore release, horizontal transport, and deposition (Isard et al. 2005; Aylor 1986, 1990), and these processes may occur over a variety of different transport scales (Schmale III and Ross 2015).

The long-distance atmospheric transport of *Fusarium graminearum* has been associated with the production of perithecia and ascospores from crop debris and the subsequent release of these ascospores into the atmosphere (Sutton 1982; Prussin II et al. 2014a; Prussin II et al. 2014b; Prussin II et al. 2015; Schmale III and Bergstrom 2005; Schmale III et al. 2005b; Schmale III et al. 2006; Schmale III et al. 2012; Schmale III and Ross 2015). Ascospores are released from perithecia at exceptional acceleration of 8,500,000 m s^{-2} (Trail et al. 2005), yet the impact of meteorological conditions on the potential variability of ascospore discharge remains unresolved. Ascospore concentrations in the atmosphere have been associated with light (Schmale III et al. 2006; Schmale III et al. 2005b; Trail et al. 2002), temperature (Del Ponte et al. 2009; Sutton 1982; Paulitz 1996), and RH (Paulitz 1996; Paul et al. 2007), but experimental work under controlled environmental conditions is needed to determine the impact of meteorological conditions on ascospore release.

Researchers have associated RH with ascospore release under field conditions (Paulitz 1996; Inch et al. 2005), but it is difficult to separate the effects of RH from solar radiation, air temperature, and other meteorological factors because many follow distinct diurnal cycles. A two-year field campaign conducted in Quebec, Canada with an active spore sampling method found that ascospore release was correlated with high levels of RH experienced during the early

evening hours and exhibited a diurnal pattern (Paulitz 1996). Field trials conducted in Manitoba, Canada, using rotorod and Burkard samplers observed a greater number of ascospores captured near midnight, when RH is higher (Inch et al. 2005). A wind tunnel study observed increased levels of ascospore release events at high RH and found that maximum discharge coincided with 100% RH (Trail et al. 2002). Convergent cross mapping causality analysis performed on two years of field data obtained in Blacksburg, Virginia, USA identified RH as a causal agent of ascospore release (David et al. 2016). Unknown is whether RH is the sole meteorological causal factor and whether the daily diurnal cycle (or an explicit threshold level) is associated with the mechanical rupturing of perithecia and associated ascospore release events.

Temperature has also been associated with ascospore release and spore germination in field and laboratory studies (Paulitz 1996; Tschanz et al. 1975; Manstretta and Rossi 2015; Gilbert et al. 2008), but the true effect has yet to be ascertained because of the interrelatedness of temperature with other confounding parameters such as RH over the diurnal cycle. A field study found that ascospore release events occurred over a large range of temperatures (11 °C to 30 °C), and that initial increases in ascospore concentration corresponded with falling temperature and rising RH (Paulitz 1996). Further field trials in which the ascospore numbers of *F. graminearum* were collected observed a daily periodicity of ascospores with the greatest numbers recovered during the cooler, nighttime periods (Fernando et al. 2000; Prussin II et al. 2014a; Prussin II et al. 2014b). The apparent tendency for ascospore release to occur at low temperatures (<20°C) supports research on germination (Gilbert et al. 2008). Laboratory investigations suggest that temperatures of 14 °C and 30 °C were required for germination in 10.4 h and 3.3 h, respectively (Beyer and Verreet 2005). The link between spore release and germination identifies similar

temperature conditions in which the ascospores remain viable and capable of causing disease. While the optimal temperatures among ascospore release (Sutton 1982; Tschanz et al. 1975; Tschanz et al. 1976), ascospore germination (Beyer et al. 2005), and disease expression (Brennan et al. 2005) varies, they also show a propensity to occur during a wide range of conditions with apparent peaks at various meteorological conditions. Consequently, research on how specific temperatures impact ascospore release under controlled conditions is needed.

We hypothesized that ascospores of *Fusarium graminearum* are discharged in greater number and at greater distances at higher RH and lower T. The specific objective of this study was to determine the effects of RH and temperature on ascospore release, in terms of both number of ascospores discharged and distances the ascospores are discharged. The results from this study may provide an improved understanding of the meteorological conditions linked to ascospore release, and may be useful to those who model and manage the spread of FHB.

Materials and Methods

Generation of perithecia of *Fusarium graminearum*

A single isolate of *Fusarium graminearum* (Fg_Va_GPS13N4_3ADON) was used in this study (Prussin II et al. 2014a). The fungus was stored at -80 °C in glycerol and inoculated onto an artificial substrate, carrot agar. The carrot agar was prepared using organic carrots with a concentration of 400 g carrots L^{-1} medium (Klittich and Leslie 1988). A single piece of autoclaved filter paper (7 cm, 09-801A, Fisherbrand, Waltham, MA) was placed on each carrot agar petri dish (10 cm) using sterilized forceps prior to inoculation. The isolate Fg_Va_GPS13N4_3ADON was inoculated on the filter paper and carrot agar petri dishes and

the cultures subsequently maintained at room temperature for ~8 days to allow for the development of vegetative hyphae. Perithecia were induced from the mycelium by adding 1 mL of 2.5% Tween 60 (P1629, Sigma, St. Louis, MO) and flattening the mycelium with a sterile rod. The cultures were maintained for 9 days under a 12/12 h light/dark cycle at room temperature to stimulate the production of perithecia on the filter paper. Prior to introduction into the testing chamber (discussed below), 4 mm by 10 mm test strips were cut from the 7-cm circular filter paper containing mature perithecia. Nine-day-old perithecia (days after the application of 2.5% Tween 60) were used in all experiments, and the number of perithecia per strip were counted prior to introduction into the chamber using a microscope (Schmale III et al. 2005a).

Controlled environmental chamber

A controlled environmental chamber was used to regulate three environmental parameters: air temperature, light, and RH. The chamber configuration consisted of 3D-printed spore discharge devices inside a polypropylene container placed within a clear acrylic vessel inside a growth chamber (Fig. 3-1). Each spore discharge device contained two discharge alleys. The growth chamber (PGC20, Conviron, Winnipeg, Manitoba) had an interior volume of 2916 L, the acrylic vessel had a total volume of 49 L (composed of a 45.7 cm cylinder capped with a dome), and the polypropylene container (1751, Sterilite Co., Townsend, MA) had a volume of 5.7 L. All ports into the acrylic vessel were plugged to prevent flow through the vessel such that experiments were performed in still air. While the growth chamber controlled light and air temperature, saturated salt solutions of sodium bromide, sodium chloride, and potassium sulfate maintained RHs of 60%, 75%, and 95%, respectively, inside the acrylic vessel. Each saturated salt solution was created by adding the pure salt to 200 mL of deionized water such that excess solute remained in the final solutions.

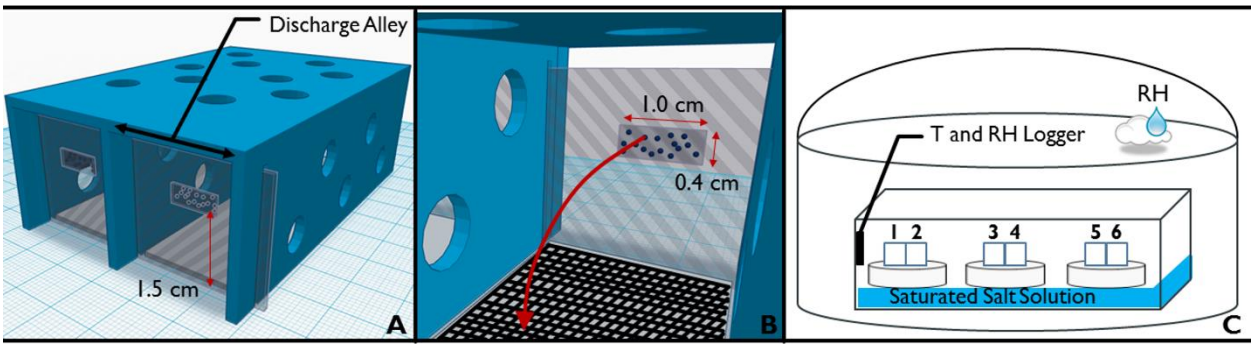

Figure 3-1: Schematic of 3D-printed spore discharge devices inside controlled environmental chambers. (a) 10 mm × 4 mm strips of filter paper with *Fusarium graminearum* perithecia were adhered to a microscope slide and oriented 1.5-cm above the floor of a 3D-printed spore discharge device, (b) perithecia were oriented to allow ascospores to be forcibly discharged horizontally (red arrow) onto a microscope slide coated with silicone grease, (c) six spore discharge alleys with strips of filter paper containing 9-day-old perithecia were placed on top of 10-cm petri dish lids and positioned inside a polypropylene container with a saturated salt solution to maintain target RH conditions and were situated within a larger acrylic vessel

Ascospores were captured in discharge alleys (Fig. 3-1) that had perithecia mounted on one end. Based on the design used by Schmale III et al. (2005a) and Aylor and Anagnostakis (1991), the devices were created with a 3D printer using an acrylonitrile butadiene styrene filament (Robo3D, San Diego, CA). Each device was composed of two side-by-side alleys measuring 29 mm x 26 mm x 81 mm (H x W x L). To promote rapid equilibration of temperature and RH inside the device, each exterior surface had five 0.7-cm holes, and one end of each channel was open. The discharge device may be reproduced using the G-code in Online Resource 1.

Temperature and RH within the chamber configuration were recorded every 30 s with a data logger (U23-001, Onset, Bourne, MA) placed inside the polypropylene containers, and the data

were offloaded onto a computer for analyses using a dedicated base station (BASE-U-4, Onset, Bourne, MA).

Measurement of ascospore release

The apparatus was designed to have elevated perithecia eject ascospores horizontally and allow them to fall to the floor of each discharge alley so that the distance traveled could be compared under different environmental conditions. Strips of perithecia (filter paper peels cut into rectangles 10 mm long and 4 mm wide) were grown on carrot agar. The perithecia testing strips were placed 1.5 cm above the bottom of the spore discharge chamber on vertically-oriented microscope slides that slid into a notch at one end of the chamber as shown in Fig. 3-1. The strips were adhered to the slides using the moisture present on the strips.

Ascospores released from the perithecia deposited on microscope slides placed along the bottom of the spore discharge devices. The slides had 1 mm x 1 mm grid lines and were coated with a thin layer of silicone grease. The numbers of ascospores released and distances traveled (analyzed from 0 to 12 mm away from the perithecia) were counted and measured manually by visualizing the slides under a microscope. The distance of 12mm was chosen as a cutoff based on previous studies of discharge distances of ascospores and the observation that nearly all of the ascospores were discharged less than 12 mm (Schmale III et al. 2005a; Trail et al. 2005).

Experimental design

Two temperatures (15 °C and 25 °C) and three RHs (60%, 75%, and 95%) were selected for investigation. Each condition (six combinations of temperature and RH) was run with six discharge alleys providing six sub-samples (Table 3-1). Each condition was also conducted

without perithecia to serve as a negative control. Each chamber trial was initiated at 1900 EST/DST and lasted 12 h (Fig. 3-2). We defined trial as the testing of one of the combinations of temperature and RH (Table 3-1). The positions of the spore discharge devices and acrylic vessels were randomly defined inside the growth chamber for each trial.

Table 3-1: Summary of treatments including number of acrylic vessels, discharge devices, and discharge alleys. There were six treatments of temperature (15 °C and 25 °C) and RH levels (60%, 75%, and 95%)

Date	Treatment	No. of Acrylic Vessels	No. of Spore Discharge Devices	No. of Discharge Alleys
Sep 17	25°C & 75% RH	1	3	6
	25°C & 95% RH	1	3	6
Sep 18	15°C & 95% RH	1	3	6
	15°C & 75% RH	1	3	6
Sep 25	25°C & 60% RH	1	3	6
Sep 26	15°C & 60% RH	1	3	6

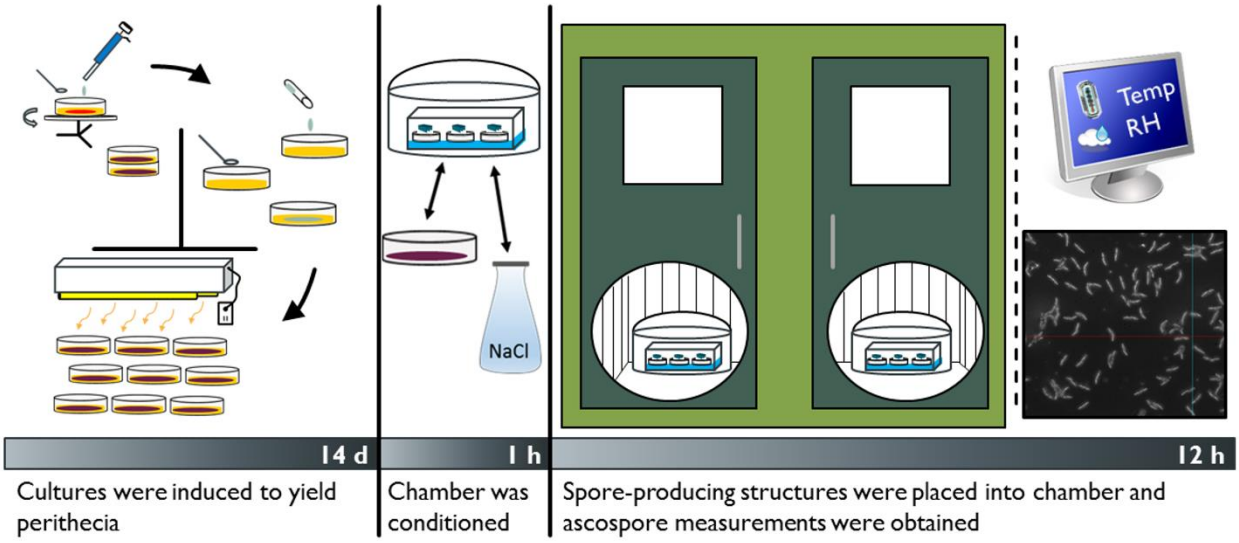

Figure 3-2: Timeline of chamber investigation. *Fusarium graminearum* cultures were induced in the laboratory to produce perithecia on carrot agar media. Perithecia were introduced into the chamber configuration where RH, temperature, and dark conditions were maintained. Custom 3D-printed spore discharge devices were used to align perithecia inside the chamber and allow for capture on microscope slides. Ascospore distance and counts were measured microscopically

Each experiment included the following steps: the desired temperature (15 °C or 25 °C) was set on the growth chamber, the RH of the growth chamber was set to 60%, the lights were turned off inside the growth chamber, the desired saturated salt solution was placed into the bottom of the polypropylene container, three spore discharge devices were placed on the lids of 10-cm petri dishes inside the polypropylene container above the salt solution, the cover was placed on the polypropylene box, the acrylic vessel was placed over the polypropylene box, and the seam between the acrylic vessel and the growth chamber floor was sealed with tape. The growth chamber and acrylic vessels were conditioned for ~1 h prior to each trial to set the appropriate growth chamber temperatures and desired salt solutions. The perithecia-containing strips were

introduced into the chamber just prior to the initiation of each trial. Table 3-1 provides the order and number of discharge alleys used per condition.

Initial horizontal ascospore velocity

The initial horizontal velocity of the ascospores released was calculated. As shown by Trail et al. (2005), the spore release event is composed of two distinct regimes: a primary regime where drag forces dominate and ascospore transport is overwhelmingly in the horizontal direction and a vertical settling regime. The settling velocity of ascospores released in still air has been determined by Schmale III et al. (2005a). The initial velocity of ascospores was calculated by assuming the horizontal ascospore discharge distances were equivalent to stopping distance. Stopping distance is the distance a particle travels due to its effective initial momentum before aerodynamic drag halts further transport (Hinds 1999). Because of the distinct drag-force dominated regime (Trail et al. 2005) and the experimental conditions of release into still air (Hinds 1999), this assumption was reasonable. The relaxation time (τ) that represents the duration a particle requires to adjust to a change in forces, was calculated using the following equation:

$$\tau = \frac{\rho_p \times d^2 \times C_c}{18 \times \mu} \quad (1)$$

where the density of the particle, ρ_p, was assumed to be that of water (1000 kg m^{-3}); the aerodynamic diameter, d, of 11.84 µm (mean) was measured using an Aerodynamic Particle Sizer (3321, TSI, Inc., Shoreview, MN); the Cunningham correction was calculated as 1.0; and the dynamic viscosity, μ, of air was calculated for each combination of temperature and RH according to Morvay and Gvozdenac (2008). The initial horizontal velocity (V_0) was calculated using the equation:

$$V_0 = \frac{S}{\tau} \quad (2)$$

where *S* was the horizontal distance an ascospore traveled within the discharge alley.

Results

Effects of temperature and relative humidity on number of ascospores released

Ascospore release was measured at temperatures of 15 °C and 25 °C and at RHs of 60%, 75%, and 95%. The actual temperatures averaged 15.2 °C and 24.7 °C, and the actual RHs averaged 63.8%, 75.6%, and 96.1%. A total of 368,106 ascospores were counted across six sub-samples at each of the six combinations of temperature and RH. Table 3-2 shows the number of ascospores released, totaled across all six sub-samples at each condition and averaged across trials. The number of perithecia on each 4 mm by 10 mm testing strip was 37 ± 3 (mean ± std. error). The greatest number of ascospores was released at 15 °C and 95% RH, on average nearly 55,000 per sub-sample, and the second greatest at 15 °C and 75% RH. Fewer than 5 ascospores, on average, were released at 60% RH at both temperatures tested. Of the six trials conducted at each set of temperature and RH conditions, three sub-samples at 15 °C and 60% RH, three sub-samples at 25 °C and 60% RH, and two sub-samples at 25 °C and 75% RH resulted in no ascospore release. In general, the number of ascospores discharged increased with RH. The samples at each condition showed some variability in total counts (e.g., a range of ascospores counted at 25 °C and 95% RH of 340 to 4540). The varying numbers of perithecia on each strip, the range of maturity of the perithecia on a single strip, and the variety of perithecia maturity from strip to strip may be contributing factors to the variability in ascospore count.

Table 3-2: Summary of total ascospore counts across six sub-samples at each combination of temperature and RH investigated

	60% RH & 15°C	75% RH & 15°C	95% RH & 15°C	60% RH & 25°C	75% RH & 25°C	95% RH & 25°C
Total Spores	17	27505	329274	26	1490	9794
Mean per Sub-sample	3	4584	54879	4	248	1632
Std. Error	1	2050	8951	2	175	681

Impact of temperature and relative humidity on ascospore discharge distances

Upon release, ascospores traveled from 0.5 up to 12 mm (farthest distance analyzed) and beyond. At 15 °C, many of the trials had ascospore release events reaching 12 mm: zero, five, and six of the six sub-samples conducted at each of the 60%, 75%, and 95% RH conditions, respectively. Four out of the six sub-samples conducted at 25 °C and 95% RH produced at least one ascospore reaching 12 mm.

The number of ascospores discharged (log scale) generally increased with increasing RH and peaked in distance traveled between 4 to 5 mm from the perithecia at both 15 °C and 25 °C (Fig. 3-3). Perithecia appeared to discharge ascospores farther at higher RH and warmer temperature (Table 3-3). Fewer ascospores were released at 60% RH (0.0048% and 0.23% of total ascospores at 15°C and 25°C, respectively) compared to the higher RH values investigated.

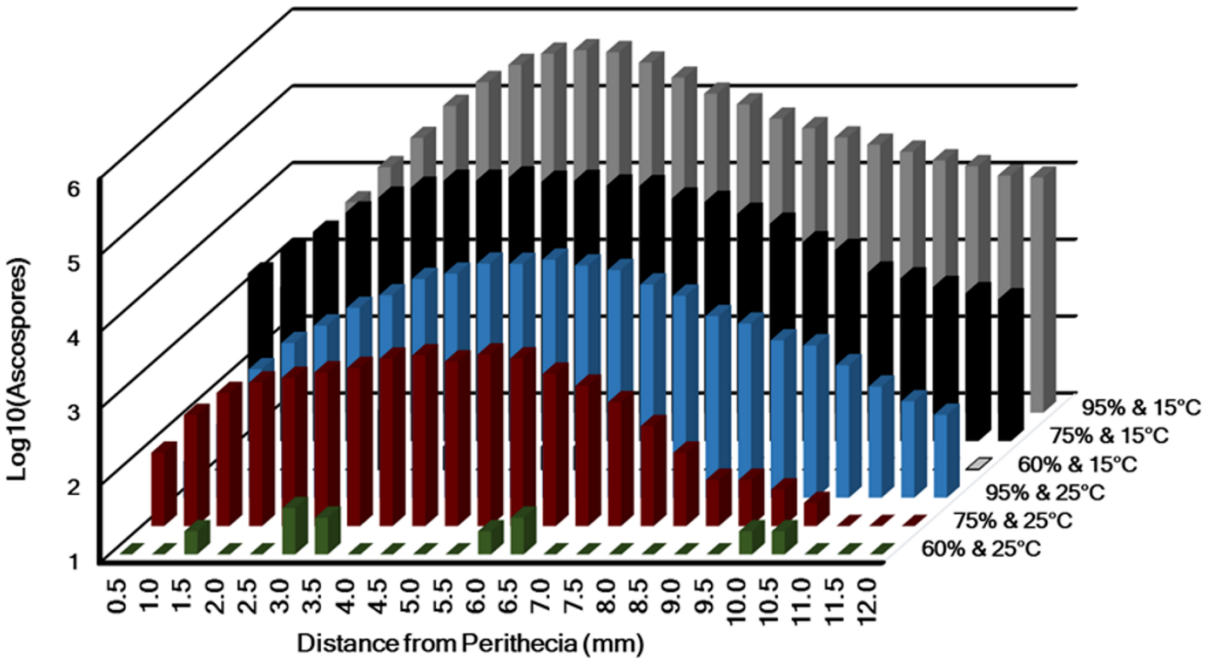

Figure 3-3: Ascospore counts at the six combinations of temperature (15 °C and 25 °C) and RH levels (60%, 75%, and 95%) investigated. Ascospore counts at each distance shown are associated with spores that were counted within a band that extended 0.5 mm behind that value. Ascospore counts were combined for six sub-samples captured at each combination of temperature and RH condition investigated

Table 3-3: Summary of mean ascospore discharge distances (in mm) at the temperature and RH combinations investigated

	15°C	25°C	All Temps
60%	3.4 ± 0.4	5.9 ± 0.3	4.9 ± 0.25
75%	5.1 ± < 0.1	4.6 ± < 0.1	5.0 ± < 0.1
95%	5.4 ± < 0.1	5.8 ± < 0.1	5.4 ± < 0.1
All RHs	5.4 ± < 0.1	5.6 ± < 0.1	5.4 ± < 0.1

A cumulative distribution function of distance discharged for each of the six combinations of temperature and RH shows a sigmoidal shape (Fig. 3-4). The curve illustrates that approximately 50% of ascospores were discharged less than 5.5 mm.

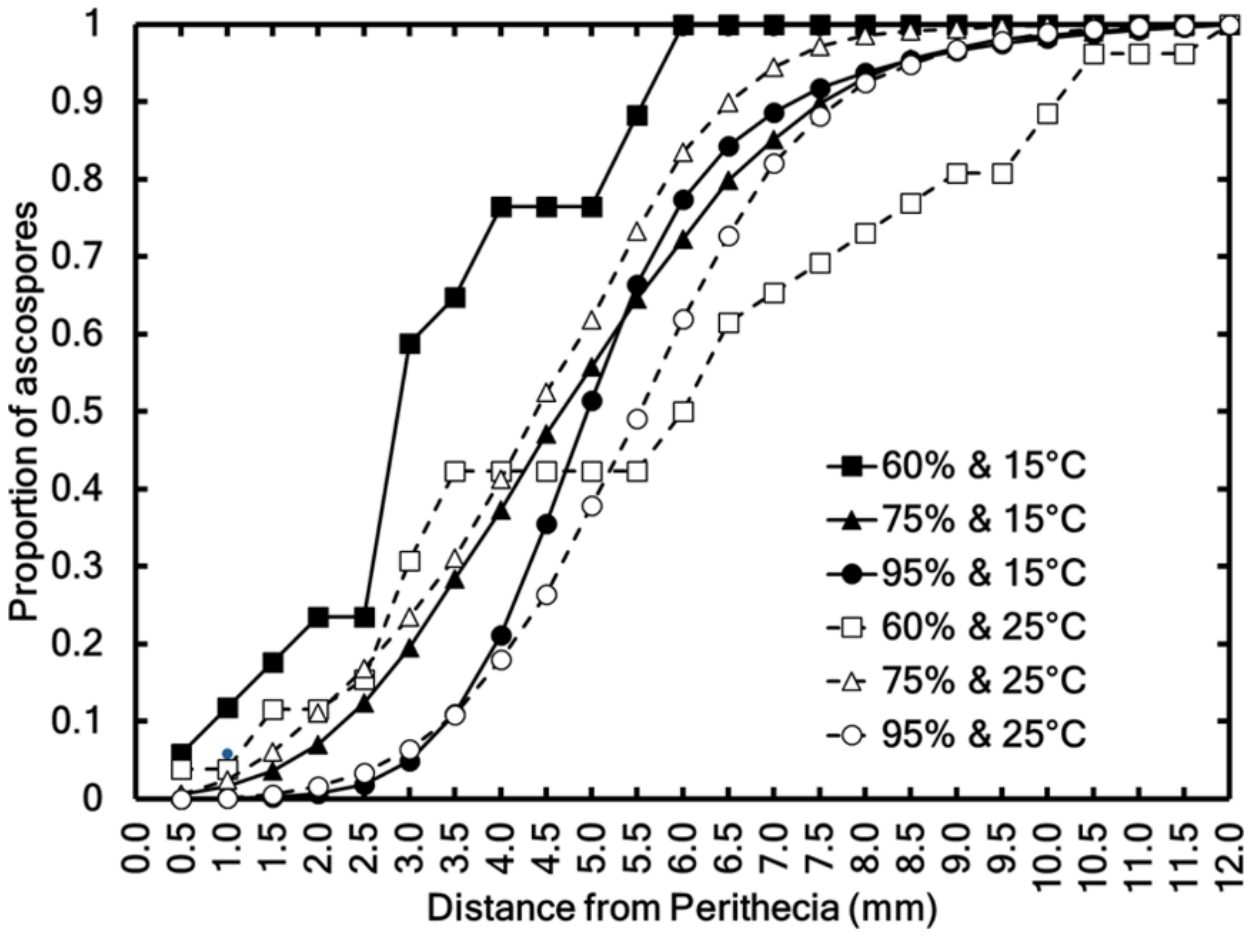

Figure 3-4: Cumulative distribution function of ascospore distance discharged from perithecia of *Fusarium graminearum*. Approximately 50% of ascospores traveled <5.5 mm and 95% traveled <8.5 mm. Ascospore counts were combined for the six sub-samples captured at each combination of temperature and RH condition investigated with 368,106 ascospores counted at the six combinations of temperature and RH conditions tested

Impact of temperature and relative humidity on ascospore discharge velocities

The mean initial ascospore discharge velocity ranged from 8 to 13 m s^{-1} and generally increased with increasing RH at both temperatures (Table 3-4). A spore that traveled 12 mm, the maximum distance analyzed, would have had an initial velocity of 28.7 m s^{-1}, and spores traveling farther than 12 mm would have had a higher initial velocity. The relaxation times calculated were between 0.43 and 0.44 ms.

Table 3-4: Summary of estimated initial ascospore discharge velocity (V_0, in m s^{-1}) at the temperature and RH combinations investigated

	15°C	25°C	All Temps
60%	7.7 ± 0.9	13.7 ± 0.8	11.3 ± 0.6
75%	11.5 ± < 0.1	10.6 ± 0.1	11.6 ± < 0.1
95%	12.3 ± < 0.1	13.4 ± < 0.1	12.4 ± < 0.1
All RHs	12.2 ± < 0.1	13.1 ± < 0.1	12.8 ± < 0.1

Discussion

New information is needed to understand the environmental conditions driving spore release of plant pathogens. Atmospheric transport models would benefit from the incorporation of more refined parameters including spore release rates (q_0) of a fungus (Schmale III and Ross 2015; Prussin II et al. 2014b; Aylor et al. 2001). This study focused on the effect of temperature and RH on the release of ascospores of *F. graminearum* and showed that both parameters influenced both the quantity of ascospores released and distance they were discharged. The present study is the first to the authors' knowledge that investigates how meteorological conditions influence ascospore release distance.

Ascospores were discharged farthest from perithecia at a RH of 95%. This research builds upon previous investigations of ascospore release distances conducted at ambient room conditions, in which mean discharge distance was between 3.5 and 4.5 mm (Trail et al. 2005; Schmale III et al. 2005a), with the latter testing ascospore release from a 1-cm launch height. Previous research using a spore discharge chamber reported mean discharge distances of 4.6 mm and 3.9 mm when investigating 6-day-old and 12-day-old mature perithecia, respectively (Schmale III et al. 2005a). These results showing larger numbers of ascospores being released farther distances at higher RH may help explain the higher concentration of airborne ascospores observed during high-RH periods of the day (Paulitz 1996; Ayers et al. 1975; Trail et al. 2002; Del Ponte et al. 2005). While an increase in the release of ascospores from a source of perithecia may be a contributing factor to higher concentrations, other factors such as atmospheric turbulence in the boundary layer (Isard et al. 2005; Oke 1987) or ascospore washout events during field trials (Aylor 1998) may be important factors within this system. Future research investigating the level of importance of these various factors on ascospore concentrations would be useful in predicting and preparing for FHB outbreaks.

The numbers of ascospores of *F. graminearum* released were highest at 95% RH at both temperatures investigated. Results of this study agree with many previous field and laboratory investigations of *F. graminearum* ascospore release showing that RH is associated with increased airborne concentrations of ascospores (Fernando et al. 2000; Inch et al. 2005; Paulitz 1996; Trail et al. 2002; Tschanz et al. 1975). Previous studies found that maximal ascospore discharge within a wind tunnel peaked at RH > 92% (Trail et al. 2002) and that ascospore discharge occurred during humid (100%) conditions within environmental growth chambers

(Tschanz et al. 1975). These results also align with field studies in Quebec, Canada, where release events were identified at RHs of 60% to 95% (Paulitz 1996). Ayers et al. (1975) found that maximum numbers of ascospores collected from wheat and corn fields in Pennsylvania occurred when RH was between 95% and 100%. These observations, along with the results from this study, suggest increased ascospore release and concentration levels during periods of high RH, such as during the nighttime (Prussin II et al. 2014a; Prussin II et al. 2015).

There were greater numbers of ascospores released at 15 °C than at 25 °C. At 75% and 95% RH, over 18x and 34x more ascospores were released, respectively. Previous studies have shown similar trends between ascospore release and air temperature. In a field experiment, the most ascospores were found between 2100 and 0600 hours, when recorded temperatures were between 13 and 22 °C (Ayers et al. 1975). Another field investigation reported that the start of ascospore release events (time when ascospore concentration exceeded 666 ascospores m^{-3}) was correlated with an increase in RH and a decrease in temperature (below ~20 °C) that occurs during the late afternoon (Paulitz 1996). A previous laboratory study employing a temperature gradient plate found that ascospore discharge was the greatest at the lowest temperature investigated (16.6 °C) and negligible above 26 °C (Tschanz et al. 1976). The relationship may be due to higher germination rates at lower temperatures (15 °C) (Gilbert et al. 2008).

The mean ascospore discharge distance was 5.6 mm at 25 °C and 5.4 mm at 15 °C. In comparison, ascospores were shown to travel 4.6 mm (6-day-old perithecia) and 3.9 mm (12-day-old perithecia) in other horizontally-released ascospore studies (Schmale III et al. 2005a). The observation that warmer temperatures resulted in longer ascospore discharge distances was

unexpected when compared to previous field investigations (Paulitz 1996; Ayers et al. 1975) and laboratory studies (Tschanz et al. 1976; Tschanz et al. 1975). One component to why ascospores travel farther at warmer temperatures may be the availability of ascospores within the perithecium and ascus. The optimum temperature for perithecium maturation was reported to be ~29 °C (Tschanz et al. 1976). Temperatures may fluctuate within large, dense crop fields resulting in temperature gradients throughout the field. A chamber study found that ascospores are released at an exceptional acceleration of 8,500,000 m s^{-2} (Trail et al. 2005) and that increased turgor pressure is the mechanism that causes the ascospore discharge event (Trail et al. 2002). It is possible that at warmer temperatures, ascospores and turgor pressure are readily available for release events. The comparatively fewer ascospores released at warmer temperatures may travel farther due to an availability of turgor pressure and a "clean" release from the asci because fewer spores are being released in close temporal proximity.

No (zero) ascospore release in some sub-samples may suggest either non-optimal meteorological conditions for release or experimental error. There were, at most, three out of six sub-samples for any given combination investigated where no ascospores were released. Since all of the testing strips originated from the same petri dish, this is not likely to be a source of experimental error. The results showed that half of the sub-samples at 60% and 15 °C, half the sub-samples at 60% and 25 °C, and a third of the sub-samples at 75% and 25 °C did not exhibit any ascospore release. *Fusarium graminearum* ascospore release has been hypothesized to require an abrupt increase in RH (Paulitz 1996) and the transition from room temperature and RH to 15 °C or 25 °C and 60% RH was not a sharp enough change to cause ascospore release in all sub-samples.

Additionally, wind tunnel studies found that while maximum discharge occurred at 100% RH, there was discharge between 40% to 100% RH (Trail et al. 2002).

The initial velocity of an ascospore determines its distance traveled, which was farthest at 95% RH in this study. Thus the highest initial horizontal velocities also occurred at 95% RH. The conditions with the largest initial horizontal velocities were 25 °C and 95% (13.4 m s^{-1}) and 60% (13.7 m s^{-1}) RH. The initial velocities were less than those reported by Trail et al. (2005), who calculated initial velocity using a smaller ascospore diameter, a generally shorter horizontal discharge distance, and from ascospore discharge distances obtained at ambient room conditions. The settling time calculated by Schmale III et al. (2005a) was between 3.1 to 3.6 s for ascospores with aerodynamic diameters of 5.5 to 7.2 µm. Of the two distinct regimes of spore transport identified (Trail et al. 2005), the drag-dominated initial phase takes up a minimal amount of time (< 0.012%) when compared to the entire settling period but is associated with the vast majority of horizontal transport. The second regime is therefore associated with the majority of the required settling time and vertical deposition. The initial velocity is associated with the turgor pressure within a perithecium, while air density impacts the drag after release. The results suggest that the vertical placement of perithecia within the spore discharge devices would have made only a minor difference in horizontal ascospore discharge distance since this distance is covered in the drag-dominated, minor-settling primary phase of ascospore transport.

This study contributes knowledge about the role of RH and temperature on the primary stage of atmospheric transport and could be incorporated to model more effectively the spread of *F. graminearum* ascospores. This study showed that ascospores of *F. graminearum* were

discharged in the greatest numbers at cooler and more humid conditions, but are transported furthest from the perithecia at warmer and more humid conditions. Ascospores traveled approximately 0.23 mm farther at 25 °C than at 15 °C and 0.39 mm farther at 95% RH than 75% RH. In order for the ascospores to be transported long distances in the atmosphere (Prussin II et al. 2014a; Prussin II et al. 2014b; Prussin II et al. 2015), the ascospores must reach turbulent air currents (Isard et al. 2005). Even small differences in discharge distances may be important for the escape of ascospores from the laminar boundary layer. Thus, any additional distance perithecia are able to discharge ascospores through the laminar boundary layer, increases their probability of reaching the turbulent layer where long-distance transport can occur. Future work should examine how the structure of the perithecium mechanistically responds to meteorological conditions. This knowledge would assist in clarifying the parameters that impact spore release rates (q_0), thus providing novel information to refine transport models and to make appropriate field management decisions.

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Chapter 4 – Compression tests of *Fusarium graminearum* ascocarps provide insights into the strength of the perithecial wall and the quantity of ascospores

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Abstract

The plant pathogenic ascomycete *Fusarium graminearum* produces perithecia on corn and small grain residues. These perithecia forcibly discharge ascospores into the atmosphere. Little is known about the relationship among the strength of the perithecial wall, the age of the perithecium, and the quantity of ascospores produced. We used a mechanical compression testing instrument to examine the structural failure rate of perithecial walls from three different strains of *F. graminearum* (two wild type strains, and a mutant strain unable to produce asci). The force required to compress a perithecium by one micrometer (the mean perithecium compression constant, MPCC) was used to determine the strength of the perithecial wall. Over the course of perithecial maturation (5 to 12 days after the initiation of sexual development), the MPCC was compared to the number of ascospores contained inside the perithecia. The MPCC increased as perithecia matured, from 0.06 N μm^{-1} at 5 d to 0.12 N μm^{-1} at 12 d. The highest number of ascospores was found in older perithecia (12 d). The results indicated that for every additional day of perithecial aging, the perithecia become more resilient to compression forces. Every additional day of perithecial aging resulted in ~900 more ascospores. Knowledge of how perithecia respond to external forces may provide insight into the development of ascospores and the accumulation of turgor pressure. In the future, compression testing may provide a unique

method of determining perithecial age in the field, which could extend to management practices that are informed by knowledge of ascospore release and dispersal.

Highlights:

- A mechanical compression testing instrument was used to determine the failure of ascocarps
- Physical resiliency and number of spores increased with age of *F. graminearum* perithecia
- The age of perithecia is important to consider in disease models

Keywords: fungus; *Fusarium graminearum*; Fusarium head blight; mechanical properties; compression

Introduction

The fungus *Fusarium graminearum* causes Fusarium head blight (FHB) of wheat and barley (Goswami and Kistler 2004). Between 1990 and 2000, FHB caused more than $3 billion in crop losses in the United States and $220 million in Quebec and Ontario (McMullen et al. 1997; Paulitz 1999; Schmale III and Bergstrom 2003; Windels 2000). Because *F. graminearum* produces the mycotoxin deoxynivalenol, the fungus causes adverse health effects (e.g., vomiting and nausea) in swine and humans if ingested in feed or finished food products, respectively (Snijders 1990; Desjardins et al. 1993; Sutton 1982). *F. graminearum* forcibly discharges ascospores from perithecia at high acceleration rates from sources of inoculum such as crop debris (Goswami and Kistler 2004; Trail et al. 2005; Guenther and Trail 2005). The ascospores can be transported > 500 m in the atmosphere to susceptible crop fields (Sutton 1982; Prussin II et al. 2014a).

Perithecia of *F. graminearum* measure 150 to 175 µm in diameter and have a perithecial wall about 25-50 µm thick (Seifert 1996). The perithecial wall is composed of three distinct layers: an 8-25 µm outer wall, a 6.5-13 µm middle layer, and a 4-7 µm inner layer. The cells transition from ellipsoidal in the outer wall to more elongated in the inner layer (Seifert 1996). The ostiole (pore through which ascospores are discharged) is delineated by cells in the upper wall (Trail and Common 2000). Inside the wall, the centrum develops, forming asci from rounded cells of the ascogenous system, and the apical paraphyses (sterile hyphae) that grow down from the upper wall (Trail and Common 2000). Ascospore-containing asci develop within the inner portion of each perithecium between paraphyses that collapse as the asci develop (Trail and Common 2000). The asci stretch upward in the perithecium, and ascospores develop within the asci in two rows (biseriate) with eight ascospores per ascus (Trail and Common 2000; Seifert 1996). The ascospores discharge through a pore at the end of the ascus that extends through the ostiole (Trail and Common 2000).

A conceptual model for ascospore release includes four essential steps, including a cue to release mature ascospores and the accumulation of sufficient turgor pressure (Trail and Seminara 2014). High levels of relative humidity (Paulitz 1996; Paul et al. 2007; Inch et al. 2005; Trail et al. 2002) and low air temperature (Del Ponte et al. 2009; Sutton 1982; Fernando et al. 2000) have been correlated with *F. graminearum* ascospore discharge. Additionally, several meteorological conditions have been identified as causal agents of ascospore release (David et al. 2016a), and the numbers and distances of ascospore release have been investigated using 3D-printed discharge devices indicating differences based on temperature and relative humidity (David et al. 2016b). A laboratory-based study of ascospore discharge identified forces of 870,000 g during

ascospore release and suggested that fluctuations in Cl^- and K^+ ions may play a role in ascospore release (Trail et al. 2005).

Little is known about the relationship between the strength of the perithecial wall, the age of the perithecium, and the quantity of ascospores produced (Sikhakolli et al. 2012). Paraphysis degeneration is necessary as perithecia mature to provide space within the structure to accommodate all relevant perithecial structures (Sikhakolli et al. 2012; Trail and Common 2000). Paraphyses maintain functional membranes such that changes in humidity may result in the swelling of the perithecia and pressure changes within the asci and perithecia (Trail and Seminara 2014). An unresolved issue is whether differences in perithecia age would result in differences in numbers of ascospores released under similar meteorological conditions. These factors would affect the pressure within the perithecium that may drive spore release.

The mechanical testing of biological materials represents a unique approach to help advance fundamental understanding of biological processes, improve the design of biological applications, and inform development of bio-inspired materials (Meyers et al. 2008). The investigation of biofilms of the bacterium *Pseudomonas aeruginosa* using a film rheometer identified a compression speed associated with biofilm failure, providing valuable information on its mechanical stability (Körstgens et al. 2001) that would be useful when designing systems to prevent biofouling of membranes (Flemming 2002). Compression testing has been used on materials ranging from the horseshoe crab *Limulus polyphemus* exoskeletons to shells of the abalone, *Haliotis refescens*, highlighting compressive forces that result in failure (Chen et al. 2008). Atomic force microscopy was used to analyze elastic properties of hyphae of the fungus

Aspergillus nidulans, showing that the elasticity of the cell wall may be impacted by conditions within the growth medium (Zhao et al. 2005). Force-deformation relationships were obtained to the point of cell failure by mechanically compressing cells of *Saccharomyces cerevisiae*, and a correlation was found between deformation and compression force at failure (Smith et al. 2000b; Smith et al. 2000a).

We hypothesized that older perithecia would be able to resist greater amounts of compressive force and would contain greater numbers of mature ascospores. The specific objective of this study was to determine the force-deformation relationship of *F. graminearum* perithecia at different ages. Perithecia ranging from 5 days old to 12 days old after the initiation of sexual development were tested to structural failure by compression forces. The relationship between age of perithecia, compression force-deformation values, and ascospore number was determined. Enhanced understanding of the association between age of the perithecium and ascospore number provides additional information on ascospore emission rate (Prussin II et al. 2014b) that will be valuable for models of the spread of FHB. The results from this study, combined with knowledge about the effect of meteorological conditions on ascospore release (Paulitz 1996; Tschanz et al. 1975; Inch et al. 2005; Reis 1990; David et al. 2016a), will improve predictions of ascospore release under field conditions and the management of FHB. In the future, compression testing may provide a unique method of determining perithecial age in the field, and could inform management practices that depend on knowledge of ascospore release and dispersal.

Materials and Methods

Generation of perithecia of *Fusarium graminearum*

Perithecia were generated from three strains of *Fusarium graminearum*: wild-type strain Fg_Va_GPS13N4_3ADON (hereafter referred to as FgVa) used in prior field studies in Virginia (Prussin II et al. 2014a; Prussin II et al. 2014b), wild type PH-1 (NRRL 31084) (Trail and Common 2000; Trail et al. 2002; Cuomo et al. 2007), and mutant of gene FGSG_4417 (a mutant of PH-1 with no asci but a fully developed perithecial wall). The generation and characterization of this mutant has been reported elsewhere (Trail et al., *submitted*).

Perithecia were generated on carrot agar (Burgess and Sydney 1994; Leslie et al. 2006; Klittich and Leslie 1988). Prior to the initiation of the cultures, a sterile 7-cm diameter filter paper (09-801A, Fisherbrand, Waltham, MA) was placed on the surface of carrot agar medium in 100-mm Petri dishes. The cultures were incubated at ambient room temperature (23°C) and relative humidity (RH, 40%). After ~6 d, the plates were flooded with 1 mL of 2.5% Tween 60 solution (P1629, Sigma, St. Louis, MO) and the aerial mycelium was flattened using a sterilized rod. The cultures were then placed under a 12 h/12h light/dark cycle at room temperature and RH until perithecia formed on the filter paper.

Experiments took place with perithecia ranging in age from 5 d to 12 d (measured following addition of 2.5% Tween 60 to the plates at 0 d). Prior to each compression trial, an individual perithecium was extracted from the carrot agar at the specified age using a scalpel and forceps. The sample was then placed onto a testing tip (described below) for compression tests.

Uni-axial compression testing instrument

A uni-axial compression testing instrument (Kammarth & Weiss, Tensile/Compression Module 5kN, Dortmund, Germany) was used to determine the force required to compress the perithecia and ultimately result in failure of the perithecial wall. The instrument is capable of applying a load of up to 5,000 N. Traditional compression tests produce two distinct regimes: a linear elastic regime indicating the relationship between stress and strain that is due to bending of the material (Flores-Johnson et al. 2008) and a plastic regime where permanent deformation occurs and the stress is reduced to zero (Lubarda and Lee 1981). The transition from the elastic to plastic regime is known as the yield point (Lade 1977) and defined as the collapse of the perithecial wall..

Each perithecium was tested in a load frame, which was modified using two 1.27-mm slotted-head, 0.32-mm pin, aluminum mounts designed for use in scanning electron microscopy (SEM) (commonly known as SEM stubs) (16111, Ted Pella Inc., Redding, CA), as shown in Figure 4-1. The failure surface was the wide, flat top of the stub, while the pin end was secured to one end of the load frame. The testing surface was composed of two specimen mounts that were adhered together head to head. One pin end was secured to the opposite end of the load frame from the failure surface. The other pin end was modified using a rotary grinder to create a smaller, flat surface that served as the testing tip. During each trial, a single perithecium was adhered to the testing tip using a thin layer of nonconductive adhesive (16079, Ted Pella Inc., Redding, CA).

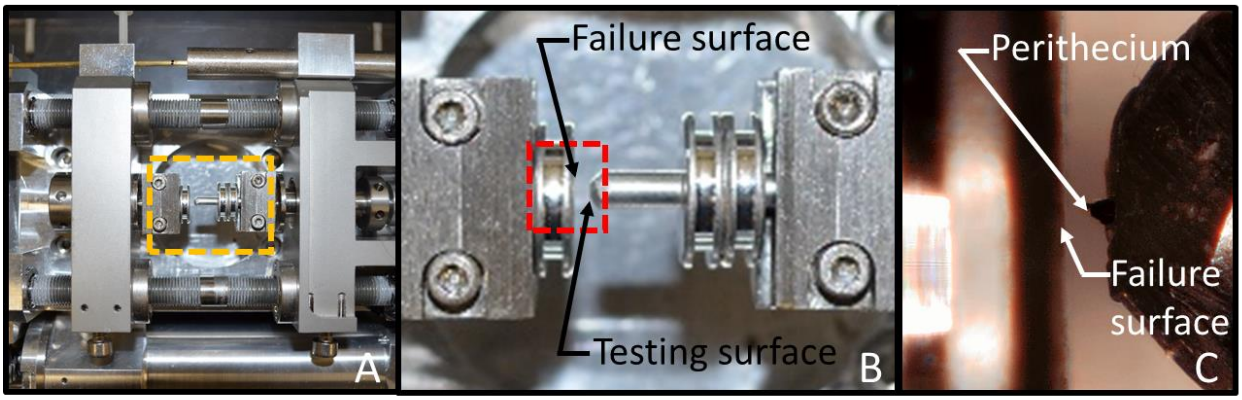

Figure 4-1: Compression testing. Plan view of (A) load frame modified using 1.27-mm slotted-head, 0.32-mm pin, aluminum mounts; (B) failure surface and testing surface (enlarged view of orange box in (A)); (C) adhered perithecium on testing surface (enlarged view of red box in (B)). The perithecium was displaced at a rate of 1 µm s^{-1} toward the failure surface until a force of 50 N was applied.

Perithecia were tested at each age ranging from 5 to 12 d (n = 12 for each age). Because testing was destructive, a different group of perithecia was used for each age of interest. The testing surface with the adhered perithecium was secured into the load frame, and a small clearance of ~0.15 mm between the perithecium and the failure surface was established prior to the start of the compression test. As illustrated in Figure 4-1, the perithecia were tested in the horizontal direction. The compression instrument had a displacement speed of 1 µm s^{-1}. The trials ended once a load of 50 N had been applied to the sample, providing ample opportunity to observe the elastic regime, yield point, and plastic regime (i.e., rupture of the perithecial wall).

Four polymer samples with known hardness properties were used as controls. The materials tested (with durometer values indicating polymer hardness) included latex (38D to 40D), gum rubber (40D), santoprene (55D), and oil resistant vinyl (70D). Each polymer sample was 1.6 mm

thick, and three replicates of each control material were subjected to compression testing according to the same protocol as used for the perithecia.

Analysis of compression data

The compression data was analyzed using MATLAB 8.4 (MathWorks Inc., Natick, MA). For each replicate, a plot of force applied (N) vs. deformation (μm) was created. An example plot is shown in Figure S1. At small deformations, the perithecium remained in the elastic region, where force increased linearly with deformation. As deformation increased, the perithecium reached the yield point, which indicated the amount of force required to rupture the perithecium. Large, non-linear changes in compression force versus displacement corresponded to the plastic zone, where the perithecium had been permanently damaged (i.e., ruptured).

Initially, the start and end of the elastic zone for each sample were determined, and the corresponding slope of the resulting line was calculated. The start was defined as where at least three sequential force measurements increased (indicating sample contact and compression), thus omitting data points corresponding to the initial void (~0.15 mm clearance) between the perithecium and the failure surface. The end of the elastic regime was defined by a displacement of 25 μm following initial contact with the perithecium. This displacement span was selected because all tests had a displacement span of at least 25 μm in the elastic regime, although data points beyond 25 μm and still within the elastic regime were excluded from the analysis. A linear fit was applied to the elastic regime, and the slope of the fitted line was calculated. This slope was denoted the mean perithecium compression constant (MPCC) (N μm^{-1}), signifying the amount of compression force required to compress the perithecium by 1 μm. The MPCC was defined as positive.

The yield point (N) was obtained by determining the compression force at which the elastic zone transitioned into the plastic zone. This point was identified by initially determining the slope between the compression force and the deformation of perithecia over 20-µm segments of the curve. The difference in slope between successive segments was calculated, and the yield point was defined as the force at which the slopes changed by >6% over each of three adjacent segments. The yield point was visually verified to fall within the elastic-plastic transition area and not the noisy region at the start of most trials. Once the location of the yield point was established, the corresponding force was determined by obtaining the median value of the three consecutive 20-µm segments used to obtain the slopes.

Quantification of ascospores

The number of ascospores contained in each perithecium was determined as a function of age. Because the procedures were destructive, different perithecia were used from those subjected to compression testing. For each of the ages investigated, a perithecium was extracted from carrot agar using a scalpel and forceps. The perithecium was placed inside of a 0.6-mL microcentrifuge tube with flat cap (02-681-240, Fisher Scientific, Waltham, MA). A metal rod was used to rupture the perithecium in a similar manner to perithecial failure during compression testing. The perithecium and released ascospores were centrifuged at 2000 g for ~5 s. The ascospores were subsequently re-suspended in 12 µL of sterile water. Ascospores were quantified using 10 µL of the re-suspended ascospore solution (n = 10 for each maturation day) placed within a hemocytometer (3100, Hausser Scientific, Horsham, PA) and quantified using a light microscope at 40x magnification.

Statistical analyses

Statistical analyses were conducted using JMP Pro 10.0.2 (SAS Institute Inc., Cary, NC). Statistical significance was defined at a level of 0.05 for all analyses. Tukey's pairwise comparisons test ($P < 0.05$) was used to determine whether differences were significant between all pairwise combinations of different ages of perithecia. Least–squares regression analysis was used to identify significant linear relationships between variables.

Results

Relationship between perithecial age and compression force

At day 5 after initiation of sexual development, the perithecium wall was fully formed and the perithecia contained some asci with mature spores. This was the first day that forcible ejection of ascospores was observed. Ninety-six perithecia representing eight ages (5 d to 12 d) were subjected to compression tests. The perithecia selected did not exhibit cirrhus formation: the exudation of asci, *en masse*, without forcible firing. The MPCC ranged from 0.060 (5 d) to 0.12 N μm^{-1} (12 d), where a larger value indicates a larger compressive force required to squeeze perithecia by 1 μm. Differences were significant (Tukey's pairwise comparisons, $P < 0.05$) between younger ages (5 d to 8 d) and 12 d: 5-day-old vs. 12-day-old ($P < 0.01$); 6-day-old vs. 12-day-old ($P < 0.01$); 7-day-old vs. 12-day-old ($P < 0.01$); and 8-day-old vs. 12-day-old ($P = 0.029$). The strong linear fit ($R^2 = 0.77$) shown in Figure 4-2 indicates that as perithecia matured from 5 d through 12 d, a larger compressive force was required to produce failure of the perithecial structure. The slope of the line indicates that for each additional day perithecia matured, they were able to withstand an additional 0.007 N of force to compress the perithecia structure by 1 μm.

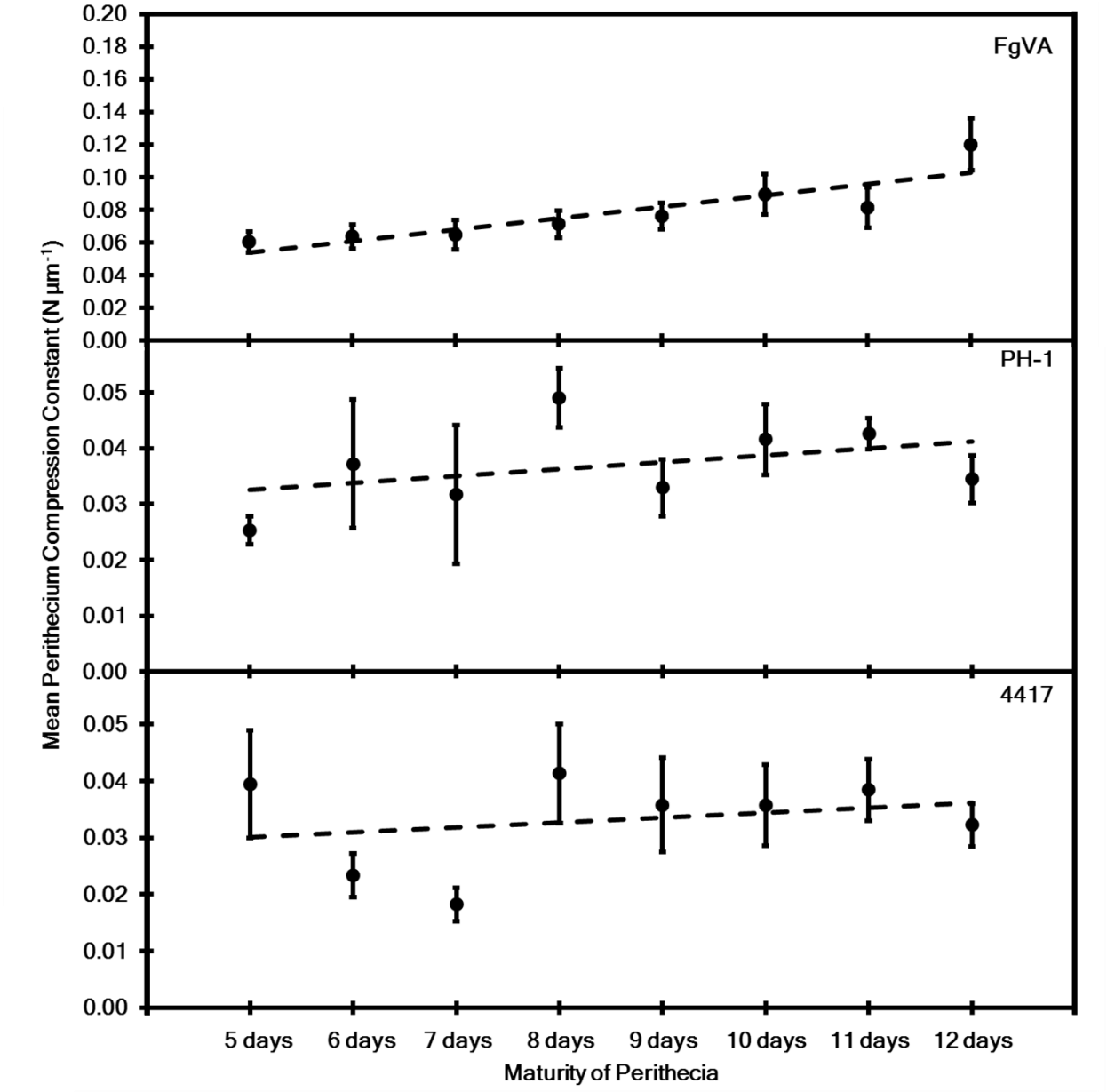

Figure 4-2: Mean perithecium compression constant (MPCC) versus perithecial age and linear relationship between the two variables for strain FgVa, parent strain (PH-1) with fully developed perithecia, and mutant strain (4417) of PH-1, which does not develop asci. The dashed line in each panel provides a linear fit with FgVa ($y = (7.0 \times 10^{-3})x + (4.7 \times 10^{-2})$) having a significant linear relationship ($P < 0.01$ and $R^2 = 0.77$) between the MPCC and age of perithecia. The linear fits associated with PH-1 ($P = 0.31$; $R^2 = 0.17$; $y = (1.3 \times 10^{-3})x + (2.6 \times 10^{-2})$) and 4417 ($P = 0.54$; $R^2 = 0.07$; $y = (8.6 \times 10^{-4})x + (2.6 \times 10^{-2})$) had positive slopes

though not significant. Older perithecia have a greater tolerance for compression failure and are more difficult to rupture.

Least-squares regression analysis identified a significant linear relationship ($P < 0.01$) between the MPCC of the isolate (FgVa) of *F. graminearum* and age of perithecia. The relationship between age of perithecia and MPCC was not significant for the mutant strain 4417 (P=0.54) and the parent strain PH-1 (P=0.31), as shown in Figure 4-2. The MPCC's for PH-1 (0.0013 N μm^{-1} per day) and FgVa (0.007 N μm^{-1} per day) had the same magnitude. The slope of the mutant strain with no developed asci was lower: 0.0009 N μm^{-1} per day. The wild-type strains of parent strain PH-1 (range of std. error values: 0.0026 to 0.012 N μm^{-1}) and FgVa (range of std. error values: 0.0064 to 0.016 N μm^{-1}) had similar variability within perithecial ages while the mutant 4417 had less variability (range of std. error values: 0.0029 to 0.0095 N μm^{-1}).

The yield point of the FgVa, PH-1, and 4417 strains generally increased with age (Fig. S2), though differences between ages and least-squares linear regression analysis were not significant. The 8-day-old yield point for the FgVa dataset was an outlier, and if it were removed from the least-squares regression analysis, a significant linear fit ($P = 0.04$) was identified.

The compression tests performed on polymers demonstrated the ability of the compression testing instrument to detect differences between materials (Fig. S3). There were significant differences between latex vs. oil resistant vinyl ($P < 0.01$); gum rubber vs. oil resistant vinyl ($P < 0.01$); and santoprene vs. oil resistant vinyl ($P < 0.01$). Differences were not detected among the materials with overlapping or close durometer values (latex vs. santoprene, latex vs. gum rubber, and santoprene vs. gum rubber).

Relationship between perithecia age and number of ascospores

The highest numbers of ascospores were found in older (more mature) perithecia. There was a significant difference in number of ascospores between the younger (immature) and older (mature) perithecia. The pairs identified as significantly different by Tukey's pairwise comparisons were 5-day-old vs. 12-day-old ($P < 0.01$); 6-day-old vs. 12-day-old ($P < 0.01$); 7-day-old vs. 12-day-old ($P < 0.01$); 8-day-old vs. 12-day-old ($P = 0.036$); and 5-day-old vs. 11-day-old ($P = 0.036$). Figure 4-3 shows the strong linear relationship ($R^2 = 0.94$) between number of ascospores and perithecia age. As perithecia aged, greater number of ascospores were produced. For every additional day of perithecial aging, about 877 ascospores were produced in each perithecium.

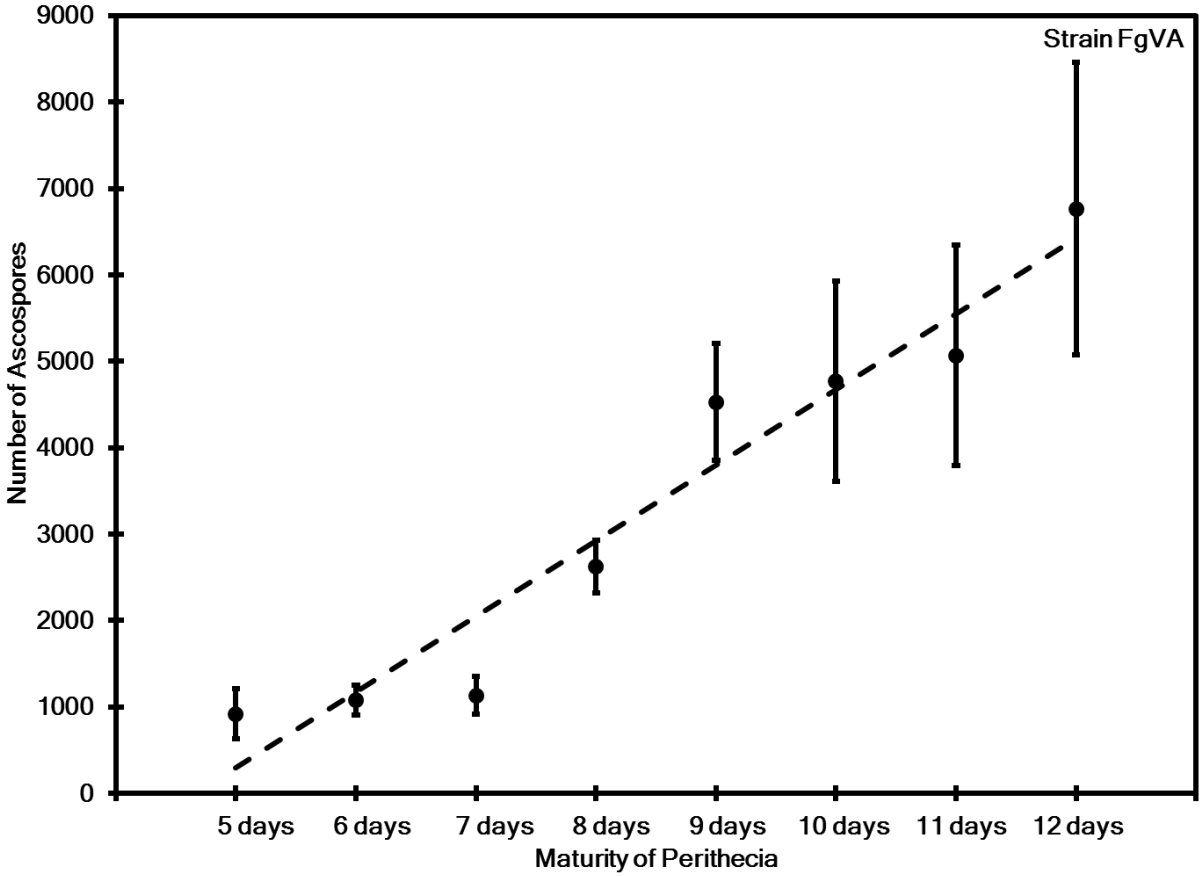

Figure 4-3: Quantity of ascospores in perithecia of different ages (5 d to 12 d) for strain FgVa. Twelve perithecia were observed for each age. There is a positive, linear relationship ($P < 0.01$; $R^2 = 0.94$; y = 877.09x – 585.69) between the two variables indicating that older perithecia contain more ascospores.

Relationship between compression force and number of ascospores

There was a linear relationship between the number of ascospores within perithecia and the perithecial structure's MPCC. The lowest number of ascospores corresponded to the lowest MPCC, and ascospore count increased with MPCC as perithecia matured (Fig. S4). An additional 1,027 ascospores are contained within a perithecium for every increase in MPCC by 0.01 N per micrometer.

Discussion

Few published studies have investigated the mechanical properties of living cells. Studies on yeast cells (*Saccharomyces cerevisiae*) found a correlation between bursting force and the deformation at failure due to compression (Smith et al. 2000b). A study of *Zea mays* (maize) found that lignin concentrations increased with age and then plateaued (Morrison et al. 1998), indicating that this complex organic polymer known for supplying structural integrity (Buxton and Casler 1993) develops over time. A light microscopy study of *F. graminearum* development observed distinct changes as perithecia development is initiated; distinct outer and inner wall layers appear around the ascogeneous system (Trail and Common 2000). The centrum (central cavity) then develops with apical paraphyses forming from the upper wall, the ostiole establishing at one pole of the perithecium, the asci developing between collapsed paraphyses, and the ascospores arranging biseriately within the asci (Trail and Common 2000). Additionally, perithecial development includes development of chitin, which provides a resilient fibrous network, comparable to steel on a mass basis, enough to withstand external forces (Munro and Gow 2001; Latgé 2007; Lenardon et al. 2010). Here, we used compression testing to rupture perithecia of *F. graminearum*. We showed that the force required to rupture perithecia of strain FgVa was associated with the number of ascospores inside the ascocarp. To our knowledge, this is the first detailed report of compression testing of fruiting bodies of a fungus.

The force required to permanently deform perithecia increased as perithecia matured. This increase in uni-axial compressive force required to cause the failure of perithecia may be attributed to its maturation that culminates with the production of asci filled with ascospores (eight per ascus) (Seifert 1996). As a perithecium matures, the density of the perithecial contents will likely increase, contributing to the overall resilience of the ascocarp. There is a potential loss

of contents through spore firing and cirrhus formation with previous research indicating peak spore firing occurring 6 to 9 days after tween application (Trail et al. 2002). A potential explanation for the increasing tolerance of older perithecia is the senescence of the cell walls and the change in perithecial wall structures as perithecia age. An investigation of the *F. graminearum* perithecial development visualized the three layers of the periderm and observed the developmental changes of each wall (Trail and Common 2000). The light microscopy study observed the thick-walled and thin-walled cells of the middle and inner periderm layers compressed as perithecia aged (Trail and Common 2000). The strength provided by the periderm has been noted in studies of carrot roots noting that removal of the periderm resulted in increased levels of damage due to tensile forces (Hartz et al. 2005). A study of cortical bones determined that orientation of fibers and density were important in determining tensile strength of the bone (Martin and Ishida 1989). The aging of perithecium and the related flattening and compressing of the middle and inner periderm layers may be factors that result in the changes in MPCC at different ages. Future work should also include the impacts of continuous ascospore firing and cirrhi formation (our tests did not tested include perithecia that produced cirrhi). The activity of an ascus may impact the nature of neighboring asci (Trail et al. 2005; Cavinder et al. 2012).

The MPCC and perithecia age were not correlated for both the wild-type parent (PH-1) and the mutant strain (4417) unable to produce asci. While the mutant strain's flatter slope was anticipated due to the lack of asci-related development that may contribute to perithecial strength, the variability of the MPCC for both the PH-1 and 4417 strains among the days provided less clarity. Chitins levels, attributed to providing strength to fungal cells (Bowman and Free 2006; Levitz 2010), decreased and rates of growth slowed in *Saccharomyces cerevisiae*

when chitin synthase was disrupted (Shaw et al. 1991). A study of the impact of chitin synthase genes in *Fusarium oxysporum* wild-type and mutant strains found that chitin content in hyphae was reduced by 10% for mutant strains that had disrupted chs1 and chs2 genes (Martín-Udíroz et al. 2004). The variation in MPCC and perithecial age for PH-1 and 4417 may be associated with differences in periderm development that contains layers that compress as the perithecium ages (Trail and Common 2000), but additional compression tests on more mutants would be needed to test this hypothesis.

Ascospore numbers generally increased with age of perithecia for strain FgVa (ascospores were not quantified for PH-1). These findings are compatible with the study performed by Trail and Common (2000), who observed that meiosis and mitotic division produced the eight ascospores per ascus. A laboratory study found that the number of discharged ascospores increased up to approximately 5 d after perithecial maturity before decreasing until no release was observed (Prussin II et al. 2014b). The highest number of ascospores per perithecium corresponded with the largest compressive force required for perithecial-structure failure. This result suggests that the maturation of perithecia produces a more resilient structure and a higher potential risk of pathogen spread. The results seem to indicate a larger ascospore source at older ages, with the timing and number of the ascospores previously also linked to meteorological conditions (Del Ponte et al. 2009; Sutton 1982; Prussin II et al. 2014b; Paulitz 1996). Perithecial age and meteorological conditions appear to be important determinants of a release event and the severity of an event. Studies have shown the ascospore-containing asci are composed of long chitin fibrils and that covalent crosslinks between chitin and β-glucan provide a scaffold linked to the shape and strength of the cell wall (Lenardon et al. 2010; Munro and Gow 2001; Ruiz-Herrera 1991;

Walker et al. 2010; Zhao et al. 2005). The forcible discharge of ascospores from asci at immense accelerations likely requires support from the rigid cell wall (Trail et al. 2005; Trail 2007; Schmale III et al. 2005a; Schmale III and Ross 2015). The perithecial wall may have the capability of withstanding outside compressive forces as well as the high forces experienced by the perithecia during ascospore release events (Trail et al. 2005).

The hardness of perithecia may provide useful information regarding the age of the perithecium, but potentially more valuably, the amount of turgor pressure and the number of developed ascospores contained within the structure. It has been shown in both field investigations (Paulitz 1996; Inch et al. 2005; David et al. 2016a) and laboratory studies (Trail et al. 2002; Tschanz et al. 1976) that meteorological parameters such as light (Schmale III et al. 2006; Schmale III et al. 2005b; Trail et al. 2002), relative humidity (Paulitz 1996; Paul et al. 2007; David et al. 2016a), and temperature (Del Ponte et al. 2009; Sutton 1982; Paulitz 1996; Fernando et al. 2000) may be required at specific levels to trigger ascospore release events. Additional research is required to fully elucidate the relationship of apparent contributors—perithecial age and meteorological conditions—on ascospore release.

In consideration of the hypothesis that asci of mature perithecia swell prior to discharge (De Bary et al. 1887; Trail and Seminara 2014) due to the accumulation of K^+ and Cl^- ions (Trail et al. 2005), the greater compression forces identified for the mature perithecia may indicate that a sufficient buildup of ions has resulted in swelling and that the exposure of the perithecia to favorable meteorological conditions may produce a release event. On the other hand, less mature perithecia may not contain a prerequisite level of turgor pressure for ascospore discharge. Thus,

the exterior compressive forces required to result in failure may reflect an array of information like age of perithecia, number of ascospores available for discharge, and the level of turgor pressure accumulated within the perithecia. Additional mutant strains of PH-1 (with variable regulation of K^+ and Cl^- ion accumulation) would likely be needed to test these hypotheses. Mechanical testing of such mutant strains, and those strains with and without asci and ascospores, would provide additional information to determine the extent the perithecial wall, the number of ascospores, the level of turgor pressure, or other parameters may impact compressive failure.

The relationship between the mechanical strength of a perithecium and the number of ascospores it contains offers a unique approach to the management of FHB. One could envision a portable compression testing instrument for field use that could provide estimates of the nature and timing of spore release to inform timely field management decisions, such as the application of fungicides. This information could be incorporated into an improved understanding of the emission rate of spores of *F. graminearum* (Schmale III and Ross 2015; Prussin II et al. 2014b; Aylor et al. 2001; Aylor 2003; Prussin II et al. 2015), particularly in regions of the world where local sources of inoculum (i.e., within field) are predominant. Moreover, this study has the potential to inform the development of fungicides that could target the perithecial wall and/or the firing of ascospores, which could offer some unique disease management strategies (Li et al. 2008; Chen and Zhou 2009; Broders et al. 2007).

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Chapter 5 – Conclusions

A knowledge gap exists in the field of FHB forecasting regarding the role of meteorological conditions and perithecial maturity on the timing and quantity of ascospore release. Various field studies have concluded that increased ascospore concentrations occur during high periods of relative humidity (Paulitz 1996; Ayers et al. 1975), but this research was the first to identify a causal relationship between specific meteorological parameters and ascospore release.

Outcomes of Research Objective #1

Determine which meteorological variable(s) influence ascospore release through causality analysis. This work introduced the power of causality analyses, including both convergent cross mapping and multivariate state space reconstruction, to investigations of *F. graminearum* ascospore concentrations in the atmosphere. We identified relative humidity, solar radiation, wind speed, and air temperature as causal agents and predictors of ascospore release from field data obtained in 2011 and 2012 in Blacksburg, VA. This work may assist cereal crops farmers and growers in identifying the meteorological parameters most pertinent to monitor when making field management decisions, such as the timing of fungicide placement. Implications may also extend to FHB modelers, who may use these results to define important meteorological parameters when forecasting the spread of FHB. Additionally, the causality analyses applied in this work would be valuable tools when investigating other plant pathogens and bioaerosols to determine explicit cause-and-effect relationships.

Outcomes of Research Objective #2

Determine the direct effects of relative humidity and temperature on the number and distance of discharged ascospores. This work provided additional knowledge of how varying levels of air temperature and relative humidity impact the number and distance of discharged ascospores. We demonstrated that both quantities were sensitive to relative humidity and temperature. The greatest number and farthest discharged ascospores occurred at the highest relative humidity condition (95%). The farthest discharged ascospores coincided with 25°C and the greatest numbers occurred at 15°C. Results from this study may be used to help improve risk assessment tools for FHB (De Wolf et al. 2003; Del Ponte et al. 2009) by providing a more complete understanding of how the environmental variables impact ascospore release events. The chamber designed and used to achieve this objective may be used to study additional relative humidity and temperature conditions, and it is compatible with the investigation of solar radiation. The chamber can be used to study spore release in the future, but could also be combined with aerosol science equipment such as an aerodynamic particle sizer or scanning mobility particle sizer to determine size and concentration of bioaerosols in real-time.

Outcomes of Research Objective #3

Mechanically compress perithecia at various stages of maturity to understand the force required to rupture the perithecia. We developed a method to biomechanically study perithecia to determine how their structural properties evolve as they mature. We demonstrated that when exposed to an external uni-axial load, the amount of compression force required to compress perithecia by one micrometer increased as the perithecia matured. We showed that more mature perithecia also contained greater numbers of mature ascospores. Greater numbers of ascospores may translate to an increased risk of FHB disease spread. This knowledge could be used to

develop a standard, handheld compression testing instrument that farmers and growers can use to test the range of perithecial maturity in their fields, thus providing useful information with which to make field management decisions.

Implications

The contribution of this work to the scientific literature includes identifying the causal, uni-directional meteorological conditions resulting in ascospore release; demonstrating that high levels of RH result in greater numbers of ascospores and farther distances discharged during release events; and showing that biomechanical compressive measurements of *F. graminearum* perithecia provide useful insight into the numbers of mature ascospores capable of being released. These contributions have advanced knowledge of *F. graminearum* ascospore release that can be applied to reduce the spread of disease.

The use of convergent cross mapping and multivariate state space reconstruction causality methods within this complex natural system may be used to parse through other similarly intricate systems to identify causal agents of an event. Researchers may incorporate these methods in the fields of environmental engineering, aerosol science, plant pathology, and epidemiology to understand the transport of a pathogenic agent, aerosol, or particle.

The knowledge that *F. graminearum* ascospore release events may be associated with relative humidity, temperature, and perithecial maturity verifies the importance of including these parameters in cereal crop management decisions and FHB models. This knowledge will provide farmers and growers of susceptible crops with an enhanced understanding of the importance of relative humidity and temperature while managing their fields. The potential for ascospore

source strength to be better approximated by leveraging knowledge of perithecial maturity via compression investigations may enable modelers, plant pathologists, and plant epidemiologists to improve the accuracy of their work. Improvement in the scientific knowledge of *F. graminearum* ascospore release events will further assist in improving the ability to predict FHB spread and to protect global cereal crops.

Recommendations for Future Work

We recommend that future *F. graminearum* ascospore release field studies use causality analysis to identify uni-directional relationships and that they are designed to obtain finer resolution data temporally. Causality analysis provides a major advantage over correlation analysis, which has dominated much of the research in the field (Fernando et al. 2000; Paulitz 1996; Trail et al. 2002). The ability to harness the power of causality analysis with *F. graminearum* ascospore field data obtained with a high temporal resolution (on the order of seconds or minutes), rather than hours (David et al. 2016; Prussin II et al. 2014a; Prussin II et al. 2014b; Manstretta and Rossi 2015), would be useful in identifying whether minute variations in meteorological condition trigger ascospore release. An experiment could include two years of field experimentation where artificial inoculum is introduced inside a wheat field and an aerodynamic particle sizer is placed at the center of the field. The aerodynamic particle sizer is capable of measuring the aerodynamic diameter of *F. graminearum* ascospores with a sampling time as low as one second providing an expected peak at the aerodynamic diameter of the ascospores and a fine temporal resolution of when ascospores are present, respectively.

This work provides information on ascospore release events as a function of relative humidity and air temperature. While the specific relative humidities and temperatures investigated based

on previous literature indicating high ascospore concentrations at these meteorological conditions (Sutton 1982; Paulitz 1996), a more complete investigation including a larger suite of conditions can be performed using the chamber developed in Chapter 4. Other conditions previous researchers have associated with ascospore release events or high concentrations include light (Schmale III et al. 2006; Schmale III et al. 2005; Trail et al. 2002), rainfall (Chen and Yuan 1984; Reis 1990; Gilbert and Tekauz 2000), and wind speed (Prussin II et al. 2014b). The growth chamber used in the experiments performed in Chapter 4 included incandescent and fluorescent lamps. For future experiments, the appropriate spectral balance to represent outdoors infrared and ultraviolet radiation would need to be achieved by adjusting the ultraviolet B radiation (280 – 320 nm): ultraviolent A radiation (320 – 400 nm): photosynthetic active radiation (400 – 700 nm) ratio and by using adequate light filters (Thiel et al. 1996). A spray nozzle was previously used to study the relationship between rainfall and *F. graminearum* (Trail et al. 2002) and *Venturia inaequalis* (Gadoury et al. 1996) ascospores and could be reproduced to test its effect in concert with other meteorological conditions. To investigate the effects of wind, a fan could be installed inside the chamber and an anemometer used to measure wind speed. An important component of further studies should also include the ability to modulate these conditions to mimic diurnal conditions that are typical outdoors and have been hypothesized as important in *F. graminearum* ascospore release events (Paulitz 1996; Ayers et al. 1975; Fernando et al. 2000; Inch et al. 2005; Osborne and Stein 2007). The ability to achieve realistic diurnal conditions would permit longer duration studies potentially allowing for the examination of the role of perithecial maturity, varying meteorological conditions, and ascospore release in a single study. For longer-duration experiments, the use of equipment capable of achieving real-time measurements with finer resolutions (e.g., aerodynamic particle sizer) would provide

valuable information on the temporal relationship between meteorological conditions and ascospore release. In our experience, nucleation of salt particles can become a concern and may be identified as false positive for an ascospore release event so these nucleation events would need to be resolved during data analysis. Additionally, the efficiency of processing of microscope slides with deposited ascospores could be improved through image processing software and custom code.

In order to potentially improve the applicability of biomechanical testing described in Chapter 4, we recommend development of a protocol to allow for compression testing to occur at various environmental conditions rather than ambient room conditions. As presented in Chapters 2 and 3, meteorological conditions have been important predictors of high ascospore concentrations (Fernando et al. 2000; Trail et al. 2002; Doohan et al. 2003; Beyer et al. 2004). The ability to investigate the relationships between perithecial maturity, compressive force, and relative humidity would provide novel information about whether perithecial swelling due to high relative humidity values is a driver of ascospore release (Trail and Common 2000; Trail and Seminara 2014; Sikhakolli et al. 2012). In addition to performing future compression studies under controlled environmental conditions, we recommend measuring parameters such as size change of perithecia over long periods of times without the application of compressive forces. The use of a high-speed camera or microscope to measure changes in perithecial size and shape with age while exposed to various meteorological conditions would provide greatly improved understanding of the development of *F. graminearum* perithecia. While high-speed camera observations have been used before in examining phenomena such as the development of electrospray (Kim et al. 2011), these cameras are designed for anticipated, rapid events. Because

there appear to be many factors involved with *F. graminearum* ascospore release, the ability to predict an instantaneous release event is challenging.

Finally, we recommend that future work should investigate the potential source strength of *F. graminearum* ascospores from a cereal crop field. Results obtained from the work described in Chapters 3 and 4 suggest that numbers of ascospores and their discharge distances are impacted by relative humidity and temperature with perithecial maturity potentially impacting the amount of ascospores available for release. While this work has provided new knowledge about ascospore release, several other stages exist within the totality of the transport cycle (Aylor et al. 2001; 1999, 2003, 1986, 1990; 2001; 1992; 1982). For ascospores to be transported long distances, they must move from the perithecia through the crop canopy and through the laminar boundary layer to the turbulent boundary layer where long-distance transport commonly occurs (De Wolf et al. 2003; Raupach and Thom 1981). Future work should address how varying ascospore discharge distances impact the proportion of ascospores that exit the canopy and laminar boundary layer. Small plots of wheat fields could be artificially inoculated with a strain of *F. graminearum* and stationary sampling sites could be set at several heights within and above the canopy to investigate the proportion of ascospores that make it through various heights. In the laboratory, potted plants can be used to simulate various canopy heights, *F. graminearum*-inoculated corn stalks can be placed at ground level, and stationary measurement sites can be used to track ascospore deposition at various heights. This information would be valuable to FHB modelers to allow for more accurate predictions of potential source strength from an impacted field.

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Appendix A: Supplementary Information to Chapter 2

Causality analyses

Convergent cross mapping

The convergent cross mapping (CCM) method (Sugihara et al. 2012; BozorgMagham et al. 2015) was used to investigate the causal relationships within this biological system and was based on transferring information from the driver time-series to the response time-series under the assumption of directional causality (from driver to response). In this causal scenario, the response time-series necessarily contains signatures (information) about the driver time-series whereas the reverse may not be true. The CCM method uses time-lagged components of the response time-series to estimate the dynamics of the candidate driver time-series. A better estimate of the driver behavior shows a stronger causal influence on the response variable. In addition, if the two variables are dynamically connected, a better estimate of the driver signal would be expected from a larger number of observations, referred to as the library of the time-series. To obtain a quantitative measure of causality, the Pearson correlation coefficient between the estimated and the original driver signals was used. In addition, to avoid spurious localized (short-term) correlated dynamics between a candidate driver and response, the recovery of the driver signal was investigated as a function of library length L. The library length describes the number of historical observations that are used to generate estimations, and can be a subset of the total number of observations N.

The first step in implementing the CCM method for two time-series $x(t)$ and $y(t)$ (the driver and response signal, respectively) was to generate the reconstructed phase spaces (shadowing manifolds) from the libraries of the two time-series with length L data points:

$$\{X^{i}{}_{L}\} = \{x(i), x(i+1), ..., x(i+L-1)\} \quad (1)$$

$$\{Y^{i}{}_{L}\} = \{y(i), y(i+1), ..., y(i+L-1)\} \quad (2)$$

for $i = 1$ to $i = N+1-L$ where N is the number of data points in the time-series and the superscript "i" denotes the i-th library. The libraries must sweep the entire length of the original time series (see **Fig. S6**). A reconstructed phase space was generated by using a proper time lag (τ) and an embedding dimension (E). The average mutual information measure was used to select the time lag (Abarbanel 1996). The embedding dimension is a measure of the number of observations used for estimation. The false neighborhood method was used to determine an optimal value for E (Cao 1997). The time-delayed vectors of the reconstructed phase spaces were:

$$X_k = (x(k), x(k-\tau), ..., x(k-(E-1)\tau)) \quad (3)$$

$$Y_k = (y(k), y(k-\tau), ..., y(k-(E-1)\tau)) \quad (4)$$

for $k = 1+(E-1)\tau$ to $k = L$ where the subscript k shows the k-th point of the i-th library of length L data points. Based on the spore release and meteorological signals, a common time lag of $\tau =$ 2 hours and embedding dimensions $E = 6$ was found.

After the reconstruction of the phase spaces from the selected libraries, for each E-dimensional point in the response reconstructed phase space (a generic E-dimensional point was denoted as Y-central in **Fig. S7**), a sufficient number of nearest neighbor points were selected and their distances, d_i, to the Y-central point were determined. Next, the contemporaneous of each neighbor point in the driver reconstructed phase space was determined. The spatial average of the designated points in the driver shadowing manifold (shown by a star in **Fig. S7**) was

determined by using d_i's as the weighting factors (Sugihara et al. 2012). This procedure was repeated for all the E-dimensional points in the response reconstructed phase space, and the correlation between the resultant points and the X-central points, the contemporaneous of the Y-central points in the X_k, was measured.

The CCM coefficient, ρ, as a function of library length L of hourly observations was defined as the average of the Pearson correlation coefficients corresponding to the libraries with the specified length. Causality is indicated by a CCM coefficient ρ that increases significantly with increasing library length and is significantly greater than zero for large library length. Higher values of the CCM coefficients indicate stronger causal influence. In this study, the convergence and relative magnitude of ρ was investigated using spore concentration as the response signal and meteorological variables as the candidate driver signals.

Multivariate state space forecasting

The multivariate state space forecasting method (Deyle et al. 2013) was inspired by a conceptual combination of the Granger causality method (Granger 1969) and the simplex method for predicting the short term evolution of deterministic chaotic time-series (Farmer and Sidorowich 1987). The multivariate forecasting method augments the information of a driver with the information of the response signal and exploits the cumulative information for a better prediction. This study applied the multivariate forecasting method and expected to observe significant improvement in the prediction of the spore concentration when the information of an influential meteorological variable, which was detected by the CCM method, was augmented with the information of the spore concentration.

In this analysis, the reconstructed phase space of the spore concentration was the same as Y_k, introduced in equation (4). The vectors of Y_k were augmented with the data of an environmental signal represented by $x(t)$. The augmented time-delayed vector was:

$$Y_{augmented} = (y(k), y(k-\tau), ..., y(k-(E-2)\tau, x(k-\Delta))) \quad (5)$$

where Δ shows the delay between the actuation of the driver and the response of the system. We used Y_k and $Y_{augmented}$ for short term (maximum 12 hours lead time) single variable and multivariate forecasting of the spore concentration, respectively (Deyle et al. 2013; Farmer and Sidorowich 1987). The statistical significance of the improvement of the root mean square (RMS) of the forecasting error between the multivariate and single variable forecasting schemes was investigated to verify the effectiveness of augmentation of the environmental data. The forecasting error in each case, multivariate and single variable, was defined as the difference between the observed spore concentration (correct values) and the forecast results.

The hourly observed spore concentrations were denoted by Y_{obs}, the hourly forecasts by Y_f and the number of available hourly forecasts by n. The RMS of the forecasting error, S, was defined as:

$$S = \sqrt{\frac{1}{n}\sum (Y_{obs} - Y_f)^2} \quad (6)$$

The number of possible hourly forecasts depends on the starting point, the lead time and the length of the time series. The starting point selected was 0700 on 11 May 2012. This provided *n*= 65 in the RMS calculations and sufficient record for forecasting purposes.

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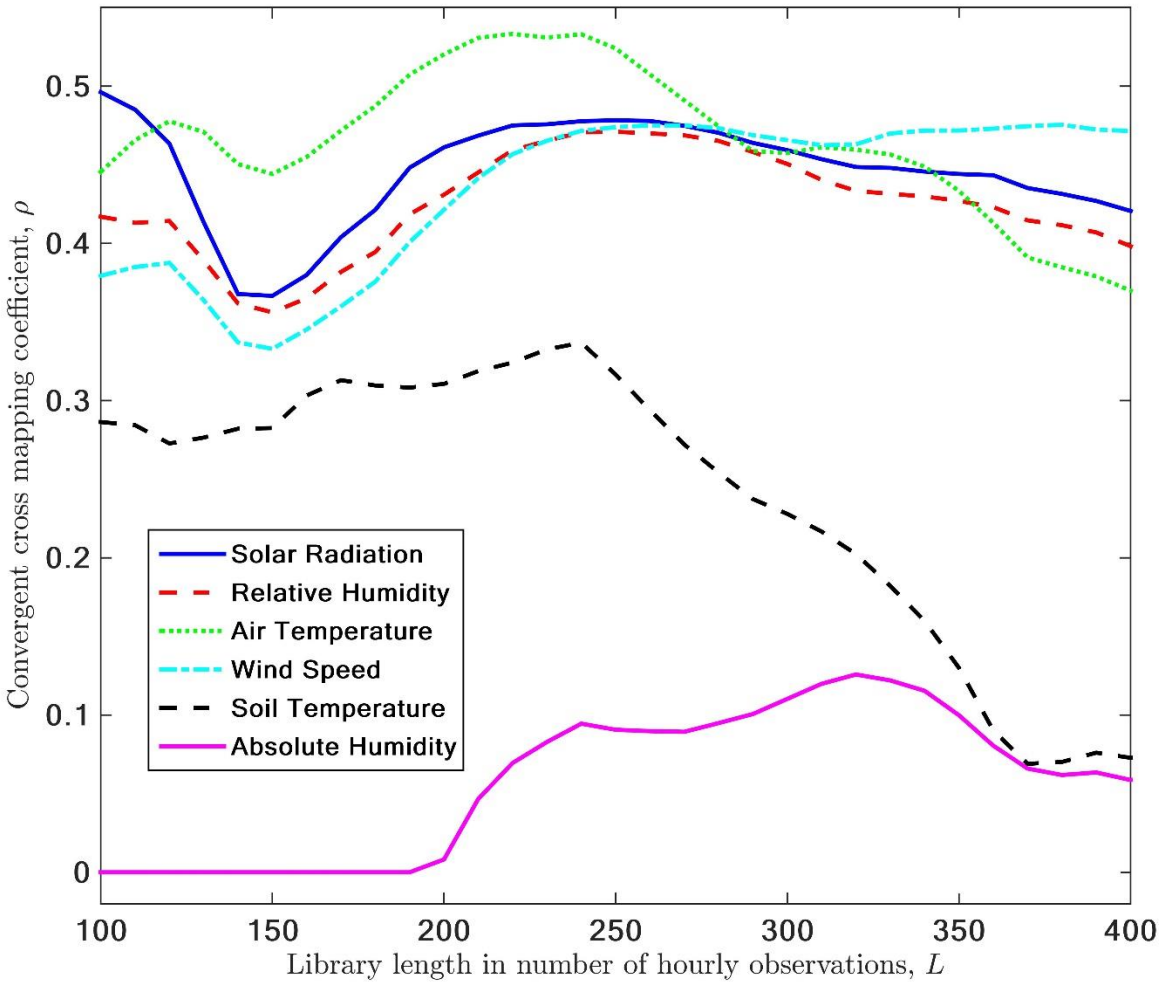

Fig. S1: The CCM coefficient, ρ, between driver signals (meteorological variables) and response signals (spore concentration) for the 2012 monitoring period. These figures identify solar radiation, relative humidity, air temperature, and wind speed as the most important controlling signals with the bifurcation identifying the meteorological conditions that have the greatest influence on spore concentrations.

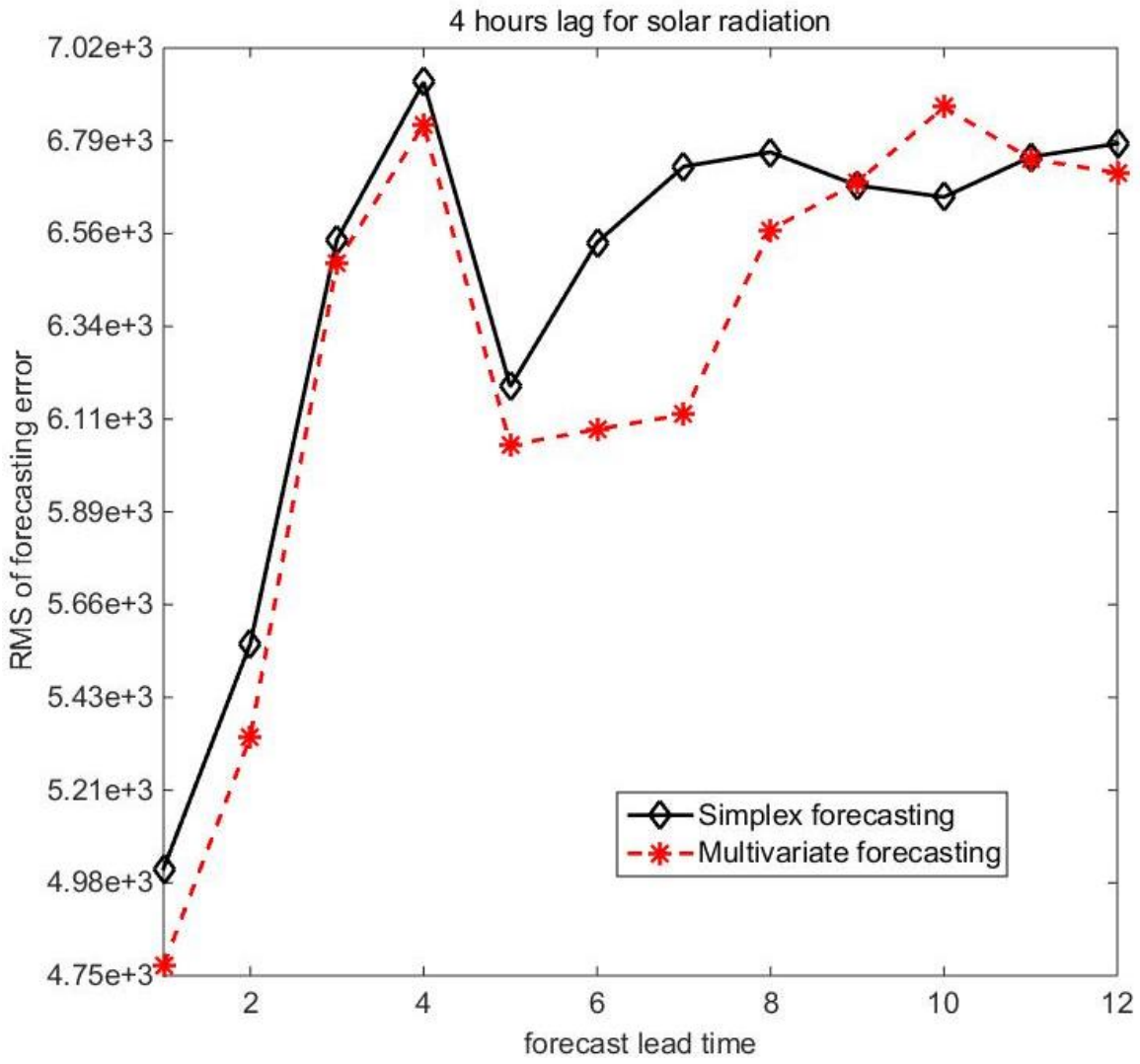

Fig. S2: Forecast root mean square (RMS) errors in cases of multivariate forecasting, solar radiation as the augmented information (dashed red line), and single variable forecasting (solid black line). A lead time of 4 hours is considered between the solar radiation signal and the response signal. Augmentation of the solar radiation improves the RMS of errors for all forecast lead times except for a 10-hour forecast lead time.

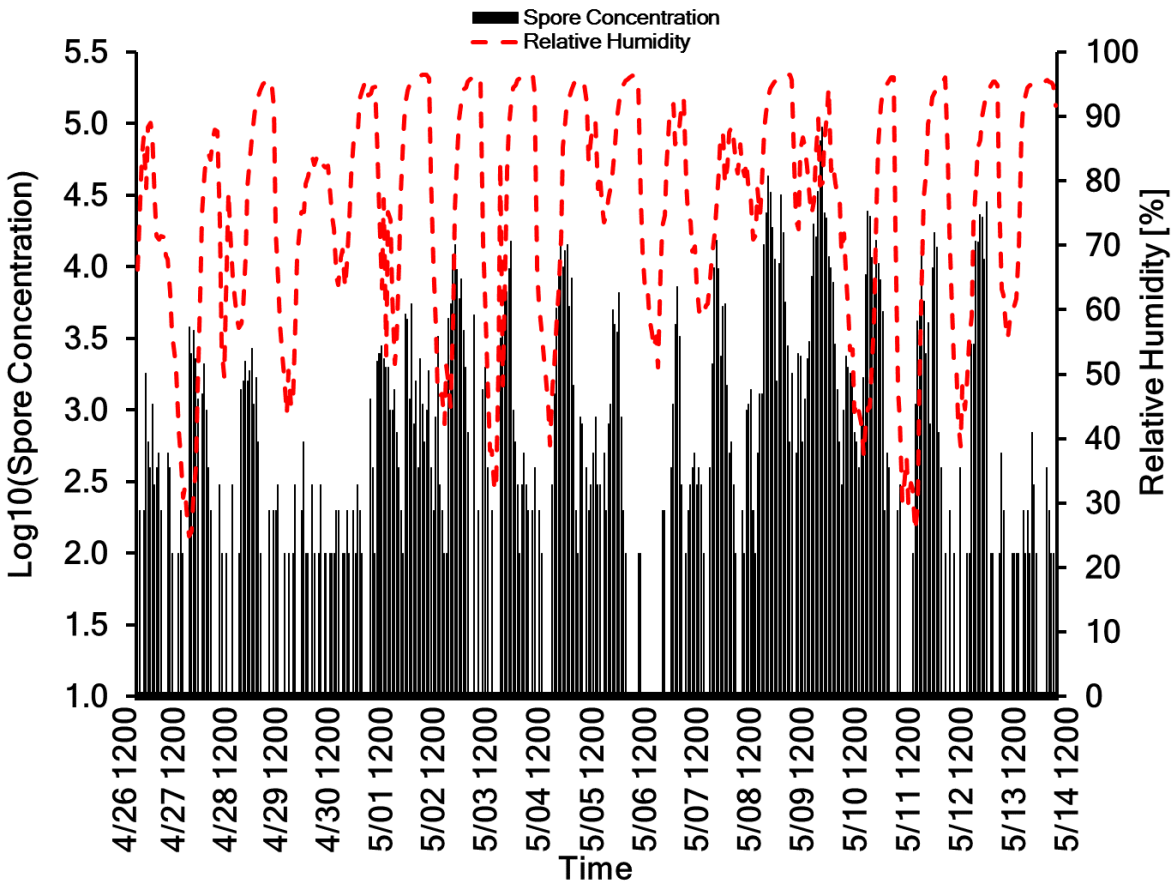

Fig. S3: Hourly spore concentration (black bar graph) and relative humidity (dashed red line) for a field source of F. graminearum between 1800 hours 26 April 2012 to 1100 hours 14 May 2012. A single strain of F. graminearum (FGVA4) was introduced within a 1-acre wheat field monitored for airborne spore concentration using a Quest volumetric spore sampler.

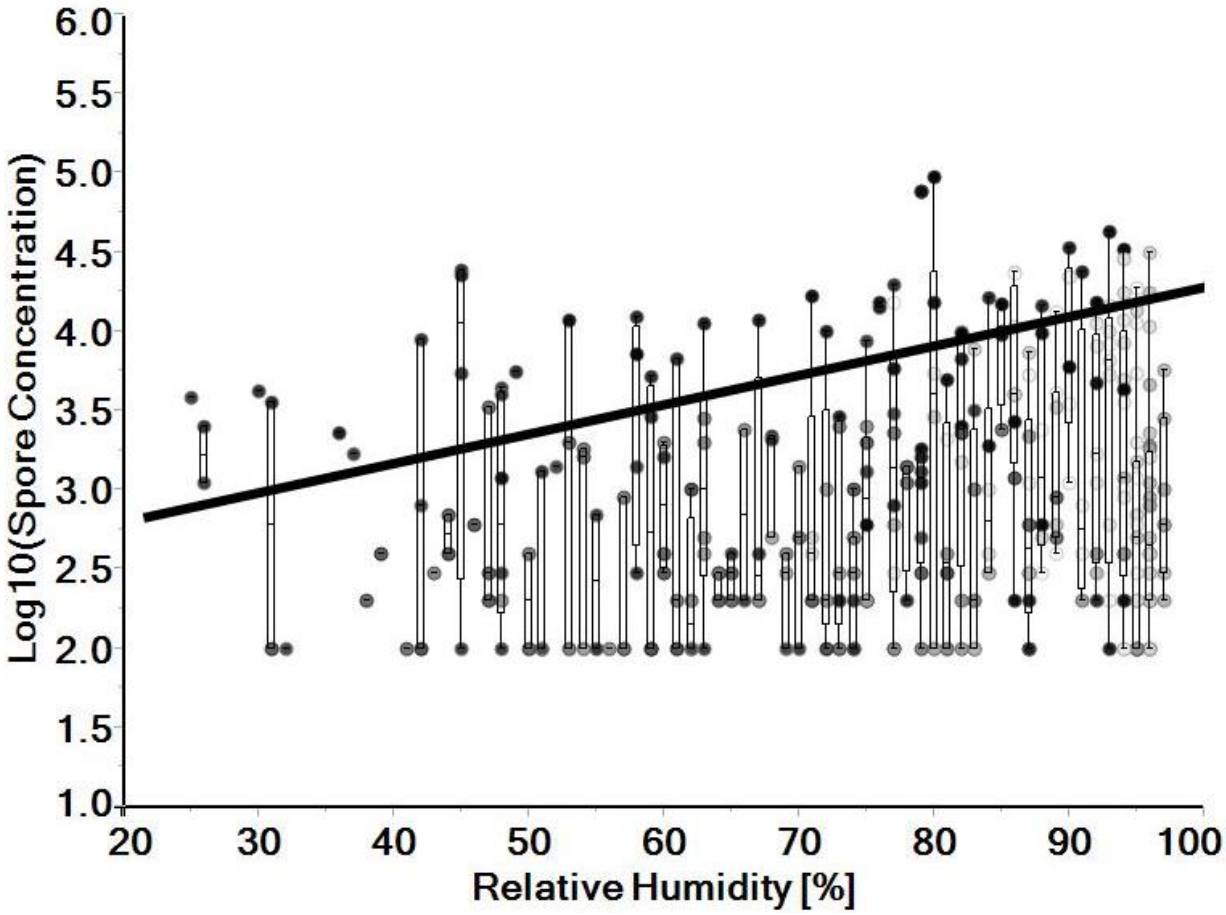

Fig. S4: Spore concentration and the relationship to relative humidity (greyscale markers) for a field-scale source in 2012. The shading distinguishes nighttime (black and white) from daytime (grey variants) events. The black line fits the highest values of spore concentration at each 1% range in relative humidity to illustrate where an apparent threshold occurs.

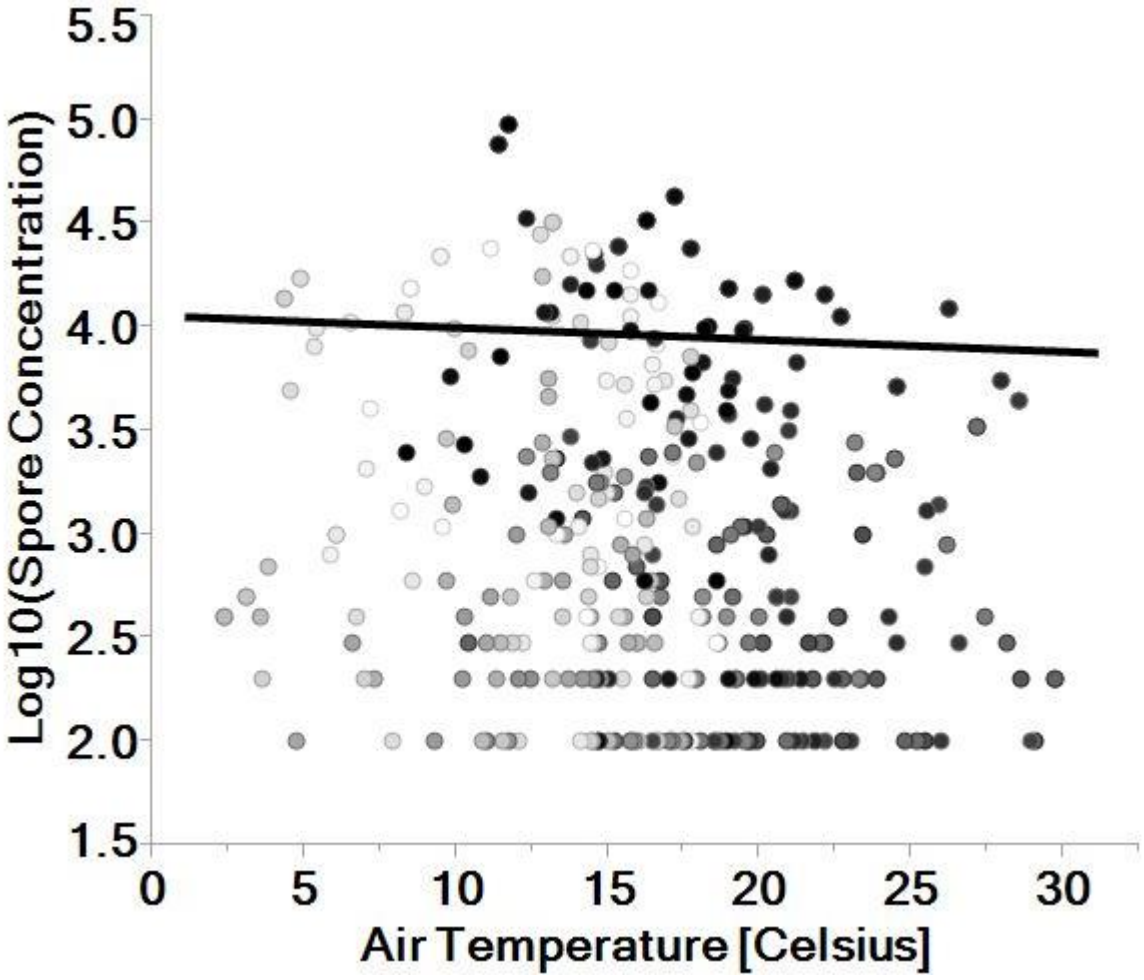

Fig. S5: Spore concentration versus air temperature for a field-scale source of F. graminearum during the monitoring period in 2012. The shading distinguishes nighttime (black and white) from daytime (grey variants) events. The black line fits the highest values of spore concentration for each 1° C range in temperature to illustrate where an apparent threshold occurs.

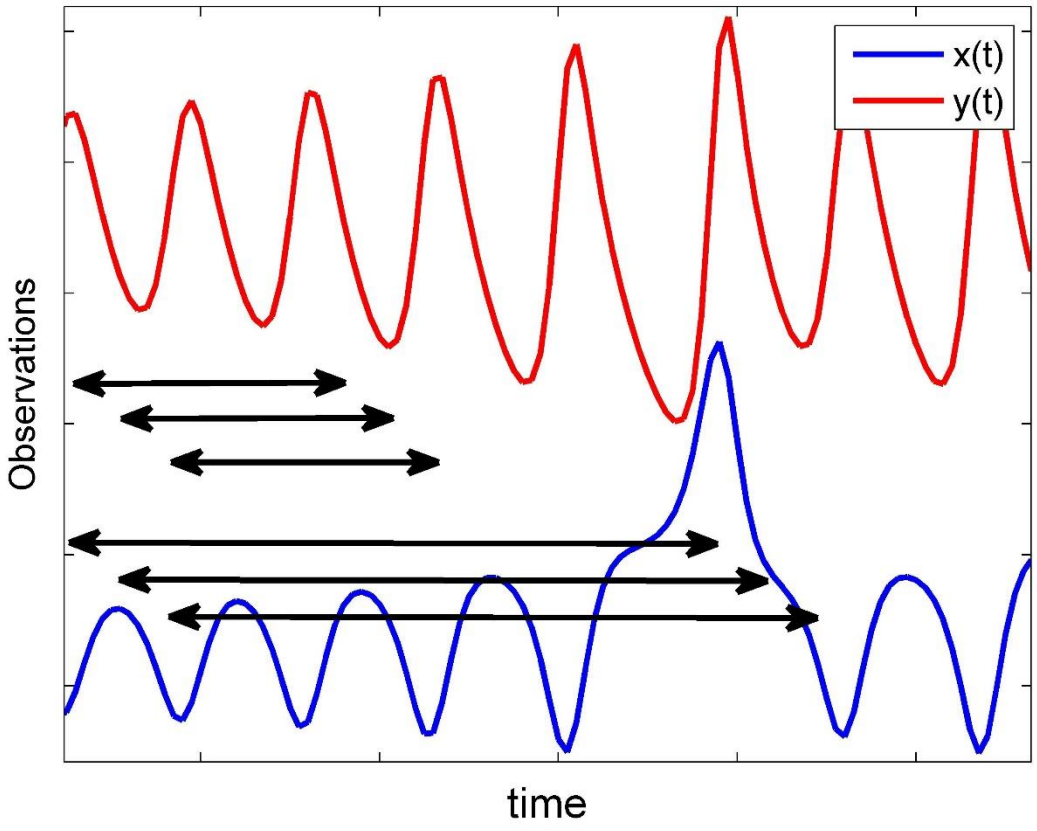

Fig. S6: Schematic of two time series indicating concept of library length. An illustration indicating two time-series of observations and three sets of libraries. For a given library length, one sweeps along the full time-series considering all possible sub-time-series, as shown schematically. Thus, for longer library lengths, there will be fewer possible sub-time-series.

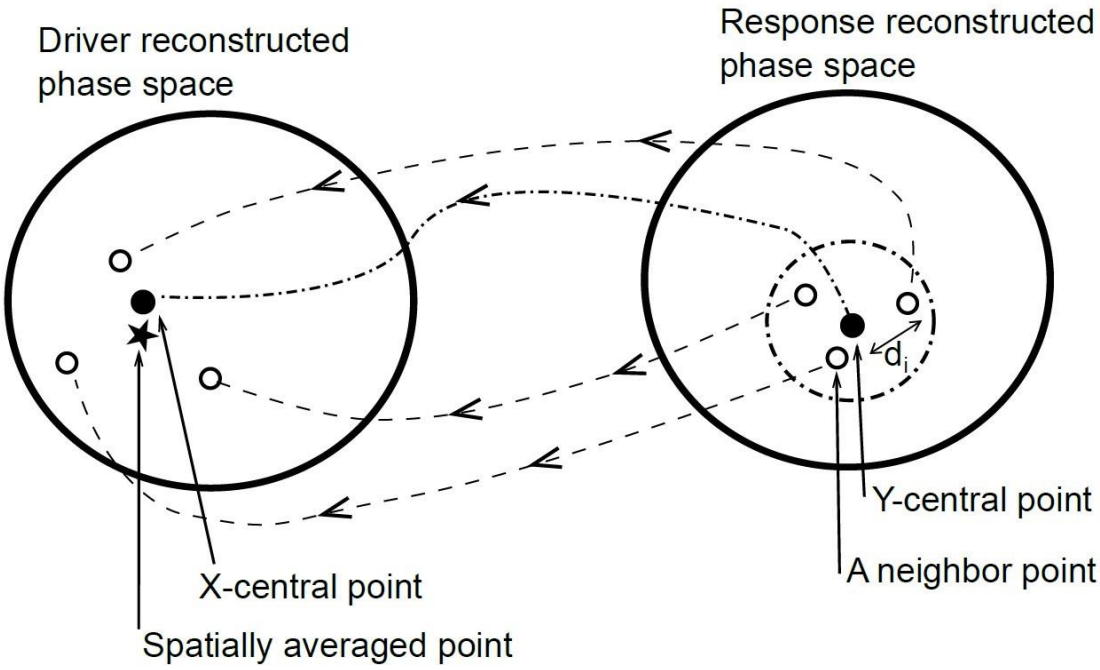

Fig. S7: Schematic of the reconstructed phase spaces of two variables and the process for calculation of ρ. For each E-dimensional Y-central point (black filled circle, right) in the response reconstructed phase space, sufficient numbers of nearest neighbor points are selected (circles, right) and their distance, d_i, to the Y-central point is determined. For each neighbor point, its contemporaneous point in the driver reconstructed phase space is determined (circles, left). These points are weighted by d_i's and averaged. The CCM coefficient is defined as the correlation between the X-central (black filled circle, left) and the recovered (star, left) points.

Table S1: Results of t-test between RMS of errors corresponding to the cases with and without augmented environmental signals.

Parameter	Analysis between RMS of errors*†ᵞ								
Delay (hours)	**0**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Solar radiation	**+1**	**+1**	**+1**	**0**	**+1**	**+1**	**+1**	**+1**	**0**
Relative humidity	**+1**	**+1**	**+1**	**+1**	**+1**	**+1**	**+1**	**0**	**0**
Air temperature	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
Wind speed	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
Soil temperature	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
Absolute humidity	**-1**	**-1**	**0**	**-1**	**-1**	**-1**	**-1**	**0**	**-1**

The analysis includes cases of augmentation of solar radiation, relative humidity, air temperature, wind speed, soil temperature and absolute humidity with the spore concentration signal.

*** 0 indicates cases that fail to reject the hypothesis**

† +1 indicates cases that reject the null hypothesis and improve the forecast

ᵞ -1 indicates cases that reject the null hypothesis and do not improve the forecast

Table S2: Results of bivariate analysis of spore concentration and meteorological variables.

Year	Indep. term	Regression equation to predict spore concentrations[γαδ]	R^2 (*P*-value)
2011	Wind speed[θ]	$\log_{10}$(Concentration) = 3.28 – 0.32*WS	0.12 (< 0.0001)
		$\log_{10}$(Concentration) = 4.95 – 0.71*WS	0.55 (< 0.0001)
	Air temperature[θ]	$\log_{10}$(Concentration) = 4.49 - 0.086*AT	0.21 (<0.0001)
		$\log_{10}$(Concentration) = 6.38 - 0.12*AT	0.59 (<0.0001)
	Relative humidity[θ]	$\log_{10}$(Concentration) = 1.00 + 0.024*RH	0.20 (< 0.0001)
		$\log_{10}$(Concentration) = -0.015 + 0.049*RH	0.66 (< 0.0001)
2012	Wind speed[θ]	$\log_{10}$(Concentration) = 3.14 – 0.13*WS	0.047 (< 0.0001)
		$\log_{10}$(Concentration) = 4.43 – 0.34*WS	0.46 (< 0.0001)
	Air temperature[θ]	$\log_{10}$(Concentration) = 3.37 - 0.025*AT	0.030 (0.0020)
		$\log_{10}$(Concentration) = 4.06 - 0.0058*AT	0.0057 (0.70)
	Relative humidity[θ]	$\log_{10}$(Concentration) = 2.58 + 0.0050*RH	0.014 (0.034)
		$\log_{10}$(Concentration) = 2.43 + 0.018*RH	0.29 (< 0.0001)

Relationships between independent model parameters versus spore concentrations for 2011 and 2012 monitoring periods. Each equation represents the linear fit predicting the concentrations of spores per cubic meter based upon the independent variables of wind speed, (m s^{-1}), air temperature (ºC), and relative humidity (%).

[γ] WS is wind speed in m s^{-1}

[α] AT is air temperature in ºC

[δ] RH is relative humidity in percent

[θ] First row is linear fit of entire data set and second row is linear fit of threshold line

Appendix B: Supplementary Information to Chapter 4

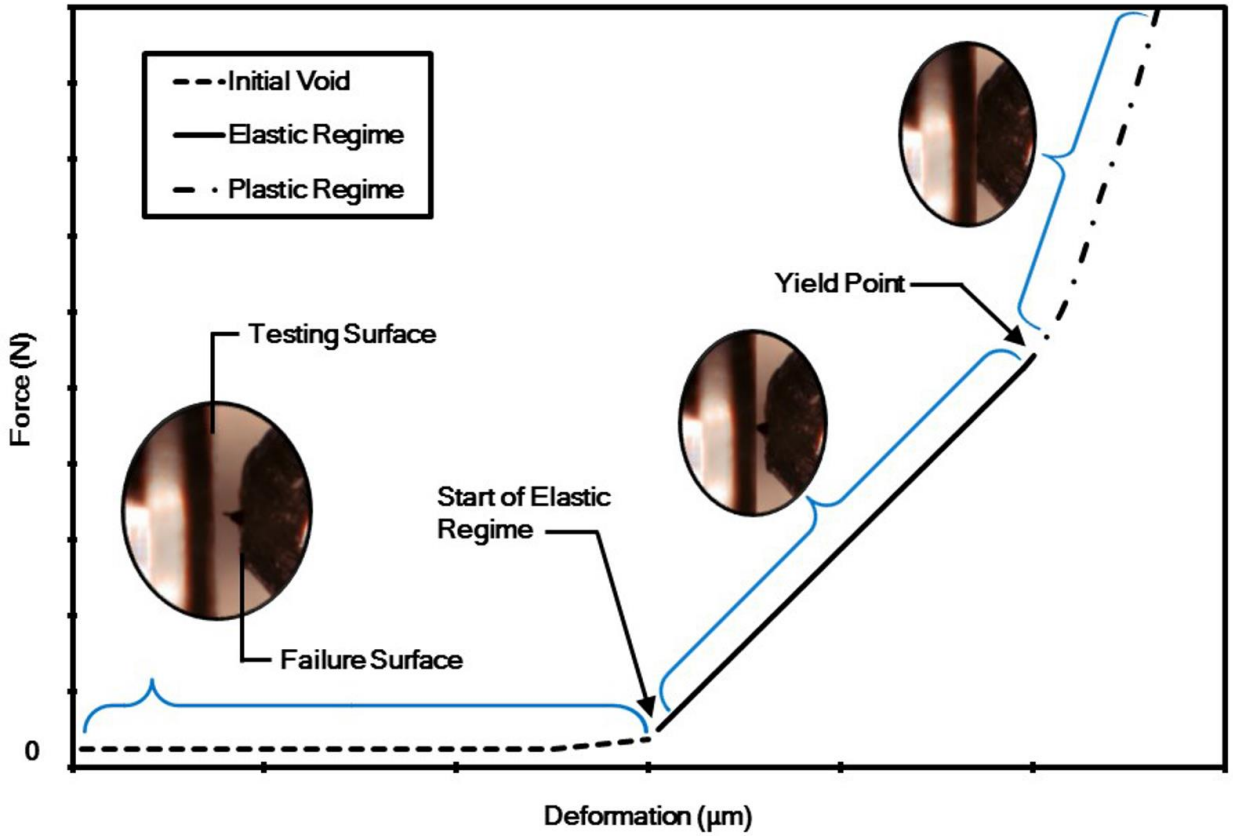

Fig. S1: A plot of force applied (N) vs. deformation (µm) with designations highlighting the elastic regime, the yield point, and the plastic regime.

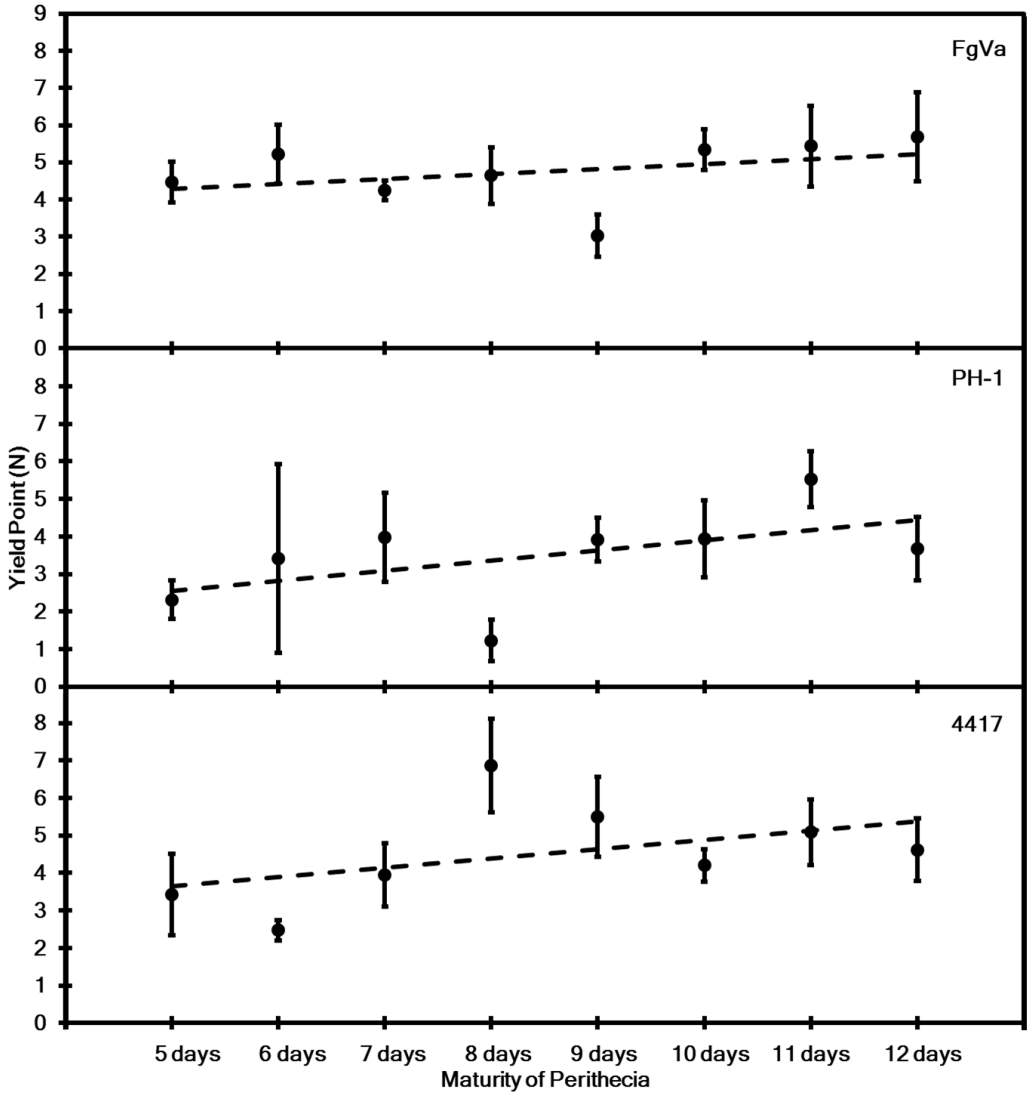

Fig. S2: Yield point of perithecia of different ages (5 d to 12 d) for strains FgVa (*P* = 0.35; R^2 = 0.14; y = 0.13x + 3.62), PH-1 (*P* = 0.19; R^2 = 0.27; y = 0.27x + 1.21), and 4417 (*P* = 0.26; R^2 = 0.20; y = 0.25x + 2.41). There is a positive, non-significant linear relationship between the two variables indicating that older perithecia appear to fail at higher compressive forces compared to less mature perithecia.

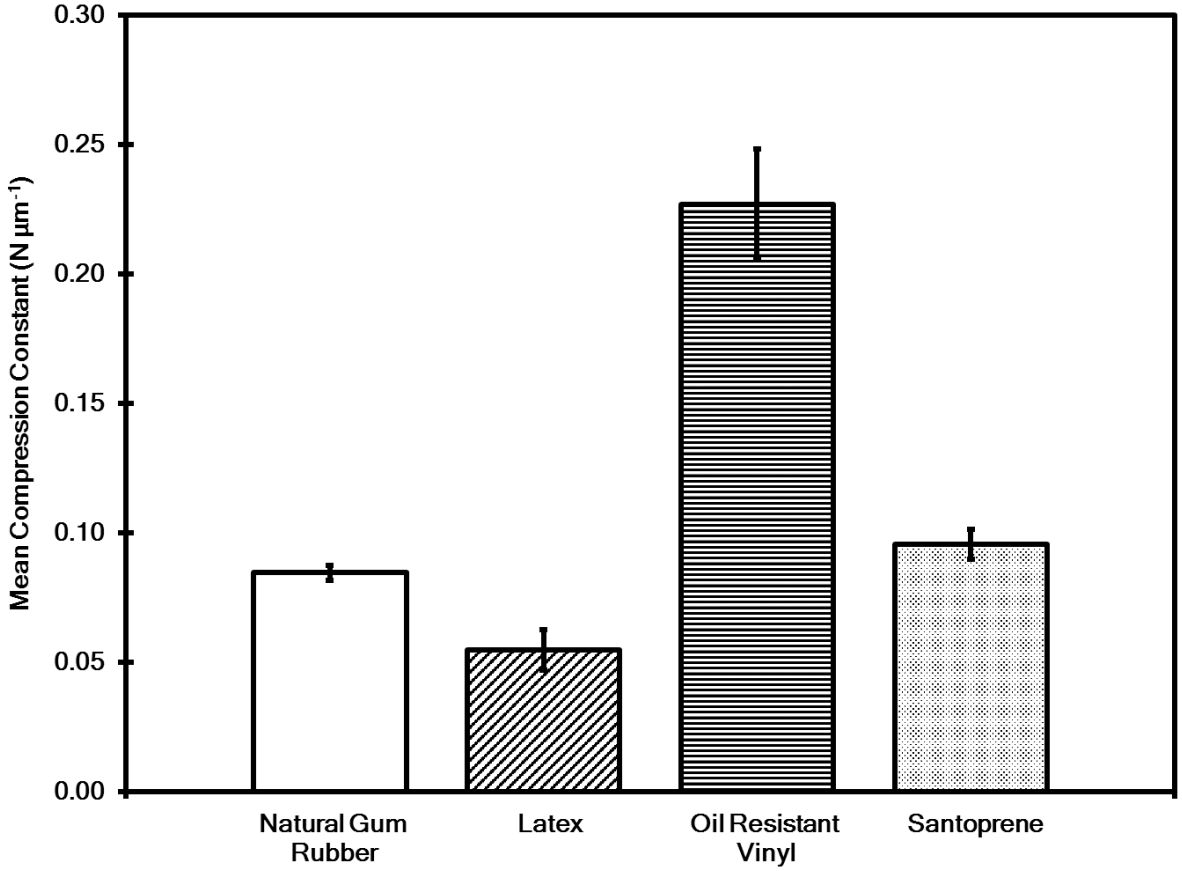

Fig. S3: Mean compression constants for polymers natural gum rubber, latex, oil-resistance vinyl, and santoprene. Tukey's pairwise comparisons test identified differences between latex vs. oil resistant vinyl ($P < 0.01$); gum rubber vs. oil resistant vinyl ($P < 0.01$); and santoprene vs. oil resistant vinyl ($P < 0.01$).

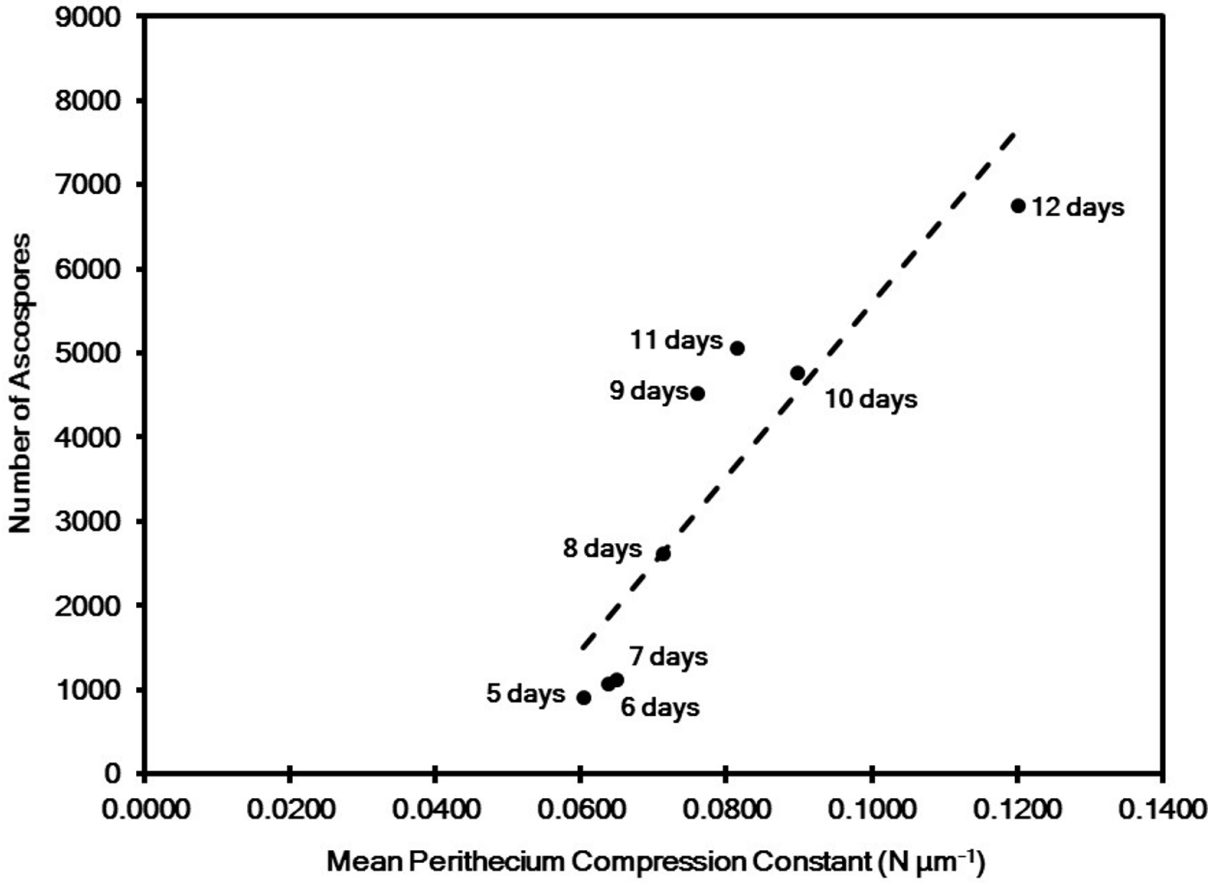

Fig. S4: Number of ascospores versus mean perithecium compression constant (MPCC) and the linear relationship between the two variables for strain FgVa. The dashed line in the panel provides a linear fit with FgVa ($y = (1.0 \times 10^5)x - (4.7 \times 10^3)$) having a significant linear relationship ($P = 0.002$ and $R^2 = 0.81$) between the number of ascospores and MPCC.