Towards early automated detection of pre-symptomatic pathogen risk: mitigating the impact of airborne fungal plant pathogens

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Abstract

Spore trapping and subsequent real-time PCR assays have been used to quantify concentrations of air dispersed fungal spores in many pathosystems. Concerns governing their application include the time it takes to achieve a diagnostic result, reliability in their application and correctly interpreting spore data. Research in this thesis builds on current methodologies, applying spore trapping with novel molecular techniques including real-time PCR and loop mediated isothermal amplification (LAMP), and trials the first fully automated spore detection system. Initially, traditional sticky tape Burkard spore traps and conventional qPCR techniques were implemented for the first time in the UK to monitor the dispersal characteristics of *Hymenoscyphus fraxinea* ascospores to help inform risk. Findings identified that large eruptions of spores can be detected during the early hours of the morning between 05:00 and 08:00 am primarily between the months of July and August, reflecting similar findings observed across Europe. A number of simple spore disruption techniques were then developed that were able to provide rapid detection of *Puccinia striiformis, P. triticina* and *Z. tritici* spores in spore trap samples using LAMP which was comparable to qPCR in detection efficiency. Infection periods were then identified using a disease risk model in combination with spore data, disease incidence and an understanding of their latency periods. This highlighted that an improvement in the disease risk model used could be achieved following the incorporation of spore data. The first fully automated spore detection system was later investigated and provided automated un-attended detection of *P. striiformis* and *P. triticina* spores. Finally, a rapid in-field detection method using LAMP was developed to investigate the latent development of *Z. tritici* in wheat leaves, that may be initiated by splash dispersed pycnidiospores. This method was able to provide an indication of the latency stage of *Z. tritici* in individual wheat leaves and as a result could help inform fungicide application. Molecular techniques are now rapid, simple and robust for incorporation into automated systems for deploying in-field. With a more translational approach between engineers and researchers emerging, the ability to provide fully automated and rapid smart sensor based networks capable of providing valuable data in real-time for informing disease management decisions is becoming more realistic.

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List of Abbreviations and Acronyms

APHA	Animal and Plant Health Agency
BIP	backward internal primer
BLAST	basic local alignment search tool
BLP	backwards loop primer
bp	base pair
B-tubulin	beta-tubulin
C	celsius
cm	centimetre
Ct	cycle threshold
DEFRA	Department for the Environment, Food and Rural Affairs
DMI	DeMethylation Inhibitors
DNA	deoxyribonucleic acid
ELISA	enzyme-linked immunosorbent assay
EPPO	European and Mediterranean Plant Protection Organisation
FERA	Food and Environment Research Agency (now Fera Science Ltd.)
FIP	forward internal primer
FLP	forward loop primer
g	gram
GS	zadoks growth stage
ha	hectare
HYSPLIT	hybrid single particle lagrangian integrated trajectory model
IAMS	integrated assessment modelling
IPM	integrated pest management
ITS	internal transcriber spacer
KOH	potassium hydroxide
LAMP	Loop-Mediated Isothermal Amplification
LFD	lateral flow device
M	molar
mM	millimolar
mm	millimetre
MEGA	molecular evolutionary genetics analysis
min	minute
mph	miles per hour
ml	millilitre
NaCl	sodium chloride
NE	North East
ng	nanogram
NGS	next generation sequencing
OSR	oilseed rape
PCR	polymerase chain reaction
PDA	potato dextrose agar
Pg	picogram
PHSI	Plant Health and Seeds Inspectorate
pmol	picomolar
PRA	pest risk assessment
QoI	quinone outside inhibitor
qPCR	quantitative Polymerase Chain Reaction
RH	relative humidity
Rnase	ribonuclease
RPM	revolutions per minute
S	second
SE	South East
SPR	surface plasmon resonance
SSSI	Sites of Special Scientific Interest
TTP	time to positive
UAV	unmanned aerial vehicles
µl	microlitre
µmol	micromolar
≤	less than or equal to
≥	greater than or equal to

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Chapter 1 . General introduction

Plants are an essential environmental, economic and social asset significantly contributing to our food supply, rural economy and biodiversity in both rural and urban environments. The increasing world population, globalisation in trade, travel, predicted climate change and potential reduction in available pesticides are just a few of the factors contributing to an ever-increasing threat to plant biosecurity and sustainable agricultural production. Invasive species pose a serious threat to global agriculture particularly from countries that are large crop producers with diverse commodities for trade (Paini *et al*., 2016).

A report by Foresite 'The Future of Food and Farming: Challenges and choices for Global Sustainability' (2011) concluded that the long-term trend of low food prices over the last century is coming to an end and food prices will rise significantly over the next 40 years. With a predicted 9 billion people inhabiting the world by 2050 the need to achieve food security is paramount. To help achieve this, investment in innovative science and technology in agronomy, agro-ecology, soil science, engineering and information and communication is needed to facilitate further sustainable intensification of food production (Godfray *et al*., 2010).

In addition to increases in threats to agricultural food production, the United Kingdom has seen a significant increase in the emergence and spread of invasive tree pathogens in recent years. Examples include *Phytophthora ramorum* and *P. kernoviae* (Sudden oak death)*, Pseudomonas syringae* (Horse chestnut blight), *Ceratocystis platani* threatening London Plane trees (*Platanus x hispanica*) and *Hymenoscyphus fraxinea* (anamorph *Chalara fraxinea)* leading to dieback of European Ash (*Fraxinus excelsior*). Further threats to UK trees have been identified in Europe, with Italy recording its first finding of Pine Pitch Canker in 2007 caused by the fungus *Fusarium circinatum* (Carlucci *et al*., 2007)*.* Such diseases pose a serious threat to the heritage, rural economy and natural ecosystems as highlighted by the UK National Ecosystem Assessment (UK NEA, 2014). Safeguarding plant health including early detection of invasive fungal pathogens is currently a high priority (DEFRA report Tree Health and Plant Biosecurity Expert Taskforce, 2013).

One important medium which many plant pathogens exploit to colonise new areas is the air (Dobbs, 1942). It is not possible to prevent the aerial dispersal of fungal spores and the only way to detect them prior to infection is by sampling the bioaerosol (Jackson & Bayliss, 2011). Aerobiological sampling technologies exist; however, their potential is not fully realised due partly to poor integration of biotechnology with aerosol science. In this research the aim is to develop and validate emerging technologies alongside traditional methodologies to bridge this gap and improve current disease risk management and control capabilities.

1.1 Importance of airborne fungal plant pathogens

The microbial fraction (pollen, algae, bacteria, fungi, yeasts) of air within the atmosphere is known as the 'bioaerosol' and is of significant environmental and public health importance. In addition to viable and non-viable microorganisms, a wide range of antigenic compounds, dander, plant and insect debris, microbial toxins and viruses can be present (Peccia and Hernandez, 2006). Viable particles can be the causative agents of plant diseases and can lead to large economic and ecological consequences (McCartney *et al*., 2003). Many fungal plant pathogens are primarily dispersed by aerial spores. Ten of the current most economically important diseases of agricultural commodities as voted by fungal plant pathologists exploit the medium of air as a means to colonise fresh plant tissue (Table 1-1).

Table 1-1 Top 10 fungal plant pathogens as voted by plant mycologists associated with the journal of Molecular Plant Pathology (Dean et al., 2012)

Rank	Fungal pathogen	Common name	Importance
1	*Magnaporthe oryzae*	Rice blast	Most destructive disease of rice world wide typically causing losses between 10 % to30 %. One half of the worlds population primary caloric intake comes from rice
2	*Botrytis cinerea*	Grey mould	Can infect over 200 plant species. Approximately 10 % (£480 million, 2001) of the worlds fungicide market is directed towards control of *Botrytis* (UIPP, 2002).
3	*Puccinia Spp.*	Rust	Three rust species can infect wheat, namely stem (black) rust (caused by *Puccinia graminis f.sp. Tritici),* stripe (yellow) rust *(P. striiformis f.sp.tritici*) and leaf (brown) rust (*P. triticina*).
4	*Fusarium graminearum*	Fusarium head blight	Can infect all cereal species and result in mycotoxin contaminated grain that at high enough concentrations is unsafe for human consumption, animal feed and malting
5	*Fusarium oxysporum*	Fusarium wilt	Complex species comprising different formae speciales which collectively infect more than 100 different hosts, causing severe loss in crops such as banana, melon, cotton and tomata
6	*Blumeria graminis*	Powdery mildew	Persistent casual agent of disease in prominent cereal crops
7	*Zymoseptoria tritici*	*Septoria tritici* blotch	Causing major economic constraints on wheat productivity. Rapidy evolving due to selective pressures including disease resistant wheat cultivars and persistant application of fungicides
8	*Colletotrichum Spp.*	Anthracnose	Causes disease in a wide variety of woody and herbaceous plants including high value crops such as strawberry, mango, avocado, citrus and banana
9	*Ustilago maydis*	Corn smut	Is not an economically impotant or devastating disease however is a model pathogen for undersanding genome evolution and host specialization
10	*Melampsora Lini*	Flax rust	Causes disease on flax and linseed. A pathogen which has provided valuable insights into the molecular basis of plant immmunity

In Europe, 5 to 10 % of the winter wheat harvest is lost annually due to the fungal plant pathogen *Z. tritici*. It is estimated to cost France, Germany and the UK combined £105 to £625 million in yield reductions annually and additional costs to control is the region of £140 to £445 million through regular fungicide applications (Fones and Gurr 2015). Substantial returns are still achievable in the region of £715 to £2,140 million (Fones and Gurr 2015). Wheat provides vital calories for human foodstuff and is used in animal feed, alcohol distillation and biofuels however, one of the biggest concerns over the increasing use of pesticide application in general is their potential detrimental effects to man and the natural environment (Aktar *et al*., 2009).

H. fraxinea was discovered for the first time in the UK in 2012 infecting common ash (*Fraxinus excelsior*). The pathogen is now widespread across the UK. With no direct control measures applicable the pathogen is predicted to cause major detrimental impacts on the rural landscape as ash is a major component of 665 Sites of Special Scientific Interest (SSSIs), and is estimated to have a commercial value of £22 million (Sansford, 2013).

In years when weather conditions favour a particular fungal plant pathogen, disease establishment can reach epidemic status. In Australia in 1971, Phoma stem canker (*Leptosphaeria maculans*) reduced the number of plantings of oilseed rape from 49,000 ha in 1972 to 2,000 ha in 1974. More recently in 2007, the UK experienced severe epidemics caused by *Sclerotinia sclerotiorum* (sclerotinia stem rot) in oilseed rape and *Puccinia triticina* (wheat leaf rust) in winter wheat crops (West *et al*., 2009). Loss in winter wheat yields in 2007 as a result of *P. triticina* in the UK has been estimated at £22.4 million (DEFRA, UK). Further threats include a relatively new race of wheat black stem rust (*P. graminis f.sp tritici)*), known as Ug99 which has been moving through Africa and crossed the red sea into Yemen and Iran. Asian soybean rust (caused by *Phakopsora pachyrhizi*) identified in the United states in 2004 came from the tip of South America transported by hurricane Ivan (Schmale and Ross, 2015). The ability of fungal pathogens to be transported over long distances in the atmosphere demonstrates certain diseases have minimal geographic boundaries. As a result of the potential impact fungal plant pathogens can have, successful control is paramount.

1.2 Control of airborne fungal plant pathogens

Control of fungal plant diseases is required to sustain the abundance and quality of food, feed and fibre produced by growers. Management can be targeted to prevent, mitigate or control plant pathogens and often relies on the application of chemical pesticides in combination with good agronomic and horticultural practices. The detection of fungal plant pathogens in bio-aerosols prior to crop infection can provide an early warning of risk to crop yields and lead to an increase in crop production and improvement of resource allocation through correctly timed control measures (Wakeham and Kennedy, 2010; Wakeham and Pettitt, 2016). The most effective disease control measures prevent the infection in the first instance (termed prophylactic treatment) and this level of protection restricts build-up of inoculum in the local area. Over the past 100 years the increasing use of applications of agricultural pesticides have significantly improved crop productivity. To achieve this level of protection there has been a tendency for chemical inputs to be over applied as an 'insurance' measure in order to reduce disease development and subsequent yield losses. This has, however, led to a number of ecological problems, for example poor ecological diversity, soil erosion and soil and water pollution (Edwards, 1989).

It is expected that climate change has the potential to exacerbate disease through increasing inoculum levels and reducing host resistance (Vaughan *et al*., 2016). For example, alterations in climate patterns, such as more frequent wet and warm conditions experienced at the time of year when winter wheat is flowering, has the potential to enhance the risk of *Fusarium* head blight epidemics in the future.

In addition, there has been a continual emergence and build-up of strains of fungal pathogen resistance. The fungus *Zymoseptoria tritici* has repeatedly evolved resistance to systemic fungicides such as the strobilurins (QoIs) and azoles (imidazoles and triazoles; DMIs). This reduced sensitivity to azoles is brought about by mutations in the sterol 14α-demethylase (CYP51) enzyme where the azoles bind (Cools and Fraaije, 2008). As a result of the high levels of resistance in field populations, succinate dehydrogenase inhibitors (SDHIs) are being favoured to manage *Z. tritici* in wheat. More recently however the development of resistance to SDHIs has been detected in strains of *Z. tritici* which were identified as having

mutations in *Sdh*C-H152R and *Sdh*D-R47W genes (Dooley *et al*., 2016). If over-use of fungicides is not reduced urgently then populations of major threats to crop yields such as *Z. tritici* will continue to evolve resistance mechanisms and become more difficult to control in the future.

To farm more sustainably, predicting the risk of disease is becoming a more important strategy for informing disease control and prevent excessive use of fungicides. In fact, forecasting the risk of disease is a key element of integrated pest management strategies (IPM) which are required to be implemented across Europe as part of the EU Framework Directive (European Union 2009). A number of disease forecasting schemes have been developed, often empirical models which are primarily based upon statistical relationships between environmental variables and the pathogen (Clarkson *et al*., 2014; Legler *et al*., 2014; Roubal and Nicot, 2016). These take into account the many intricate relationships between pathogens and abiotic factors influencing the pathogen's life cycle, in particular maturation of fruiting structures and the production and dissemination of spores. There are also about an equal number of mechanistic models that have been published which take into consideration many more aspects of a disease cycle compared to empirical models (Erick *et al.*, 2008). One of the most critical components of disease forecasting for foliar pathogens is the infection sub-model as without infection there is no disease cycle. Duration of leaf wetness or humidity determines infection success and can be used to form a simple generic infection model that can be applied to any fungal foliar pathogen (Magarey *et al*., 2005). In addition to infection success, attempts to model and predict pathogen reproduction including inoculum maturation, rate of inoculum production (De Wolf *et al*.,2003; Orlandini *et al.*, 2003; Del Ponte *et al.*, 2005; Langston *et al.*, 2002) and dispersal (Holtslag *et al.*, 2004; Luo and Zeng., 1995; Xu *et al.*, 1995) have been made. For many pathogens the data required to develop and validate pathogen reproduction models is not available, resulting in few effective models in use. In the absence of inoculum information over the period an infection risk is made, there is potential for erroneous predictions of disease being made in circumstances when conditions are conducive however no spores are present. Disease prediction models driven by weather variables are scalable and can be operated on a field or regional basis which can be downloaded from local weather

stations. A regional-scale disease prediction system to provide predictions for multiple pests and diseases has been developed for fruit and vegetable crops in the western United States harnessing weather data every 15 minutes from over 2000 stations (Thomas *et al*., 2002). Weather station data can be analysed quickly, and risk predictions made in an almost real-time scenario and made available to users. There is great potential for plant disease models to assist in maintaining global food supplies by influencing management strategies at the field scale. Advances in technology have stimulated innovations in model applications. It can be expected that their uptake will increase because of improved performance measured through increased yield and food production (Erick *et al*., 2007). One area of study which is beginning to benefit from recent advances in technology and has great potential to assist in the development and validation of disease prediction models, is fungal spore monitoring. To date, few examples of spore-based disease forecast schemes exist and even fewer which implement molecular diagnostics (West *et al*., 2008). Monocyclic diseases such as *L. maculans* and *S. sclerotiorum* could be monitored to detect their initial ingress into a cropping system. Forecasting risk of disease from polycyclic diseases such as *P. striiformis* with secondary dispersal and infection mechanisms would benefit from spore detection initiated after crop emergence. Early understanding of disease ingress would assist in determining the potential epidemic severity that may develop if conditions are conducive during the yield contributing developmental stages of the crop. As a result, an improved understanding of a crops pre-symptomatic disease status can be determined and managed earlier.

1.3 Fungal spore dispersal

Ascomycetes can produce prolific amounts of spores (Ingold, 1999) from their parent tissue (condiophore, acervulus, ascus, etc.) which are intended to colonise new healthy plant substrate. For spores to become aerosolised and go on to begin infection they need to overcome a thin column of air surrounding all natural surfaces, known as the laminar boundary layer (Weston, 1988). The thickness of this layer can vary from being a few millimetres thick during turbulent conditions to a few metres on calm nights (Griffin, 2007). Natural spread of ascomycetes occurs as a result of

asexually produced (e.g. conidia) and sexual produced spores (e.g. ascospores). Mechanisms of release are generally an active or passive process (Trail, 2007). Active release can occur following an increase in hydrostatic pressure as a result of water uptake as is characteristic for release of ascospores from the ascus. Synchronised spore release is a common characteristic in many fungal species. Forcible synchronised ejection of *Sclerotinia sclerotiorum* spores can reach 10 cm in height and move around obstacles in the air currents it creates (Roper *et al*., 2010). When active release is not possible or does not occur, spores can become airborne via deliquescence. *Calonectria crotalariae* spores during very wet weather ooze out at the ostiole and any dispersal is a result of rain splash under subsequent drier, damp conditions (Rowe and Beute, 1975). Spores of certain *Basidiomycetes* (rusts and smuts) and at certain life cycles in *Ascomycetes* (conidiospores), need to overcome bonding forces between them and the surface to which they are attached to become aerosolised. The force triggering dispersal can also be a result of electric charges, impaction of other particles or inertia. These forces can be provided by factors such as wind, rain and physical disturbance (Jones and Harrison, 2004).

Successful establishment of a pathogen relies on take-off, flight and deposition. Spore dispersal as previously discussed only takes into account the initial release of the spore into the air. Once in flight many abiotic factors are inflicted upon individual spores influencing the extent of dispersal and viability. Many succumb to drag (Roper *et al.,* 2010) and become deposited on a substrate they cannot proliferate on or are washed out due to rainfall. The process of spore liberation, drift and deposition, influenced by various ecological and environmental factors, needs to be understood in more detail when considering disease management decisions based on spore detection (Schmale and Ross, 2015).

1.4 Spore trapping

Spore traps sample air particulate matter by active or passive impaction onto a collecting surface and have been used in horticulture and agriculture to understand the aerial dispersal characteristics of fungal spores (Hirst, 1953; West *et al*., 2015). A wide range of spore trap types exist and can be selected for dependent on the

intended application. Bayliss and Jackson (2011) highlight the considerable variation in their trapping efficiency. Some of the most commonly used spore traps in plant pathology epidemiology studies include the slit-type volumetric Hirst-type spore trap, multi-vial cyclone (Burkard Manufacturing Company Ltd) and Rotorod sampler (Sampling Technologies) (Figure 1-1). These spore traps can be set up remotely to sample over long durations and sample low volumes of air in the region of 10 to 16.5 L/minute, with the exception of the Rotorod which is capable of 50 L/min. These types of spore traps have been used extensively to monitor fungal spores in many pathosystems.



Figure 1-1 Example of a traditional spore trap (Spore Watch Sampler, Burkard Scientific, left) and a Burkard multi-vial cyclone (right). Both used frequently to study airborne dispersal characteristics of fungal plant pathogens

Devices capable of sampling large volumes of air also exist such as the Jet spore trap (Burkard) and the Coriollis Recon (Bertin technologies) with the ability to draw in the region of 600 and 850 L/min of air respectively.

There have been numerous investigations comparing the efficiencies of the various spore traps available. Aizenberg *et al.,* (2000) compared impaction spore traps which included the Burkard personal volumetric air sampler with an Air-O-cell sampling cassette (Zefon International) both comprising microscope slides as the impaction surface and a button aerosol sampler which collects spores on a filter. It

was concluded the Burkard was able to collect significantly more spores while the Button sampler was effective at sampling particles less than 5.1 µm. One study suggests suction style spore traps in this case a Burkard outperformed two impaction traps (Anderson I-STG and MAS-100) when sampling for *Penicillium citrinum, Candida famata* var. *flarei* and *Rhodotorula* spores (Li and Lin, 1999).

Many factors need to be taken into consideration when selecting a spore trap for a trapping study, primarily the value of the crop, the volume of air sampled by the spore trap and the dispersal characteristics of the pathogen (West and Kimber, 2015). High volume spore traps are considered to have a greater chance of intercepting air parcels containing spores that may have been subject to a large degree of dilution in the atmosphere (West *et al*., 2017). This type of spore trap would be preferred when monitoring for rare exotic pathogens. For plant pathology purposes, air at crop height at the point where most spores are entering or leaving a crop transition, is considered a suitable sampling position (Lacey and Venette, 1995). A number of studies have erected spore traps on the top of buildings, a position used routinely when monitoring outdoor pollen (Khattab and Levetin, 2008; Lang-Yona *et al*., 2012). At elevation, fungal spores have potential to originate from a wide range of microclimates and when samples are collected in conjunction with a sampler at ground level can be used to infer the presence of common plant pathogenic spores over a regional scale (West *et al*., 2017). However, sampling at height and at increasing distances from source can reduce the chance of sampling target spore species and severely limit attempts at accurate quantification of risk.

More recently, the rapidly developing industry in unmanned aerial vehicles (UAVs) has resulted in attempts to sample plant pathogens at increased heights and distances. The airborne sporangia of potato late blight were captured through the use of two UAVs in the lower atmosphere approximately 25-45 m above infected fields (Techy *et al*., 2010). This vertical and horizontal layer sampling of the lower atmosphere using UAVs is now considered an important tool to track long-distance movement of plant pathogens. Atmospheric transport models such as HYSPLIT (Hybrid Single Particle Lagrangian Integrated Trajectory Model; Draxler, 1998) and IAMS (Integrated Assessment Modelling; Isard, 2005) when combined with inoculum data can be used to forecast disease spread and therefore assist growers in making

plant disease management decisions. Such models are used by the North Carolina Plant Disease Forecasting Centre and the Integrated Pest Management-Pest Information Platform for extension and education (Schmale and Kimber, 2015).

1.5 Detection of airborne fungal spores

Conventional methods for identifying and enumerating air-borne fungi involve microscopic spore counting or cultural techniques. These methods however are time consuming and laborious (Williams *et al*., 2001; Calderon *et al*., 2002). Molecular diagnostics for the detection of plant pathogens has been available since the 1970s and has evolved gradually ever since. Mumford *et al*., (2006) provided a detailed review of the evolution and future potential of molecular phytodiagnostics. One of the most widely adopted molecular methods is quantitative polymerase chain reaction (qPCR) and is currently extensively used for diagnostic purposes, providing a highly sensitive and specific method for targeting low concentrations of nucleic acids using oligonucleotides (primers and probe) designed to unique sequences of base pairs associated with the target microorganism. As a consequence of high specificity and sensitivity, qPCR is becoming imbedded in official protocols of the European Plant Protection Organisation for the certification, production and assessment of healthy plant materials (Sanzani *et al.,* 2014). Methods for extracting DNA for qPCR have been developed which maintain high sensitivity and specificity from a wide range of substrates including soil, water and air (Woodhall *et al*., 2012; Parker *et al*., 2014; Scibetta *et al*., 2012). Currently, laboratory diagnostics can be used to detect almost any pathogen and can be optimised for high throughput testing (Adams *et al*., 2012).

In the case of airborne plant pathogens there is a need to detect their presence early and monitor subsequent spore dispersal activity which may lead to an epidemic, if weather conditions are favourable for infection. Inoculum-based disease monitoring and forecasting is likely to benefit control of air-borne diseases that cause yield reduction in high value crops such as fruits and vegetables. Monitoring more exotic pathogens that rarely cause epidemics is not cost-effective (Madden *et al*., 2007). Due to the time-consuming nature and the requirement of

experienced personnel to carry out conventional analysis of sampled airborne propagules, many trapping devices have been used in conjunction with molecular methods (West *et al.,* 2008). Analysis is simplified following the implementation of DNA extraction methods directly from the trapped propagules (Jackson and Bayliss, 2011). PCR overcomes difficulties in assessing closely related spore types and structural impairment, which has been shown to underestimate airborne concentrations of fungi by 1-2 orders of magnitude compared to qPCR (Yamamoto *et al*., 2010). Molecular detection provides the ability to not only detect species but also strains expressing mutations. This provides a capability of detecting resistant strains within a population. Fungal resistance in different pathogens has been determined using allele-specific probes (*Rhynchosporium secalis, Venturia inaequalis, Botrytis cinerea*) PCR-restriction fragment length polymorphisms (*Cladobotryum dendroides, Mycosphaerella fijiensis*) and allele-specific PCR (*Uncinula necator, Erysiphe graminis* f. sp. *hordei*) (McCartney *et al*., 2003),

There is also growing interest in detection of multiple potential pathogens or newly emerged pathogens using next generation sequencing (NGS). NGS has the capacity to sequence complex environmental samples by generating large numbers of nucleotide sequences and requires no prior knowledge of the host or pathogen. However, interpretation and sorting of the vast amount of data generated takes considerable time, understanding and skill (Dewoody *et al*., 2013). Analysis of a single sample for plant viruses using NGS has been estimated to cost £1000 (Adams *et al.,* 2009), although advances in technology continue to reduce costs. As a result, NGS is not commonly used in routine diagnostics but is instead used for fundamental genome-based research and supporting development of new diagnostic, molecular-based technologies (Wakeham and Pettitt*,* 2016).

1.6 In-field detection of fungal plant pathogens

Early and rapid detection of plant pathogens is an important step to manage a plant disease. Methods for on-site testing are continually developing due to the benefits that carrying out a diagnostic test at point of source provides. Limitations in the complexity of equipment available that are typically used in the laboratory

environment to prepare and test the sample are restrictive. Many attempts to carryout on-site detection do however exist; for example, real-time PCR using the Cepheid SmartCycler was able to successfully detect *Phytophthora ramorum* in infected plant material (Hughes *et al.,* 2006). Uptake however has been limited due to the multiple manipulation steps still involved and the high costs associated with the equipment (Tomlinson *et al.,* 2005). Many other research efforts have directed their attention to the development of techniques that can be applied in-field. A biosensor-based approach for the detection of *P. striiformis* uredospores was achieved using a mouse monoclonal antibody and an SPR (Surface plasmon resonance) sensor (Skottrup *et al*., 2007). Application of this technique is restricted by the availability and time required to develop spore species-specific antibodies.

A molecular technique capable of amplifying large quantities of DNA without the requirement of power intensive thermo-cycling known as loop mediated isothermal amplification (LAMP) is beginning to find application in field. The method utilizes 4 or 6 primers and a DNA polymerase with high strand displacement activity to amplify DNA at a constant temperature between 60-65 ^{o}C (Notomi *et al.,* 2000; Mumford *et al.,* 2006). PCR inhibitors have been shown to have minimal detrimental effect on the sensitivity of LAMP (Poon *et al*., 2006). Visualisation of target DNA can be determined by the resulting large concentration of the by-product magnesium pyrophosphate precipitate (Mori *et al*., 2001) or optical sensors detecting universal dye bound to double stranded DNA produced in the LAMP reaction (Genie, Optisense). LAMP has also been used to enhance the specificity of immunological techniques such as lateral flow devices (LFDs) (Tomlinson *et al.,* 2007). LFDs in plant pathology are commonly used as a first line rapid screening test capable of providing a result within 5 to 10 minutes. A *Phytophthora* genus-specific LFD is regularly used by the Animal and Plant Health Agency (APHA) and Plant Health and Seeds Inspectorate (PHSI) to detect and monitor *Phytophthora ramorum* and *P. kernoviae* and has enabled effective management of the disease in horticultural nurseries through quarantine and containment measures (Lane *et al*., 2007).

1.7 In-field automated pathogen detection

The use of automated technologies in the laboratory is a common practice especially where large sample numbers are processed. Most of these systems are actually semi-automated comprising a number of dedicated pieces of equipment distributed through a laboratory or a number of rooms in the same laboratory. Typically, they still require some user input to move samples from one stage in the process to another yet take out some of the repetitive procedures involved. To handle continual large numbers of samples Forensic and Pharmaceutical companies are able to invest in fully automated robotic workstations such as the *Freedom EVO* liquid handling robot (Tecan), Qiagen M48 BioRobot or QIAsymphony SP. (Stangegaard *et al*., 2011).

Many of the processes involved in either spore trapping or molecular detection are automated. Very few successful examples exist where the two technologies have been combined into a fully automated system. Most research in this area has been for bio-warfare and homeland security purposes. For example, the USA developed a non-portable autonomous pathogen detection system known as the 'Biobriefcase' Patent US20070107539 in response to attacks using letters containing anthrax spores in 2001. More recently a miniature virtual impactor has been used to successfully trap and detect *Sclerotinia sclerotiorum* spores in combination with a biosensor targeting oxalic acid. This process is fully automated and is able to transmit the data wirelessly (West and Kimber, 2015). However, the device is specific to sclerotinia and cannot be adapted to other targets. In order to have an effective role in disease risk detection, devices will need to be able to simultaneously detect multiple threats.

1.8 Conclusions

The rising cost of food production and increasing threats from invasive and indigenous plant pathogens is driving the need for improved technologies capable of providing early detection. Spore traps have been used extensively in plant pathology

and have great potential to assist management practices directed towards reducing the impact of airborne plant pathogens. Many uncertainties in their operation and interpretation of results remain which need addressing to improve reliability and confidence in making associated potentially expensive management decisions. To achieve this bridges in technology are required including, but not exclusively, in the disciplines of plant pathology, aerobiology, molecular diagnostics and mechanical engineering.

1.9 Aims and objectives of this research

The aim of this research was to develop and apply more novel spore trapping and molecular diagnostic methods to detect airborne fungal plant pathogens early. Initially sticky tape Burkard spore traps and qPCR techniques were implemented to monitor the spore dispersal characteristics of *Hymenoscyphus fraxinea* in the UK (Chapter 2). Rapid spore lysis methods were then developed and validated to enable a more rapid detection method of airborne spores in spore trap samples using LAMP for in-field application (Chapter 3). Further validation of the methods developed were then carried out in-field alongside the gold standard qPCR (Chapter 4). The feasibility of detecting airborne plant pathogens on days when infection was highly likely to have occurred was later investigated using a disease forecast model (Chapter 5). Finally, a rapid in-field extraction and detection method using LAMP was developed to investigate the latent development of *Z. tritici* in wheat leaves which may provide a useful additional level of testing when considering the proposed limitation in spore traps to sample splash dispersed conidia (Chapter 6).

Chapter 2 . Monitoring the ascospore dispersal characteristics of *Hymenoscyphus fraxineus* in the UK

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2.1 Abstract

The ascomycete *Hymenoscyphus fraxineus* responsible for the widespread dieback of European ash (*Fraxinus excelsior*), was confirmed in England in October 2012. The pathogen exhibits a highly efficient means of dispersal through a massive synchronised release of ascospores. The release of ascospores of *H. fraxineus* was studied and quantified in England over a three-year period at three sites of infection to identify spatiotemporal ascospore dispersal characteristics and relationships with weather variables. The protocol used to sample and quantify *H. fraxineus* ascospores incorporated Burkard volumetric spore traps and a real-time quantitative polymerase chain reaction (qPCR) assay. The method proved highly sensitive, being capable of detecting DNA for quantification from as few as 10 ascospores. Accuracy of the qPCR was assessed by carrying out comparative spore counts on sticky tapes from which a strong linear relationship was observed (R^2=0.897). Variation in temporal ascospore dispersal was identified between sites within the same year and was primarily driven by temperature and rainfall. Findings indicate the ability of the pathogen's sporulation structures to develop and release ascospores in considerable numbers in response to favourable conditions. Once conducive conditions have been met hourly testing of spore tape segments identified the majority of spores are released between 05:00 and 08:00 am. Relationships between timing and intensity of spore release and meteorological conditions could provide valuable data for the development of predictive models and subsequently help inform disease management strategies.

2.2 Introduction

Dieback of common ash (*Fraxinus excelsior*), caused by the ascomycete *Hymenoscyphus fraxineus* (anamorph *Chalara fraxineus*) was first recorded in Poland and Lithuania in the early 1990s (Kowalski and Holdenrieder, 2009). Most likely originating from Asia the pathogen has since spread rapidly throughout Europe (Zhao *et al*., 2013). Primary infection is understood to be caused by the deposition of ascospores on ash leaflets, released from apothecia overwintering on *Fraxinus* rachises lying on the woodland floor. Following infection, symptom expression on host leaflets occurs in late summer in the form of necrotic lesions. The disease then progresses into the rachis and side shoot (Gross *et al*., 2014). A dark brown necrotic lesion can frequently be observed between the stem and trunk while a cinnamon-brown discolouration in young saplings is prominent as the infection spreads through the xylem. In severe cases death of the tree can occur within a few years depending on its age (Pautasso *et al*., 2013). After leaf fall, the pathogen overwinters in the leaf rachis and its life cycle is completed through the release of ascospores in the following year to infect fresh *Fraxinus* leaflets (Kiristis and Cech, 2009; Kowalski and Holdenrieder, 2009). In autumn at low temperatures conidia (spermatia) are produced on the petiole (Kowalski and Bartnik, 2010). Indirect evidence suggests that conidia may play a role in sexual reproduction following the detection of *H. fraxineus* DNA in the air samples during the absence of ascospore producing apothecia (Dvorak *et al*., 2015). More recently work *in vitro* has shown conidia are capable of germinating on ash leaves and can infect seedlings via leaves or soil (Fones *et al*., 2016).

The distance ascospores of *H. fraxineus* can disperse has not been extensively studied. Current evidence suggests the majority of ascospores deposit within 50 m of the infected ash stand but can be detected up to 400 m away (Chandelier *et al*., 2014). Indirect evidence does however support the potential for longer distance dispersal. The disease front in Norway was reported to progress between 20 to 30 km each year over a period of 2 years and it was suggested that ascospores were driving this spread (Solheim *et al*., 2011). Further evidence supports the potential for long distance dispersal following the identification of the

disease on the islands of Gotland and Åland in the Baltic sea. As it is highly unlikely infected ash plants were imported to these islands it is believed the disease came from Sweden situated 40 km west (Sansford, 2013). Although it is widely accepted ash saplings infected with *H. fraxineus* were imported and planted in the UK, an additional route of entry from ascospores being blown from infected areas in Europe across the English Channel from continental Europe has been postulated, especially because of the high incidence of the disease in the eastern regions of the UK and many wider environment infections as identified in a national survey of *Fraxinus excelsior* in the UK carried out by the Forestry Commission in 2012. The University of Cambridge modelled the potential spread of *H. fraxineus* ascospores which strongly supports the likelihood of airborne incursion by wind across the Channel from Europe (Chalara Management Plan, DEFRA, 2013).

In Europe a diurnal synchronised pattern to ascospore release has been identified. Numbers peak in the early morning with seasonal dispersal covering a period between June and October (Timmermann *et al.*, 2011; Hietala *et al*., 2013; Chandelier *et al.*, 2014; Dvorak *et al.*, 2015). Temperatures above 12 °C favoured ascospore release in Belgium with high levels of deposition occurring within 50 m of an ash stand (Chandelier *et al*., 2014). Sufficient air humidity was required for successful development of apothecia and a long duration of leaf wetness enhanced ascospore release 11 - 12 days later (Dvorak *et al*., 2015). Gaps in our knowledge exist in terms of the specific factors influencing the timing and intensity of ascospore release of *H. fraxineus* as current studies have been limited to either a single site in a given year or restricted to particular sampling days.

To date predictive models have been used extensively to inform disease management decisions in agriculture and horticulture. Such models rely on extensive epidemiological studies to provide the data used to base their predictions. A common limitation in such models is inoculum data monitored over consecutive years in order to incorporate substantial variation in observed spore numbers and weather conditions. At a local and regional scale spore samplers and meteorological equipment can been used to understand inoculum source, concentration and the influence of biotic factors required for spore release and infection (Prados-Ligero *et al*., 2003; Ganthaler *et al*., 2015). These are important factors which can be included

in predictive models for disease risk forecasting contributing to their accuracy and reliability.

Accurate measurements of airborne spore concentrations can be determined using spore traps (Hirst, 1953; Aylor *et al.,* 2001). Historically, microscopy-based methods were used to analyse spore trap samples where spores have been deposited via impaction onto a collection surface (Kennedy *et al*., 2000). However, significant limitations exist not only in time taken to examine the tapes but also degradation of spore structure (Godish *et al*., 2007). More recently real-time PCR has been incorporated to speed up the screening process and take advantage of the heightened sensitivities that it can provide (Fraaije *et al*., 2005; Luo *et al*., 2007; Rogers *et al*., 2009; Duvivier *et al*., 2010; Fountaine *et al*., 2010).

The objectives of this study were to monitor spatial-temporal patterns of the ascospore release of *H. fraxineus* using volumetric spore traps and real-time PCR over a 3-year period at infection sites across the UK. Weather data from both local and regional meteorological monitoring stations were used to investigate possible correlations between spore dispersal and weather variables to identify factors driving spore release. The data obtained were evaluated for use in the development of predictive models for informing assessments of disease risks and supporting decision making by government policy makers on management strategy.

2.3 Methods

2.3.1 Spore trapping and monitoring locations

Three Burkard 7-day volumetric spore traps (Burkard Scientific, Uxbridge, UK) were placed at two sites where infection had been confirmed and visual necrotic symptoms were present on nearby stands of *Fraxinus* (Figure 2-1). One trap was placed at a site in the North East (NE) of England and two traps positioned 750 m apart in separate woodland stands in the South East (SE1 & SE2). Woodland in both locations was comprised predominantly of mature mixed deciduous trees with a larger concentration of *Fraxinus* in the South East location.



Figure 2-1 Burkard volumetric spore trap and meteorological monitoring equipment situated at the woodland site in the North East of the UK (top left), necrotic trunk symptoms right and apothecia protruding from an ash rachis (bottom left).

Spore traps were programmed to sample air continuously from June through to September in 2013, 2014 and 2015. The spore trap orifice was positioned above the woodland floor at a height of approximately 1 m and sampled air at a rate of 10 L min^{-1}. Air spora impacted onto a melinex tape containing an adhesive coating of petroleum jelly. The tape was secured to a replaceable drum rotating at 2 mm hr^{-1} to provide a continuous sample of air spora over the entire sampling period. Power to the sampler was maintained by replacing the battery during weekly site visits when the drum was also replaced and returned to the laboratory for analysis. A total of 3 potted ash saplings (bait plants) approximately 2 m high were randomly

positioned in a 4 m radius around the spore trap at the site in the North East to identify the initial appearance of leaf necrosis caused by *H. fraxineus*.

2.3.2 Preparation of air sample tapes for DNA analysis

Weekly sections of tape were cut into 7 daily segments (48 mm long). These were then cut longitudinally splitting the segment into two equal strips. One half was placed in a 5 ml transport tube for archiving and the other half in a 2 ml screw cap tube for molecular testing. Hourly samples were obtained by dividing the archived 48 mm segment up into twenty-four 2 mm pieces. These were then placed in 2 ml screw cap tubes for DNA extraction.

2.3.3 Extraction of nucleic acids

Due to the large number of spore trap samples obtained the method established needed to be quick and maintain high levels of sensitivity throughout. To each sample 1 g 0.5 mm zirconia silica beads, 500 µl CTAB lysis buffer (120 mM sodium phosphate buffer pH8, 2 % CTAB, 1.5 M NaCl, 2 % antifoam B emulsion) and 10 µl RNase 'A' were added. Samples were then mixed on a horizontal Vortex Genie for 2 min then placed in an oven at 65 °C for 20 min. Pulverisation was carried out by subjecting each sample to 60 s on a Precellys 24 (Bertin technologies, France) at 6500 rpm. The resulting supernatant was then centrifuged to produce a clear lysate and passed through a Nucleospin Plant 2 Kit (Machenery-Nagel, Germany).

2.3.4 Real-time polymerase chain reaction

Reactions were performed using an *H. fraxineus* species specific dual-labelled probe (Cfrax-P CTCTGGGCGTCGGCCTCG) and primer pair (Cfrax-F ATTATATTGTTGCTTTAGCAGGTC & Cfrax-R TCCTCTAGCAGGCACAGTC) based on internal transcribed spacer sequences (Ioos *et al*., 2009). An Applied Biosystems 7500 real-time PCR machine was used to test each sample in duplicate using Environmental MasterMix (TaqMan® Environmental Master Mix 2.0) with the

following cycling conditions, 50 °C for 2 min, 95 °C for 10 min, and 45 cycles of 95 °C for 15 s and 65 °C for 1 min. PCR runs also included positive control standards comprised of DNA extracted from 10, 10^2,10^3, 10^4 and 10^5 ascospores to generate a calibration curve for quantification of sample spore numbers. An extraction and a PCR negative control was included to monitor for cross contamination.

2.3.5 Preparation of spore standards and determination of the limit of detection

Ash rachises were collected from the infection site and placed in humidity chambers at 20 °C to encourage apothecial development. Once mature, apothecia were dipped into 0.1 % Tween solution to encourage ascospore release. Ascospore numbers in suspension were then calculated using a haemocytometer and used to seed melinex tapes coated in petroleum jelly prior to DNA extraction at spore numbers ranging from 10 to 10^5. A serial dilution of DNA extracted from an ascospore concentration of 10^5 was carried out down to 0.1 to determine the limit of detection.

2.3.6 Comparative spore counts

To validate the accuracy of the qPCR, comparative tests were conducted on 26 of the archived half tape segments each representing 24 hrs of sampling from across the spore trapping period in 2013 and 2014. Analysis of the tapes was carried out by conducting spore counts using a light microscope at 400 X magnification. Tape segments were secured to a microscope slide using two drops of water and stained with a mixture containing cotton blue and lacto glycerol. Three transverses were conducted on each tape and the total number of *H. fraxineus* ascospores counted. This number was then used to calculate the total spores collected across the tape segment. Pictures were taken using a digital CCD camera (Infinity 2, Lumenera).

2.3.7 Meteorological data

Accompanying each spore trap was a data logger (Gemini, UK) set to record temperature and humidity every 10 minutes throughout the sampling period at each site. Additional representative hourly weather data for the spore trapping locations was acquired from the DataPoint service operated by the UK Meteorological Office (http://www.metoffice.gov.uk/datapoint). The nearest available locations were – Wattisham 31.3 km southeast of SE1 and SE2 and Leconfield 14.2 km southeast of NE. The additional weather variables obtained included wind speed (mph) and rainfall (mm).

2.3.8 Statistical analysis

Statistical analysis was carried out using Spearman's rank correlation coefficient to identify significant relationships between weather variables and spore trap data. A Kruskal-Wallis one way analysis of variance test was used to identify differences in the seasonal pattern of ascospore release between sites. All statistics were carried out using the standard stats package in R v3.2.2 (R Core Team, 2015).

2.4 Results

2.4.1 Temporal patterns in *H. fraxineus* ascospore dispersal at NE

In 2013, the first *H. fraxineus* ascospores were detected on 4 July at the site in the North East. Daily spore detections remained relatively low until three distinctive periods when high ascospore numbers were recorded on the 6, 13 and 25 August at 2,333, 4,549 and 7,863 ascospores m^{-3}/day^{-1} respectively (Figure 2-2).

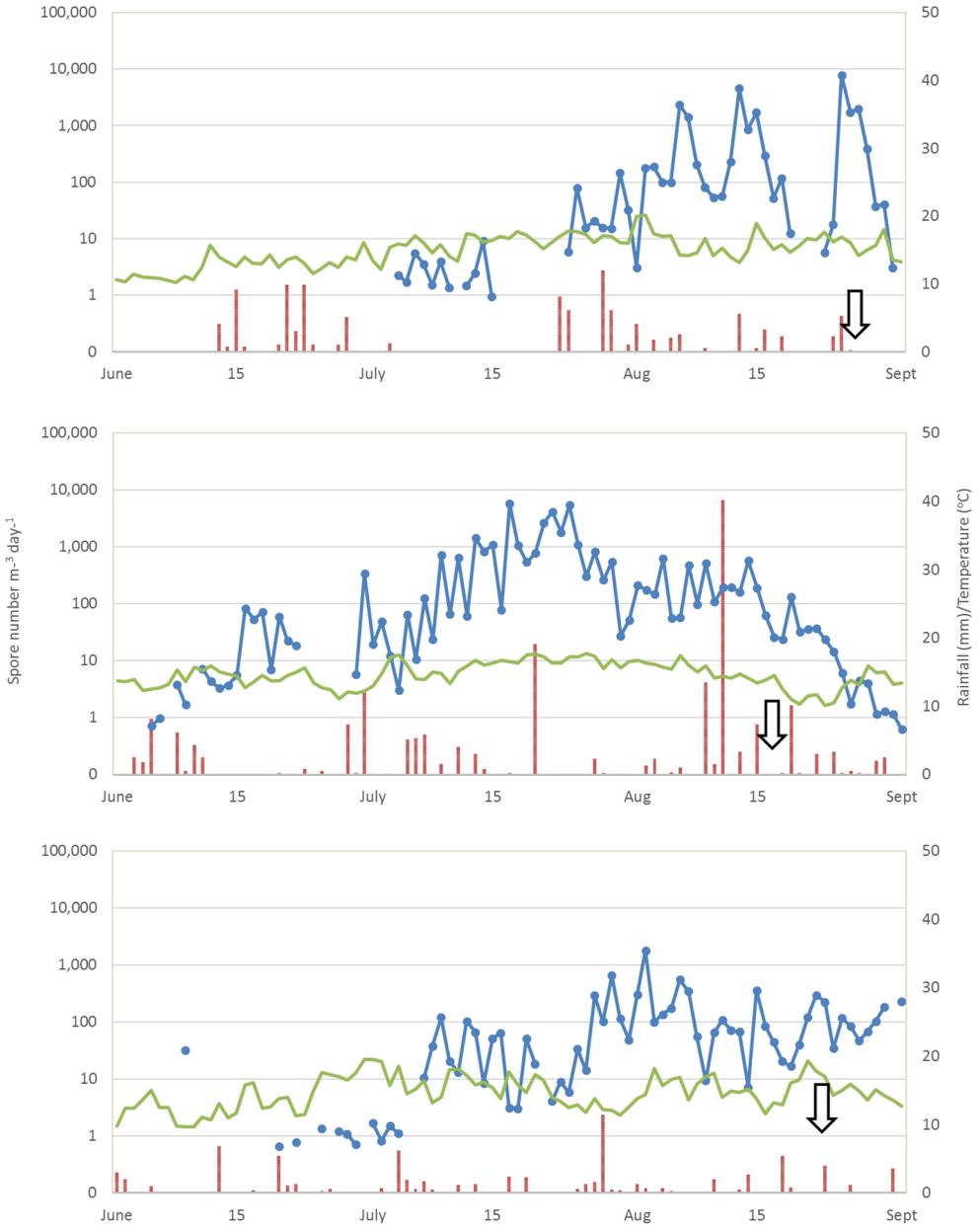


Figure 2-2* H. fraxineus *ascospore dispersal characteristics at NE in 2013 (top), 2014 (middle) and 2015 (bottom) from June to September in relation to daily rainfall (bar) and the day when initial disease symptoms were observed on baited ash samplings (downward pointing arrow)

In 2014 initial ascospore detection was made earlier than in 2013 occurring on 5 June. Large numbers of spores were released daily throughout July peaking on 17 July with ascospores m^{-3}/day^{-}1 reaching 5,695. In 2015, initial ascospore detection was made on 9 June and peak ascospore numbers released m^{-3}/day^{-}1 were 1,790 on 2 August. Average monthly temperature for June in 2013 was 12.5 °C, approximately 1 degree lower than 2014 and 2015 (Figure 2-6).

The year the highest number of spores were detected in the North East was in 2014 with approximately 35,167 ascospores m^{-3}/day-1 (Figure 2-7). The majority of these spores were released in July totalling approximately 30,302 ascospores m^{-3}/day^{-1}. Total monthly rainfall and average monthly relative humidity was at its highest in June, July and August in 2014 (Figure 2-6).

The year the least number of ascospores were detected at NE was in 2015 with 7,518 ascospores m^{-3}/day-1 (Figure 2-7) and were mainly released in August. Total monthly rainfall from June to July and average monthly relative humidity was much lower in 2015 compared to 2013 and 2014 (Figure 2-6).

Spearman's Rank correlation analysis showed a positive statistically significant correlation between spore number released m^{-3}/day^{-1} and temperature each year (NE 2013 r=0.4703 & $P \leq 0.001$, NE 2014 r=0.534 & $P \leq 0.001$, NE 2015 r=0.181 & $P \leq 0.10$) and some in year specific correlation with wind speed (NE 2015 r=0.258 & $P \leq 0.10$, NE 2014 r=0.307 & $P \leq 0.10$) and relative humidity (NE 2015 r=0.497 & $P \leq 0.001$). The first symptoms of *H. fraxineus* in the bait plants were recorded on 1 September, 20 and 24 August in 2013, 2014 and 2015 respectively (Figure 2-2). Initial symptoms appeared on ash leaflets in the form of necrotic spotting. In the following year cinnamon brown discolouration was observed on the ash sapling stems (Figure 2-3).



Figure 2-3 Symptomatic ash sapling. Necrotic spotting on ash leaflets (left) and stem discolouration (right)

2.4.2 Temporal patterns in *H. fraxineus* ascospore dispersal at SE1

In 2013 initial ascospore detection was made on 19 June. The highest number of ascospores recorded was on 9 August with 23,884 ascospores m^{-3}/day^{-1} (Figure 2-4). A period of 10 days when ascospore numbers exceeded 1000 m^{-3}/day^{-1} was subsequently recorded.

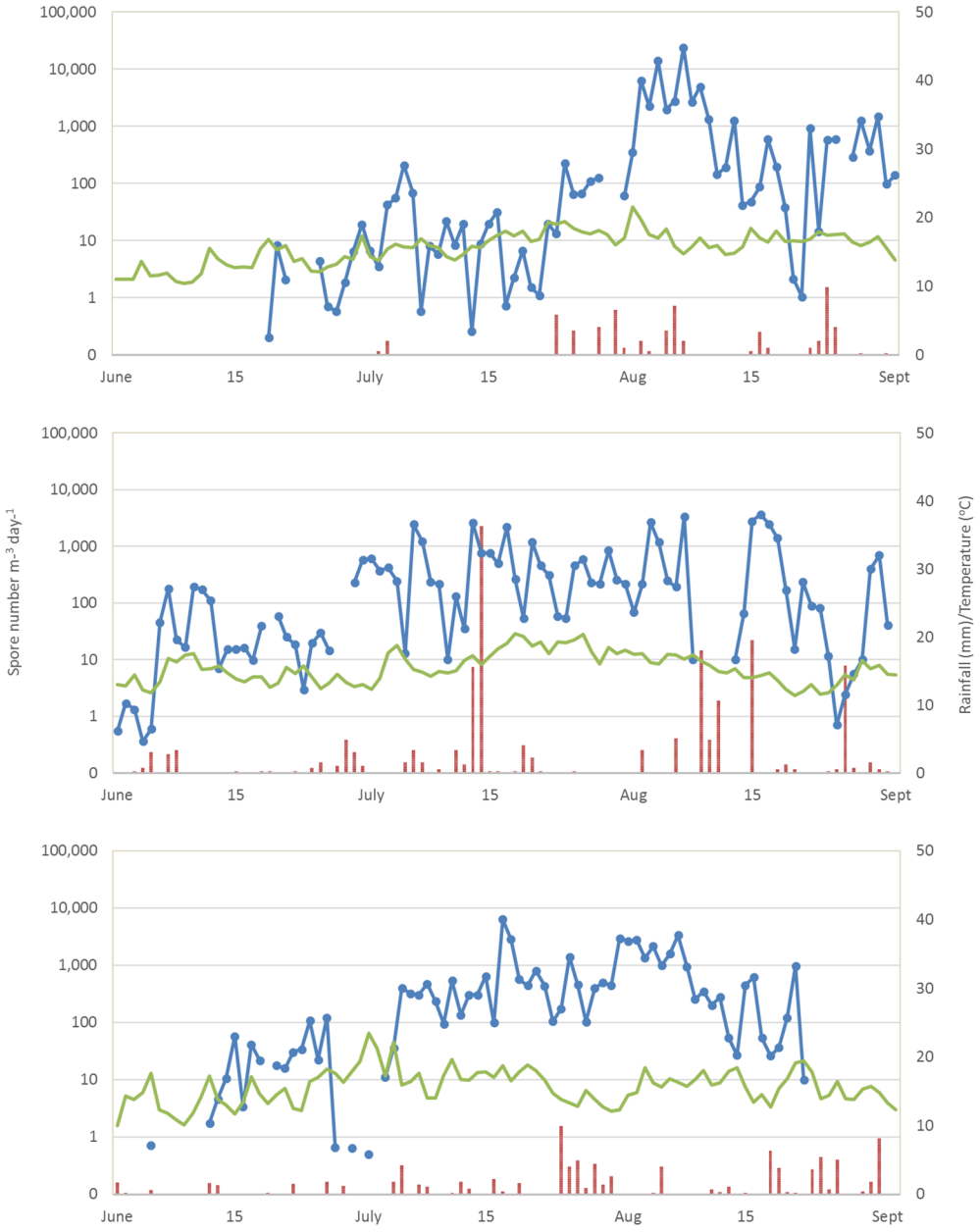


Figure 2-4* H. fraxineus *ascospore dispersal characteristics at SE1 between 2013 and 2015 from June to September in relation to daily rainfall (bar)

In 2014, initial ascospore detection was made earlier than in 2013 with low numbers recorded in the South East from 7 June. Peak sporulation events were recorded on 9 of August with ascospores m^{-3}/day^{-}1 reaching 3,637. In 2015, initial ascospore detection was made on 14 June and the most ascospores released m^{-3}/day^{-}1 was 6,361 on 17 July.

Consistently high numbers of ascospores were detected at SE1 over the 3 years. The highest number of ascospores detected at SE1 was in 2013 totalling 69,654 ascospores m^{-3}/day^{-}1, 68,463 of which were detected in August (Figure 2-7). A total of 39,773 and 41,779 ascospores m^{-3}/day^{-}1 were detected in 2014 and 2015 with relatively similar numbers of ascospores released in both July and August. The months of June and July in 2013 were considerably much drier than 2014 and 2015 with 2014 experiencing more rainfall throughout the 3 months studied (Figure 2-5). Spearman's Rank correlation analysis showed a positive statistically significant correlation between spore number released m^{-3}/day^{-1}, average daily temperature each year (SE1 2013 r=0.508 & $P \leq 0.001$, SE1 2014 r=0.408 & $P \leq 0.001$, SE1 2015 r=0.19 & $P \leq 0.10$), relative humidity in 2013 (r=-0.356 & $P \leq 0.001$) and rainfall in 2013 (r=0.282 & $P \leq 0.01$).

2.4.3 Temporal patterns in *H. fraxineus* ascospore dispersal at SE2

The first significant detection of ascospores in 2013 was made on 24 July when the equivalent of 6,661 ascospores per m^{-3}/day^{-1} was detected. A number of smaller release events were identified 8 days later, peaking at 1,066 ascospores on 4 August (Figure 2-5).

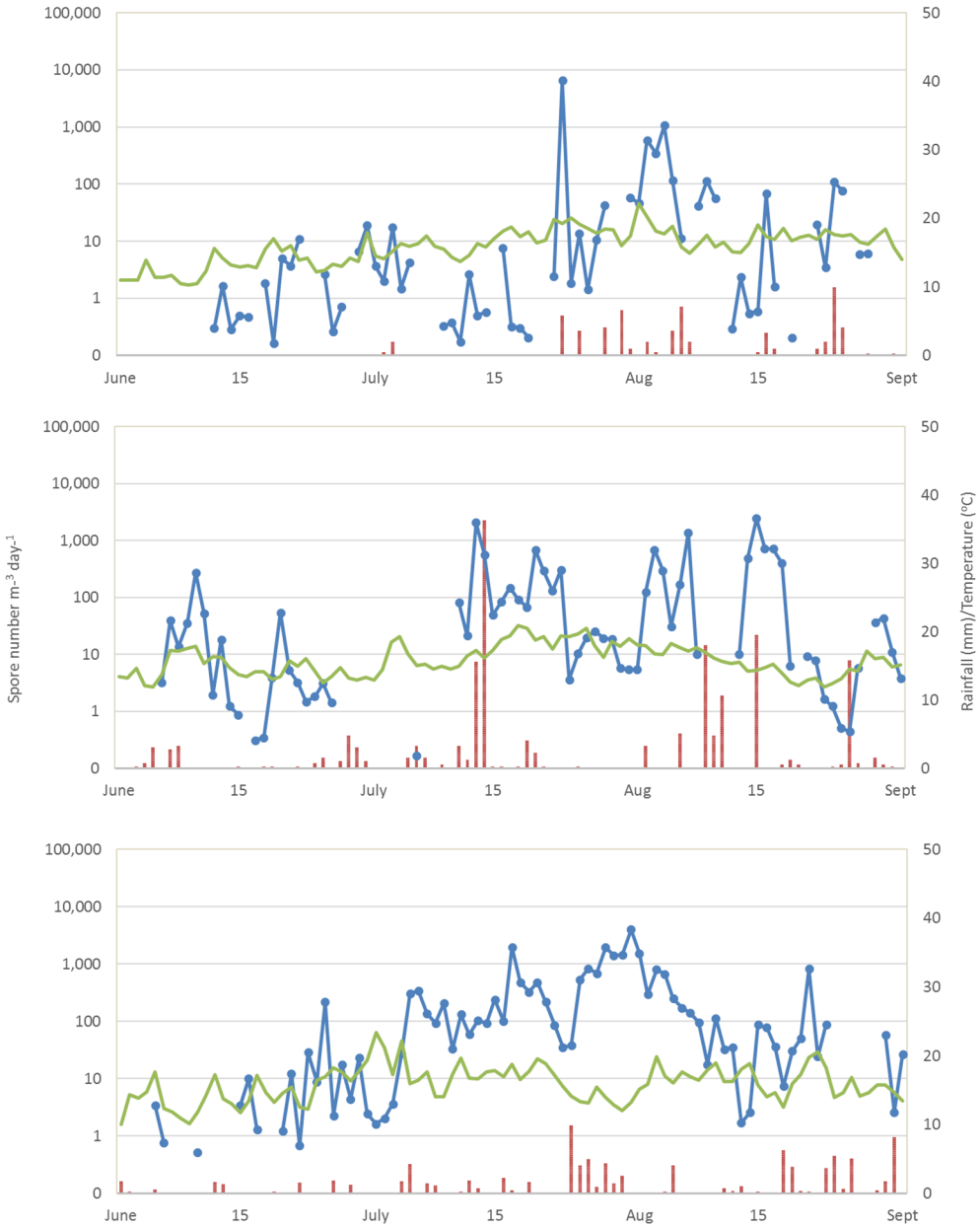


Figure 2-5* H. fraxineus *ascospore dispersal characteristics at SE2 between 2013 and 2015 from June to September in relation to daily rainfall (bar)

In 2014 peak sporulation events were recorded on 10 of August with ascospores m^{-3}/day^{-1} reaching 2,453. In 2015, initial ascospore detection was made on 16 June and peak ascospore numbers released m^{-3}/day^{-1} were 4,002 on 31 July.

Overall a gradual increase in the total number of ascospores detected at SE2 was observed year upon year. In 2013, 9,570 ascospores m^{-3}/day^{-1} were detected, of these 6,833 ascospores m^{-3}/day^{-1} were released in July (Figure 2-7). In 2015 22,184

ascospores were detected m^{-3}/day^{-1} and 16,391 of these were released in July. In 2014, 12,842 ascospores m^{-3}/day^{-1} were detected and dispersed relatively equally between July and August. Spearman's Rank correlation analysis showed a positive statistically significant correlation between spore number released m^{-3}/day^{-1}, average daily temperature (SE2 2013 r=0.413 & $P \leq 0.001$, SE2 2014 r=0.522 & $P \leq 0.001$, SE2 2015 r=0.267 & $P \leq 0.10$) and relative humidity in 2015 (r=0.201 & $P \leq 0.10$) and rainfall in 2013 (r=0.224 & $P \leq 0.10$).

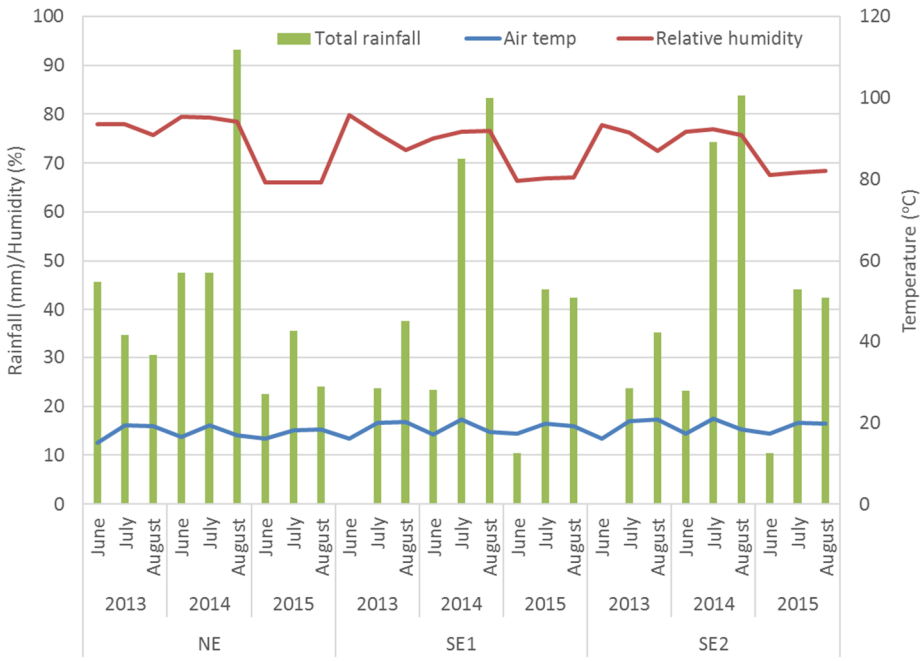


Figure 2-6 Average monthly temperature, humidity and total monthly rainfall (bar) for each site between 1 June and 1 September 2013 to 2015

2.4.4 Patterns in *H. fraxineus* ascospore dispersal

The highest numbers of spores detected across the three-year period of study was at SE1. There were marked variations in the number of ascospores released each month at the sites over the three-year period (Figure 2-7).

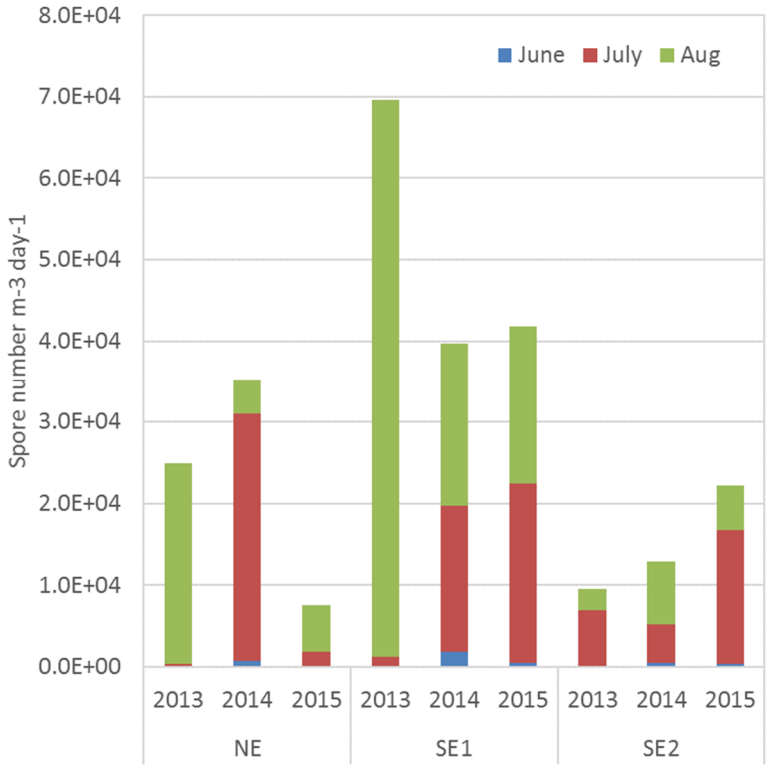


Figure 2-7 Total number of spores released per month across all sites and years

A Kruskal-Wallis test did not reveal any significant differences in the seasonal pattern of ascospore release between sites. There were however two significant differences between temporal spore dispersal profiles occurring between 2013 and 2015 at the North East site (chi-squared = 73.484, *P*=0.021) and between SE2 in 2014 and SE1 in 2015 (chi-squared = 67.231, *P*=0.017).

Over the three years of sampling 60 % of all ascospores detected were released in August and 38.5 % in July. In total *H. fraxineus* DNA was detected on 619 days out of the 828 days on which spore trapping took place. Over the days detection was made, 7.7 % of days had ascospore numbers between 10^3 and 10^4 m^{-3}/day-1, 22.7 % between 10^2 to 10^3 m^{-3}/day-1 and 40.7 % between 10 and 100 m^{-3}/day-1.

2.4.5 Daily patterns in *H. fraxineus* ascospore dispersal

A total of 20 tape segments from a 24-hour sampling period (00:00 AM to 23:59 PM) across the sporulation period between 2013 and 2014 were selected to quantify

hourly ascospore dispersal (Figure 2-8). Across all hourly samples tested, 56.8 % of ascospores detected were released between 05:00 and 07:00 AM and 36.9 % between 07:00 and 08:00 AM**.** Low numbers of spores (≤10 m^{-3}/day^{-1}) were detected over a longer time period and in some cases occurring for a full 24-hour period (data not presented).

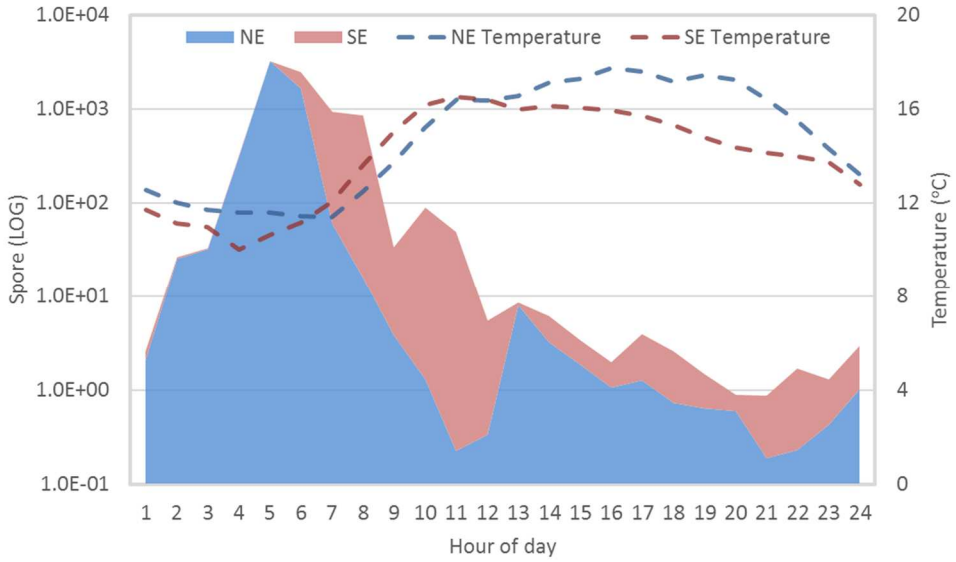


Figure 2-8 Total ascospore numbers $m^{-3}/hour^{-1}$ detected over a period of 20 days from two sites between 2013 and 2014 alongside average hourly temperature

A negative statistically significant correlation was observed between time of day and hourly spore number released (r=-0.316, *P*≤0.001 in 2013 & r=-0.239, *P*≤0.001 in 2014). Relative humidity had a significant correlation with hourly spore number in 2013 (r=0.227, *P*≤0.001 at SE) but not in 2014. Temperature was significantly correlated with hourly spore number at the North East site in 2013 (r=0.385, *P*≤0.001) and the South East site in 2014 (r=0.387, *P*≤0.001).

2.4.6 Quantification and sensitivity of detection

A set of standards was produced from samples seeded with known ascospore numbers from 10 to 10^5 and was included in each test run to enable the generation of a standard curve and trendline for quantification. An estimation of sample

ascospore number was then made by extrapolating the Ct value against the trendline. A highly reproducible linear relationship between Ct and log-concentration of ascospores was identified throughout (Figure 2-9). Sensitivity of the method was determined by testing a serial dilution of DNA extracted from 10^5 ascospores. Although detection was achieved following a five-fold dilution, a 90 % level of detection was identified after a four-fold dilution and would represent the amount of DNA extracted from 10 ascospores, producing a Ct value of approximately 33.

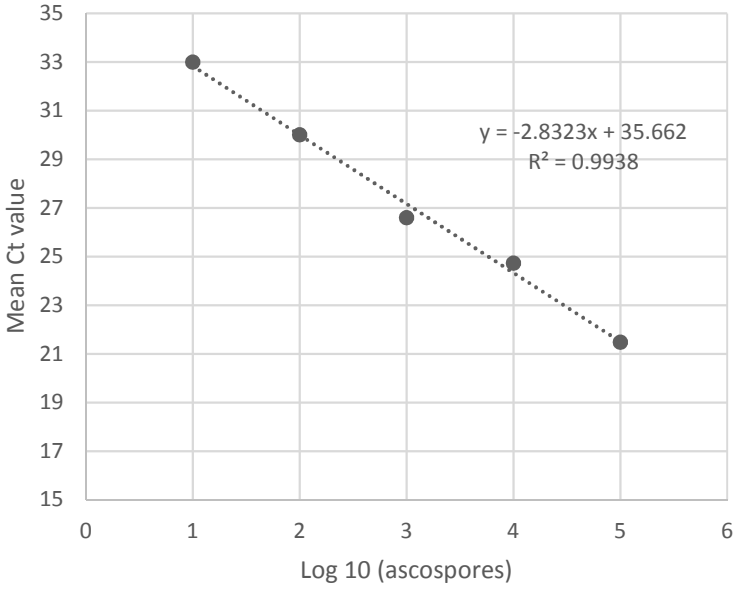


Figure 2-9 An example of a trendline produced showing the linear relationship between the log number of* H. fraxineus *spores and the real-time PCR cycle threshold value

2.4.7 Comparison between conventional spore counts and real-time PCR

Staining tape segments with cotton blue dye enabled efficient identification and counting of the hyaline *H. fraxineus* ascospores (Figure 2-10).

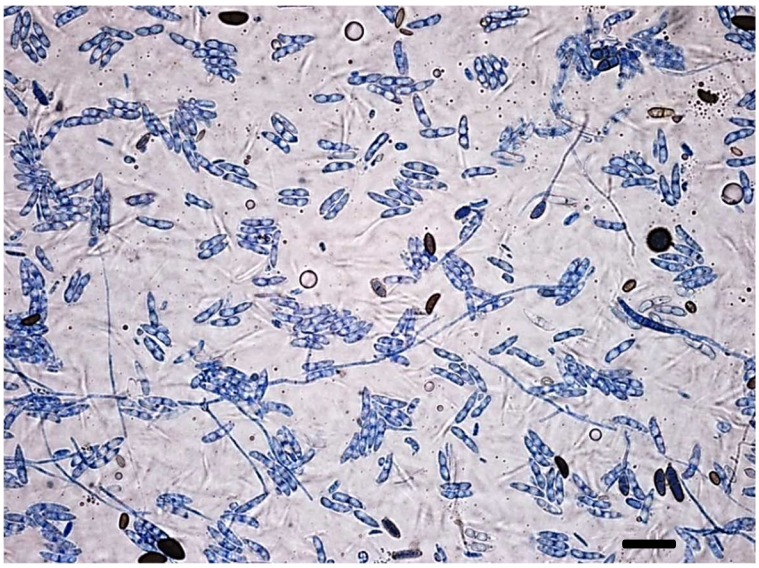


Figure 2-10 Ascospores of* H. fraxineus *impacted on a melinex tape using a volumetric spore trap stained with cotton blue and observed at 400x magnification

Spore counts were made following three transverses on each of the 26 daily segments of tape collected between 2013 and 2014. A strong positive relationship between counts and predicted real-time PCR spore numbers (R^2 = 0.897) was observed (Figure 2-11).

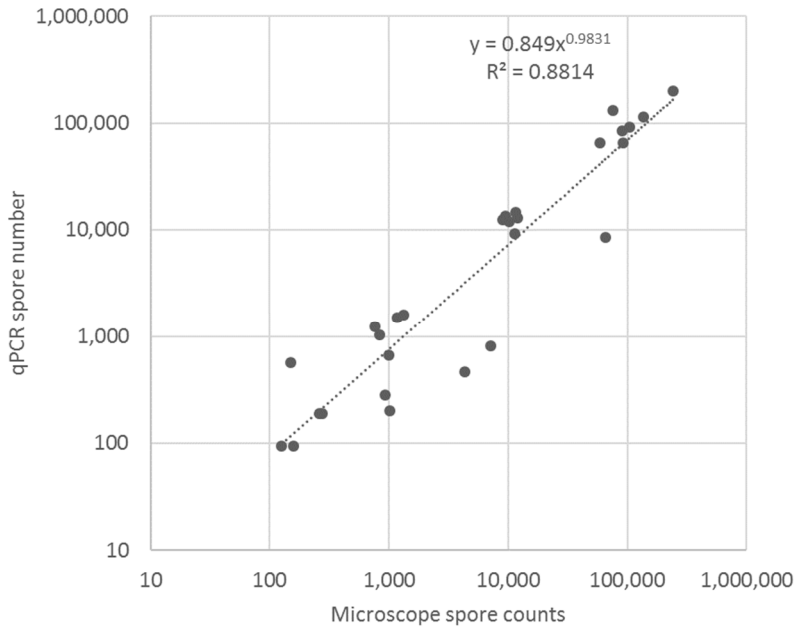


Figure 2-11 Relationship between spore counts and estimates following qPCR from 26 tape segments sampled using a volumetric spore trap

2.5 Discussion

Management of air-dispersed notifiable plant pathogens following widespread infection poses significant challenges for stakeholders and policy makers. In such cases research is crucial to the understanding of a pathogen's dispersal capability for informing disease management decisions both present and in the future. In this investigation the dispersal characteristics of the ascospores responsible for the rapid spread of ash dieback across Europe were studied in England, where European ash is the country's third most prevalent tree species (National Inventory of woodland and trees, Forestry Commission, 2001). Methods of spore trapping and quantification of air-dispersed *H. fraxineus* ascospores have previously been developed and attempts have been made to model relationships with weather variables (Timmermann *et al.*, 2011; Hietala *et al.*, 2013; Chandelier *et al.*, 2014; Dvorak *et al.*, 2015). To build upon previous research this study implemented 3 suction traps and accompanying meteorological monitoring equipment for sampling air continuously throughout the spore dispersal period over three consecutive years at two sites in England with confirmed infection. Highly sensitive qPCR was then used to quantify the concentration of DNA into spore numbers using a set of DNA standards prepared from known numbers of ascospores. Comparisons with weather variables were also made. The strong positive relationship (R^2=0.897) between qPCR spore estimates and spore counts using traditional microscopic techniques and the high sensitivity of the assay highlights the suitability of the methods for accurate and targeted measurement of pathogen spore loads in the air. The methods developed in this study are able to routinely detect DNA in spore trap samples from as few as 10 *H. fraxineus* ascospores. Comparable to methods published by Dvorak *et al,* (2015).

Overall, a seasonal episodic pattern to ascospore release was observed, with the majority of the ascospores detected in July and August each year. This timing of ascospore release has been well documented in Europe (Timmermann *et al.*, 2011; Hietala *et al*., 2013; Chandelier *et al.*, 2014; Dvorak *et al.*, 2015). Results from hourly tests indicate over 90 % of spores detected in the hourly samples occurred over a 2 to 3 hour period between 05:00 and 08:00 AM. Similar observations were

made in Norway (Timmerman *et al*., 2011; Hietala *et al*., 2013). Timmerman *et al.* (2011) suggested ascospore maturation occurs primarily at night when air humidity is high and release spores in the presence of a morning dew to prevent desiccation and increase chances of germination and infection. Results from this study indicate the majority of ascospores released when humidity was high predominantly 100 % and temperatures were at a daily low but on the rise. A study carried out by Brook (1969) identified larger numbers of *Venturia inaequalis* ascospores are released in light compared to the dark. A measure of light levels was not monitored in this study but sunrise at the site in the South East would have occurred approximately 10 – 20 minutes later than the North East. Results from this study show the earliest ascospores released were detected in the North East between 01:00 and 02:00 am. In contrast the earliest ascospores were detected in the South East was between 03:00 and 04:00 am. This suggests sunrise could be a significant contributing factor to the initiation of *H. fraxineus* ascospore release.

Frequent detections of *H. fraxineus* ascospores were made with 10 to 10^2 ascospore m^{-3}/day^{-1} on 40.7 % of the days when sampling took place. As the suction trap was positioned at a height of 1 m this indicates ascospores readily escape the relatively calm woodland floor boundary layer and do not necessarily rely on wind to facilitate dispersal as only two site specific correlations were identified between spore release and wind speed. Assuming trees require a high dose of spores to become infected (Timmermann *et al*., 2011), then during low spore dispersal periods infection will most likely occur on nearby ash tree leaflets contributing to a build-up of inoculum year on year until the source of low level ash branches and associated leaf foliage is depleted. During the weekly site visits in the North East bait plants closest to high volumes of apothecia frequently failed to produce leaflets in the second year and severe stem discolouration was observed indicating successful infection had been caused by *H. fraxineus*. Furthermore, severe trunk necrotic lesions could be observed on a number of ash trees in the immediate area yet their canopies were still able to produce leaf year on year.

Necrotic leaf symptoms caused by *H. fraxineus* were primarily observed towards the end of August on the baited ash tree leaflets 3-4 weeks post primary peak ascospore release activity. In 2014 symptoms were observed earlier in the

middle of August and is likely to have been initiated by the high numbers of ascospores released towards the end of July in comparison to other years.

Days when large numbers of ascospores (>10^3 m^{-3}/day^{-1}) were detected were less frequent over the 3 years, occurring on approximately 8 % of the days sampling took place. The site which had the most days when large numbers of spores were released was SE1. An accurate measure of infection at the woodland sites could not be taken as many trees were mature and well above visual inspection levels. The large release events were usually characterised by an increase in spore activity before and after the main release. Similar findings were highlighted in the Czech Republic with spore release occurring over a period of 1-2 days (Dvorak *et al*., 2015). The significant concentration of airborne inoculum recorded on these days could facilitate fresh infections at greater distances from the source, in particular in the North East in 2013 and 2014 where wind speed was significantly correlated with spore counts.

The large variations in numbers of ascospores detected per day across the 3 sites reflect similar findings in other spore trapping studies carried out in Europe. The highest number of spores detected in a single day in Norway was approximately 4.7 x 10^6 spores m^{-3} of air (Timmerman *et al.,* 2011) compared to 5.4 x 10^5 m^{-3} spores in Belgium (Chandelier *et al.*, 2014) and 4.83 x 10^{11} m^{-3} spores in the Czech Republic (Dvorak *et al*., 2015). These numbers suggest higher levels of disease were present at these sites at the time of air sampling in comparison to the UK sites where the highest number recorded on a single day was approximately 6.9 x 10^4 at SE1. This could be directly related to the time infection has persisted in the different woodlands where the studies have taken place and how favourable local climatic conditions are for facilitating the life cycle of *H. fraxineus*. The UK is the most recent country out of these to have the presence of the pathogen confirmed so could be considered at an earlier cycle of infection.

Temperature was the most closely correlated weather variable with spore counts. The highest concentrations of inoculum occurred when average monthly temperatures exceeded 16 °C. Hourly testing indicates rising temperatures between 10 and 14 °C shortly after the daily minimum were optimal for release.

Statistical analysis did not indicate any significant differences in the seasonal pattern of when ascospores were released between the three spore traps within the

same year. This is likely because *H. fraxineus* spores were detected on the majority of days spore trapping took place when not taking into account the number of spores released on those days. There was however one statistically significant difference in temporal spore release between 2013 and 2015 at NE1. The number of detections made at NE in 2013 was considerably low compared to 2014 and 2015 and furthermore compared to all other sites and years. Factors governing the delayed spore release in 2013 could be accounted for due to the low average monthly temperature in June and reduced frequency of days with recorded rainfall in July. It was noted at the site in the North East how apothecia maturation was progressing in July 2013 however their vigour faded following consecutive days when no rainfall was recorded towards the end of the month. Apothecia then recovered during August resulting in 3 distinctive spore release periods when rainfall became more frequent. As a result, larger numbers of spores were released when conditions became favourable. The same pattern can be seen at the sites in the South East in 2013. In general, it is understood that temperature and water availability affect the growth of the fruiting structure and therefore the inoculum potential (Jones and Harrison, 2004). Dvorak *et al*., (2015) indicate high relative air humidity is important for apothecium development and high leaf wetness enhances ascospore release 11-12 days after apothecia have matured. In 2014 and 2015 rainfall was more frequent throughout the summer presumably providing more favourable humid and wet conditions for uninterrupted apothecial maturation and active ascospore release compared to 2013. June and July are therefore important months in apothecial maturation of *H. fraxineus* and the localised weather conditions across this period can have a significant influence on the timing and extent of ascospore release.

Although the development and release of ascospores is highly synchronised primarily to the early hours of the morning between July and August predicting the release would prove difficult due to the variability in correlation between spore numbers and weather variables monitored in this study. Visual observations made while visiting the trapping locations noted apothecia at varying stages of development. Additional factors such as microclimate will have undoubtedly influenced the nature of apothecial maturation and spore release. Forest canopies have a major impact on microclimates such as temperature and solar radiation in

summer due to canopy development (Morecroft *et al*., 1998). Gross (2012) suggests near UV-light exposure is necessary for *H. fraxineus* apothecial development. Unfortunately, a measurement of UV was not taken, but it is a factor that is likely to be highly variable, influenced by the high dieback of the woodland canopy and subsequent development in ground flora due to increased light penetration. Rainfall is a common trigger for spore release in many ascomycetes providing moisture to enable the build-up of turgor pressure within the ascus and mechanical force from the impact of the droplet alone. Gottwald (1997) demonstrated active ascospore release by a number of ascomycetes occurred a few minutes after simulated rain and numbers reduced as the surface dried out but with no effect of red/infrared radiation. In a woodland environment, light showers may not impact beyond the forest canopy whereas heavy or even light sustained rainfall can lead to continual wetting on the woodland floor beyond the rainfall event itself. For a more accurate measure of rainfall in a woodland environment a rain gauge would be more appropriate than using local met station data. It's clear a number of conditions primarily temperature and moisture drive apothecial maturation and ascospore release. To understand the relationship in more detail measurements would need to be made closer to the woodland floor and at multiple locations within the same woodland due to the complex and changing microclimate environments that exist. Additional measurements such as leaf wetness and soil temperature may be able to provide more accurate correlations with spore release.

The early warning of spore release at infected sites or detection of viable spores has potential to facilitate more timely disease management strategies. Future predictive models for notifiable woodland biosecurity threats such as *H. fraxineus* need to take into account factors such as the large variation in climate within woodland ecosystems and incorporate variability associated with apothecial maturation. This work has demonstrated traditional spore traps in conjunction with molecular detection can provide detailed high-resolution data with regards to the influence of biotic factors on spore dispersal characteristics.

In the future further, technological advances in spore traps and detection techniques could deliver autonomous or in-field detection of spores. This would lead to a considerable reduction in the amount of laboratory testing that would be required

to provide continual surveillance of woodland biosecurity threats. As a result, such technology could potentially provide a result much sooner which can be acted upon rather than awaiting a laboratory based test result. Such data could then be used to identify the source and potential deposition of spores as a result of air parcel movement by combining spore trap results with atmospheric transport models such as HYSPLIT (Hybrid Single Particle Lagrangian Integrated Trajectory Model; Draxler, 1998) and IAMS (Integrated Assessment Modelling; Isard, 2005). Such data can be used in the development of pest risk maps for directing disease management decisions.

Author contributions

Conceptualization: IB JT

Data curation: IB MT

Formal analysis: IB MT

Funding acquisition: JT

Investigation: IB

Methodology: IB JT

Project administration: IB JT

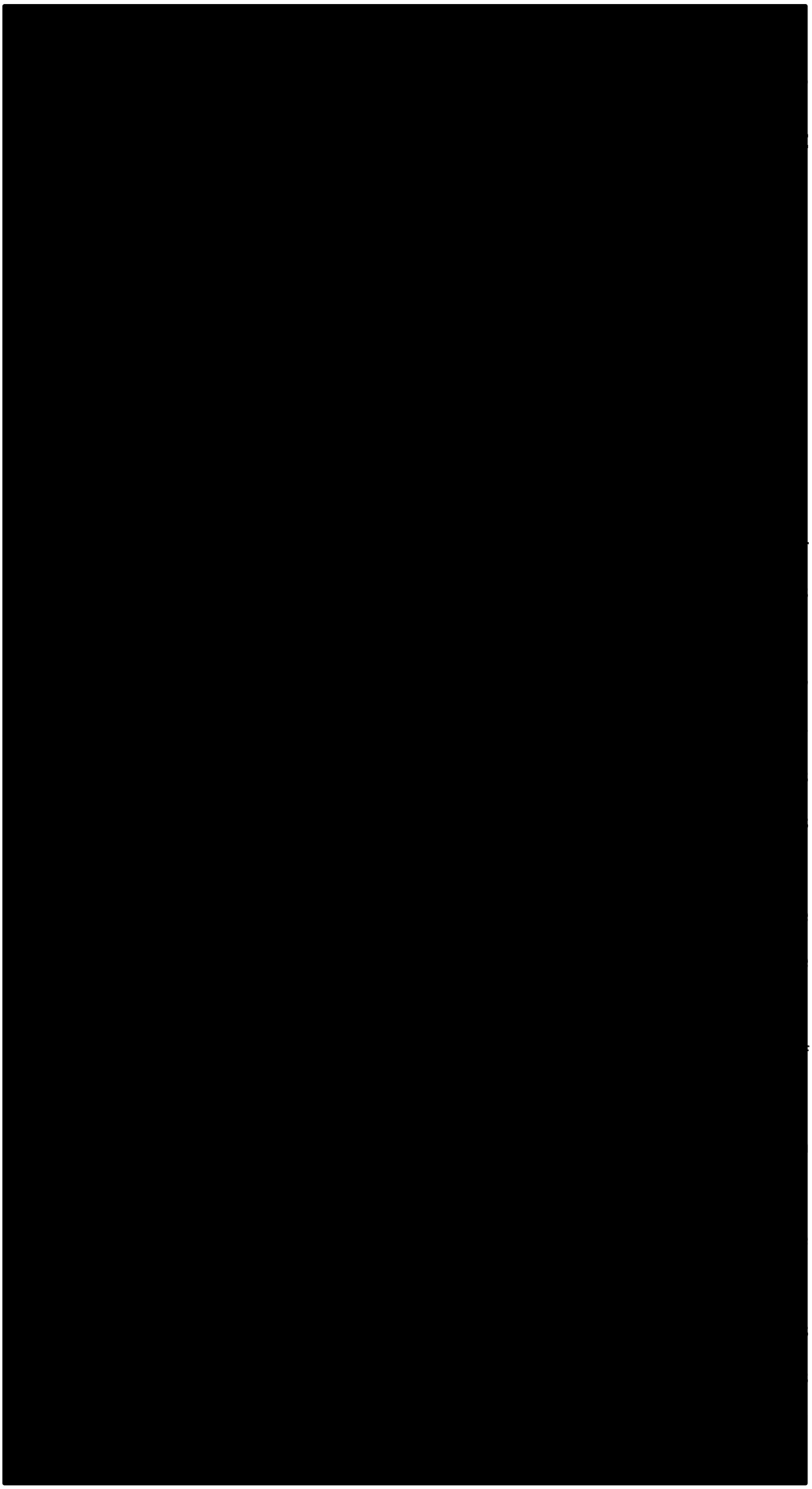
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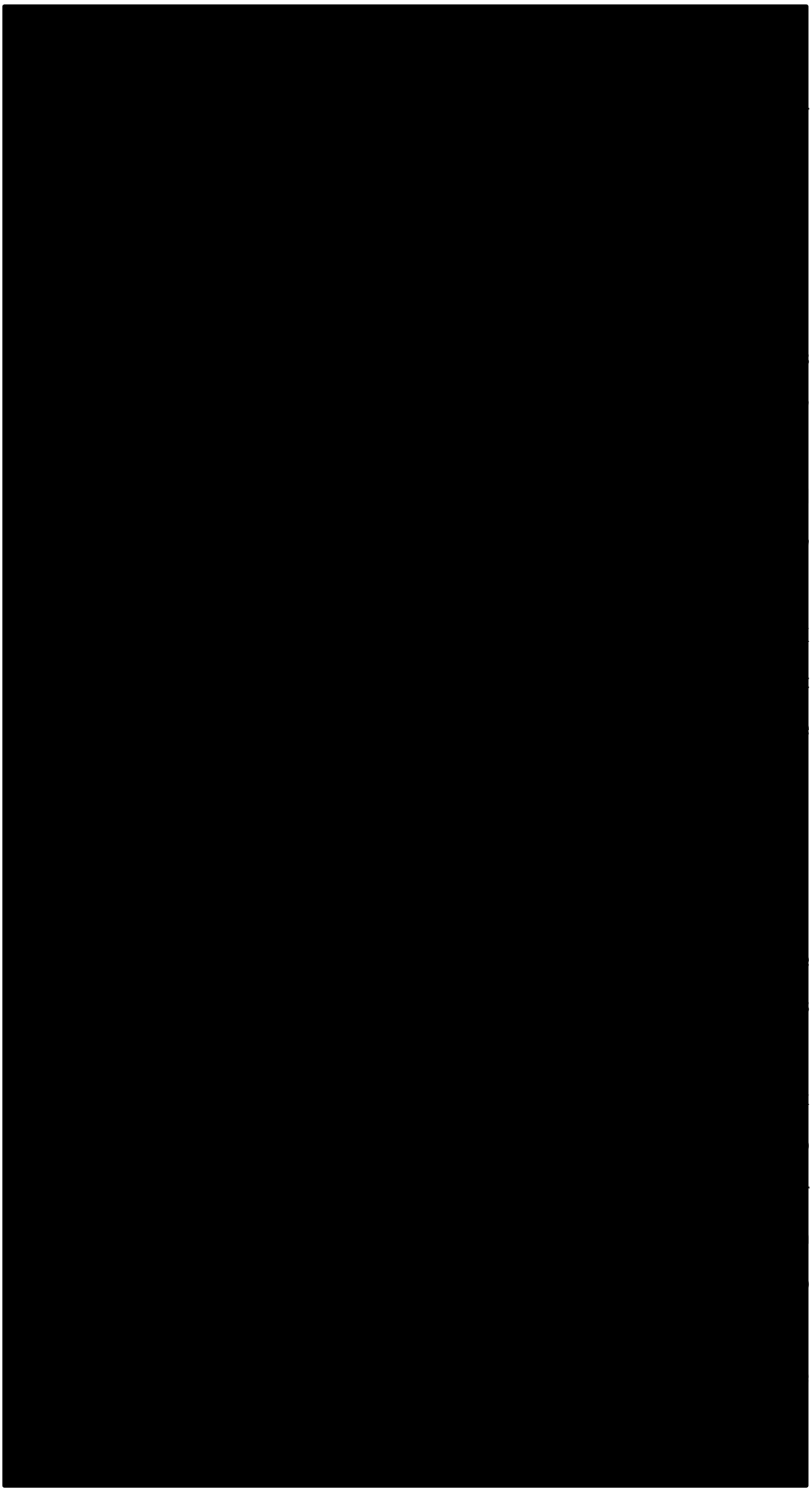
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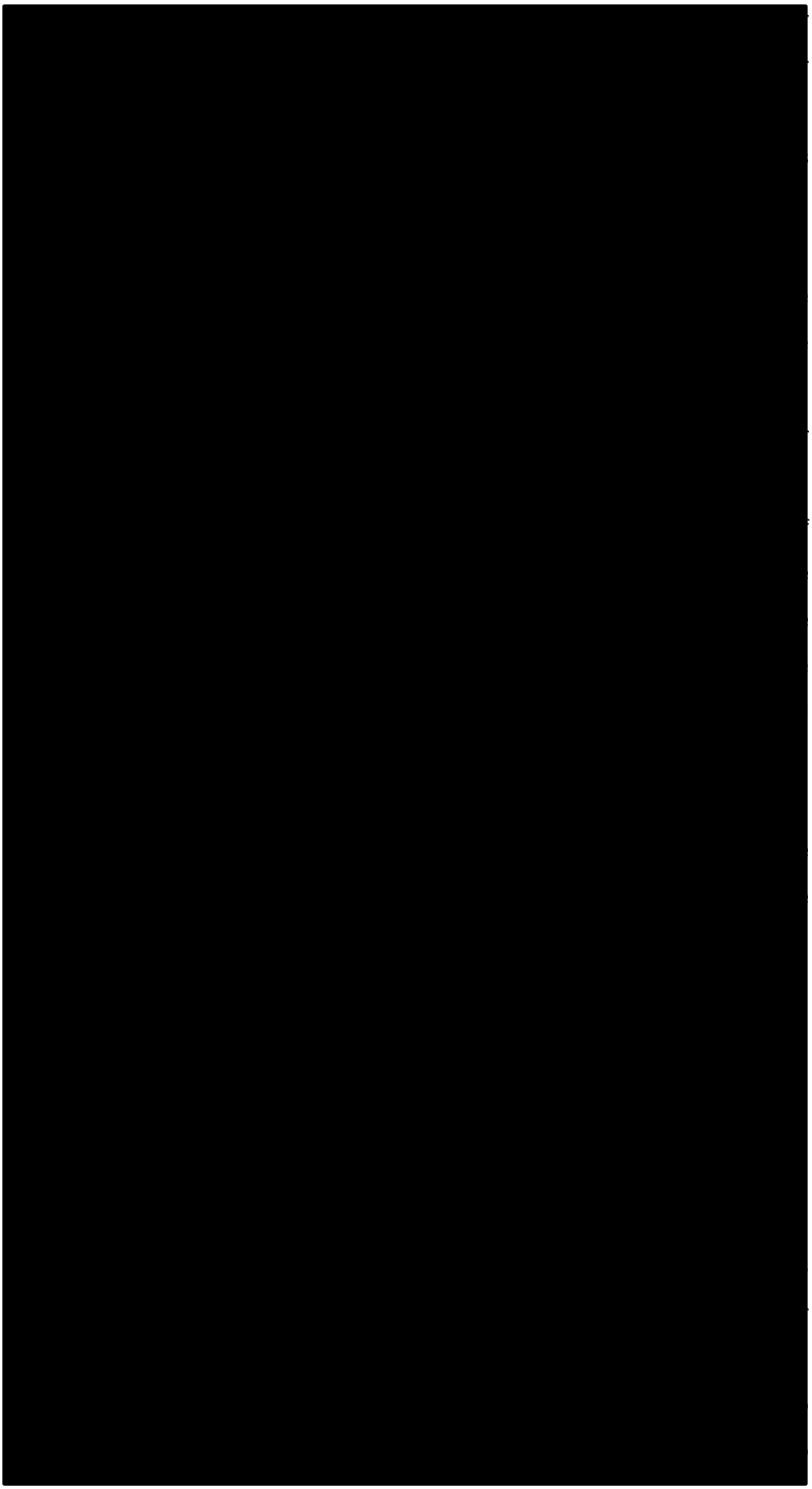
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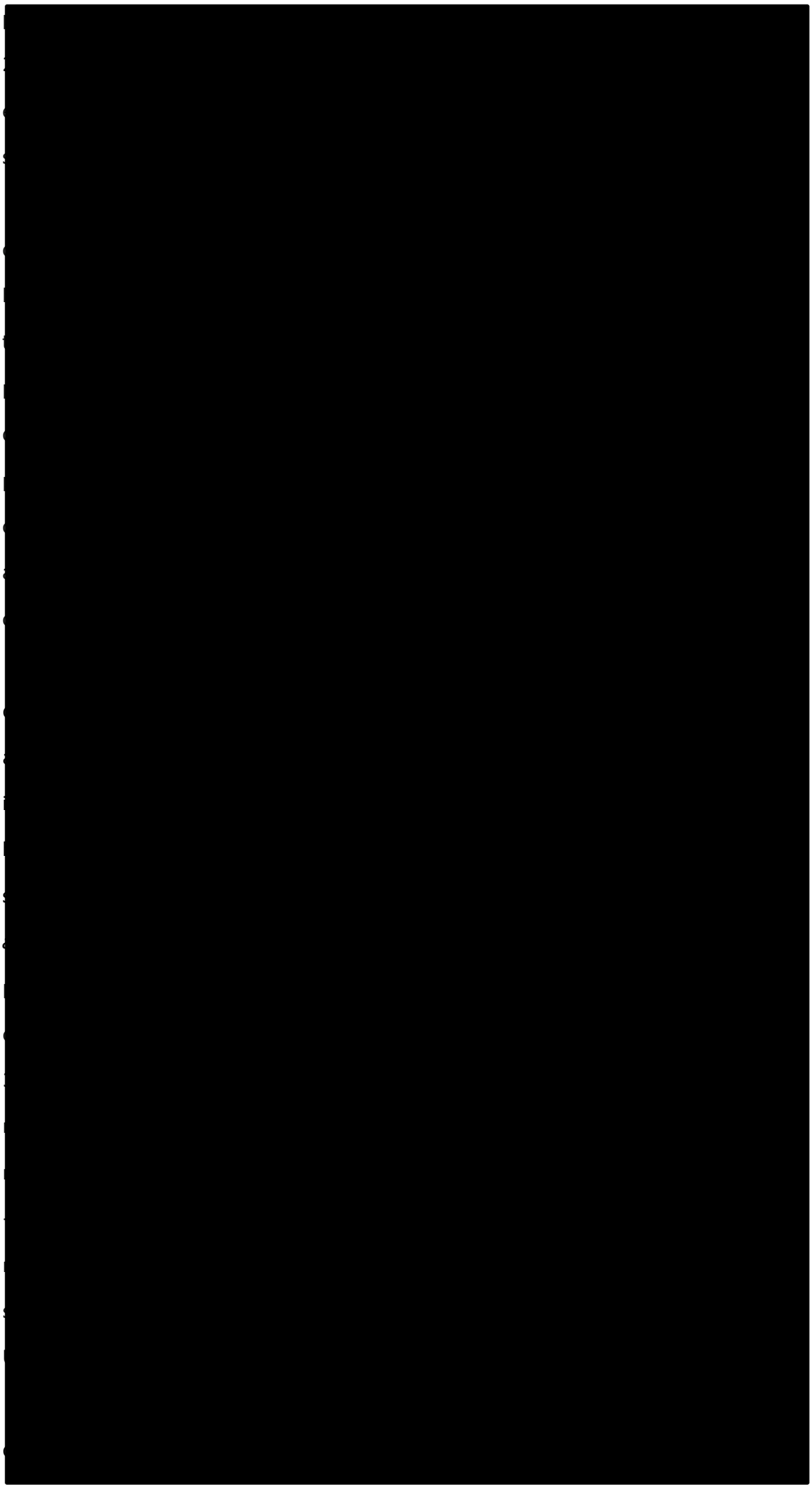
Writing – original draft: IB

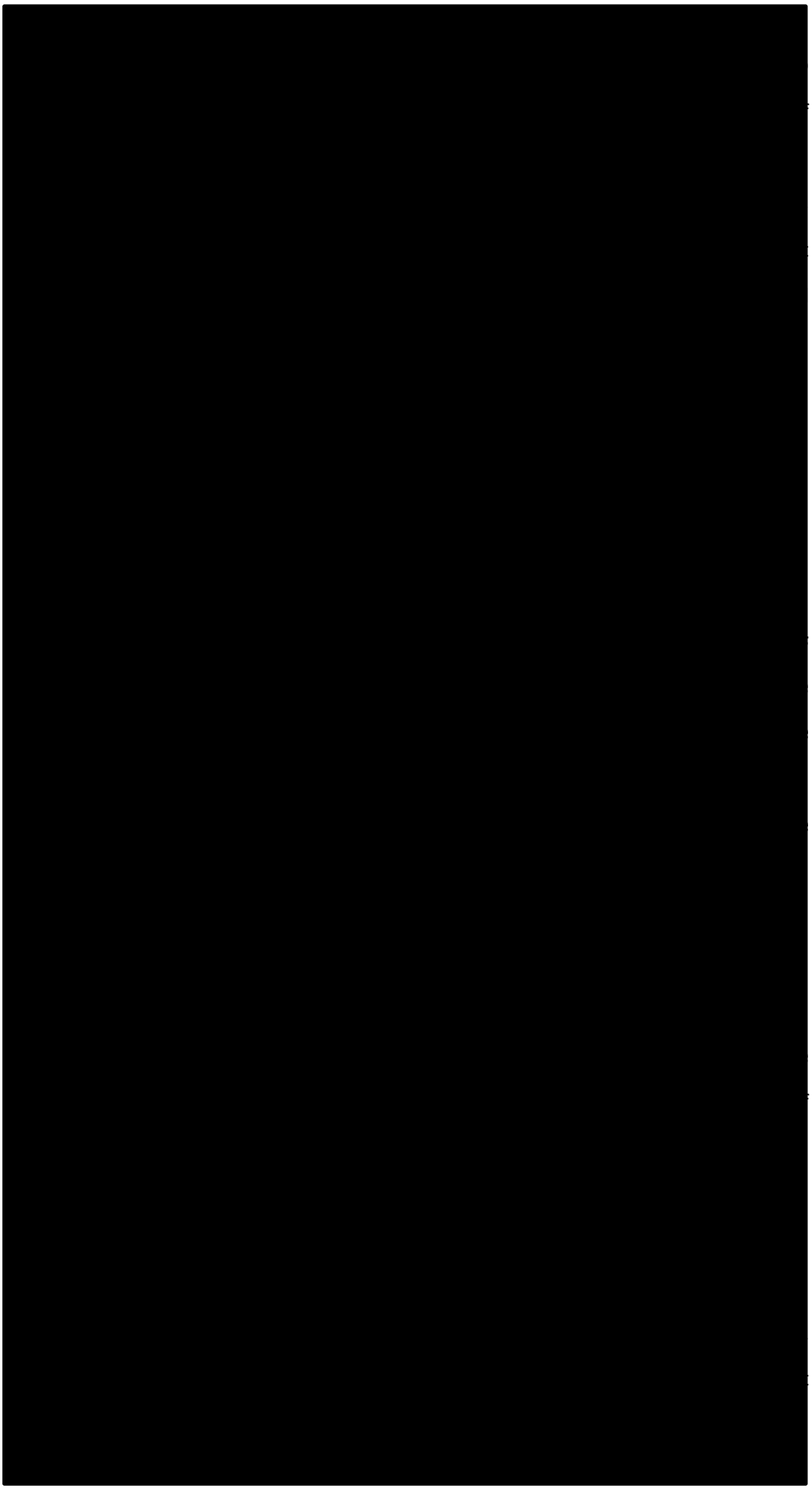
Writing – review & editing: IB JT MT NB MD

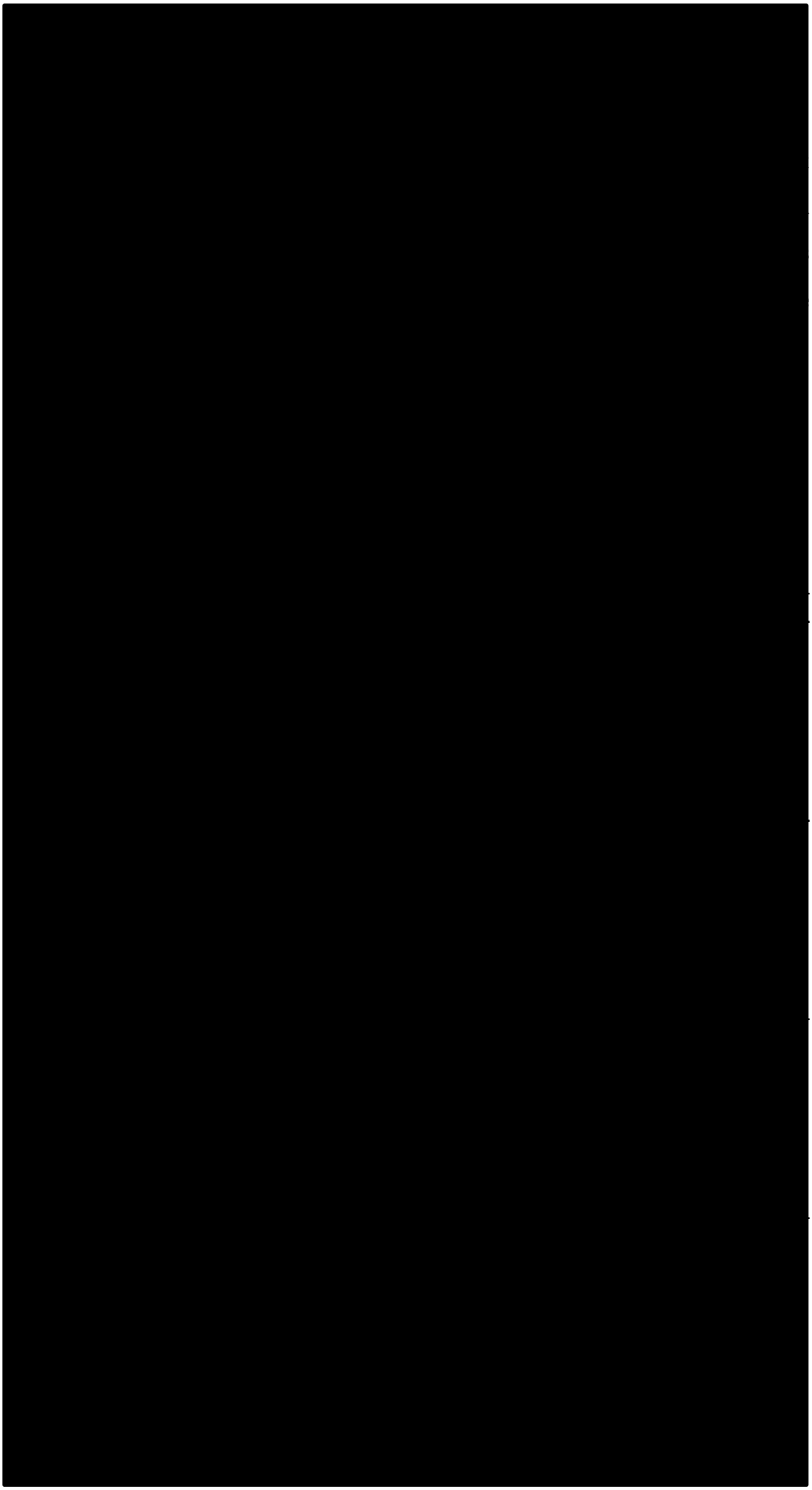


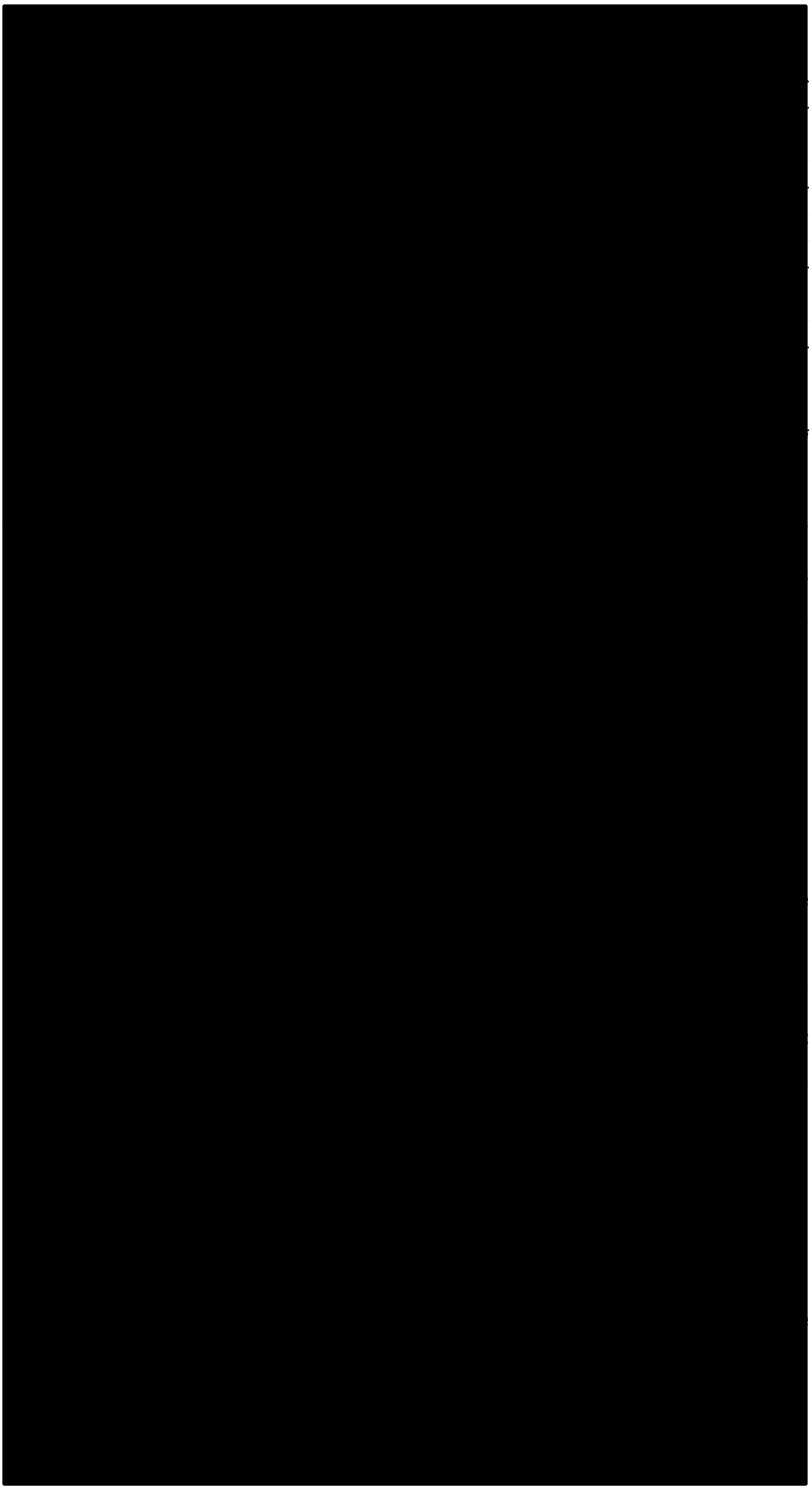


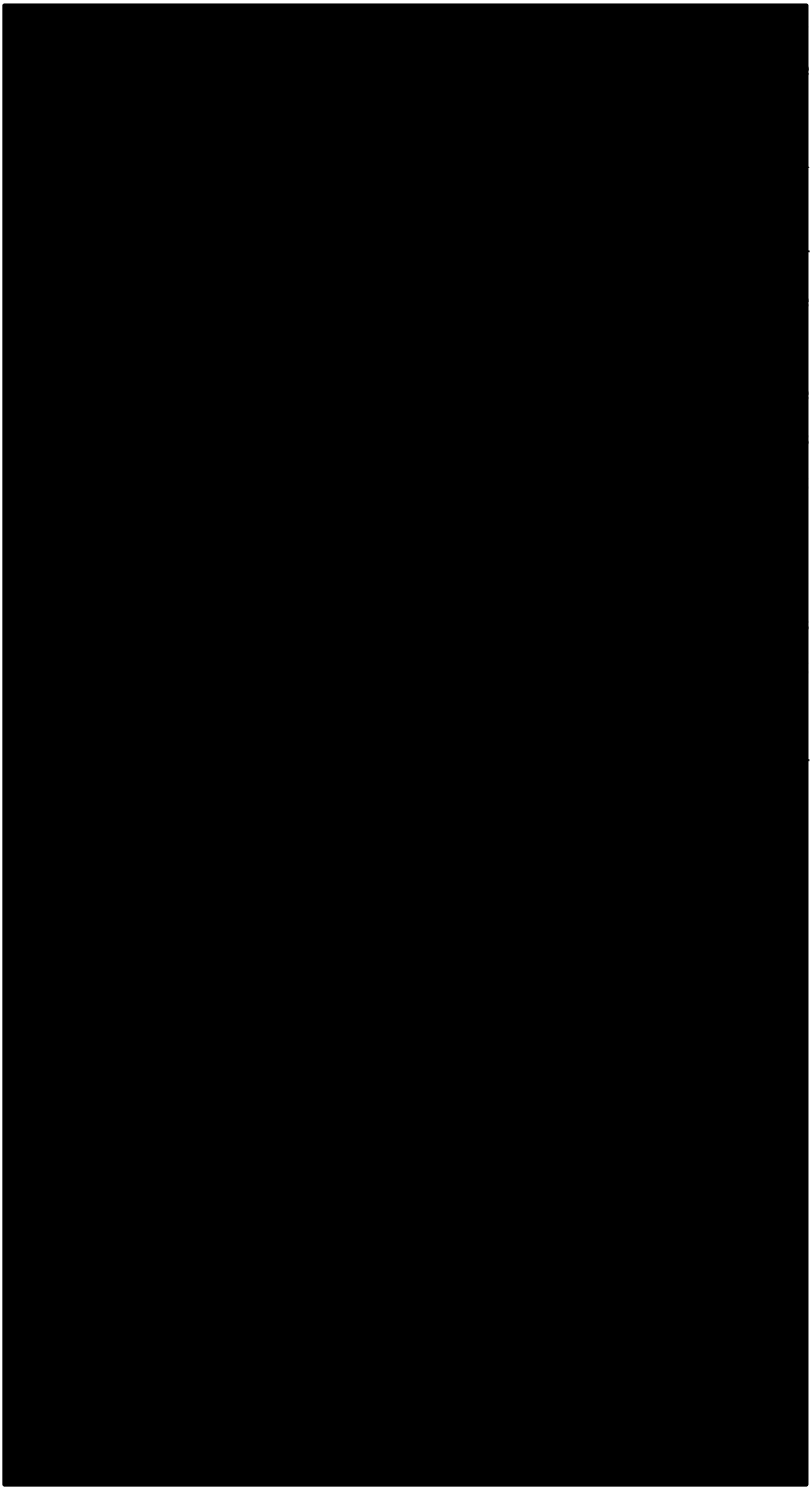


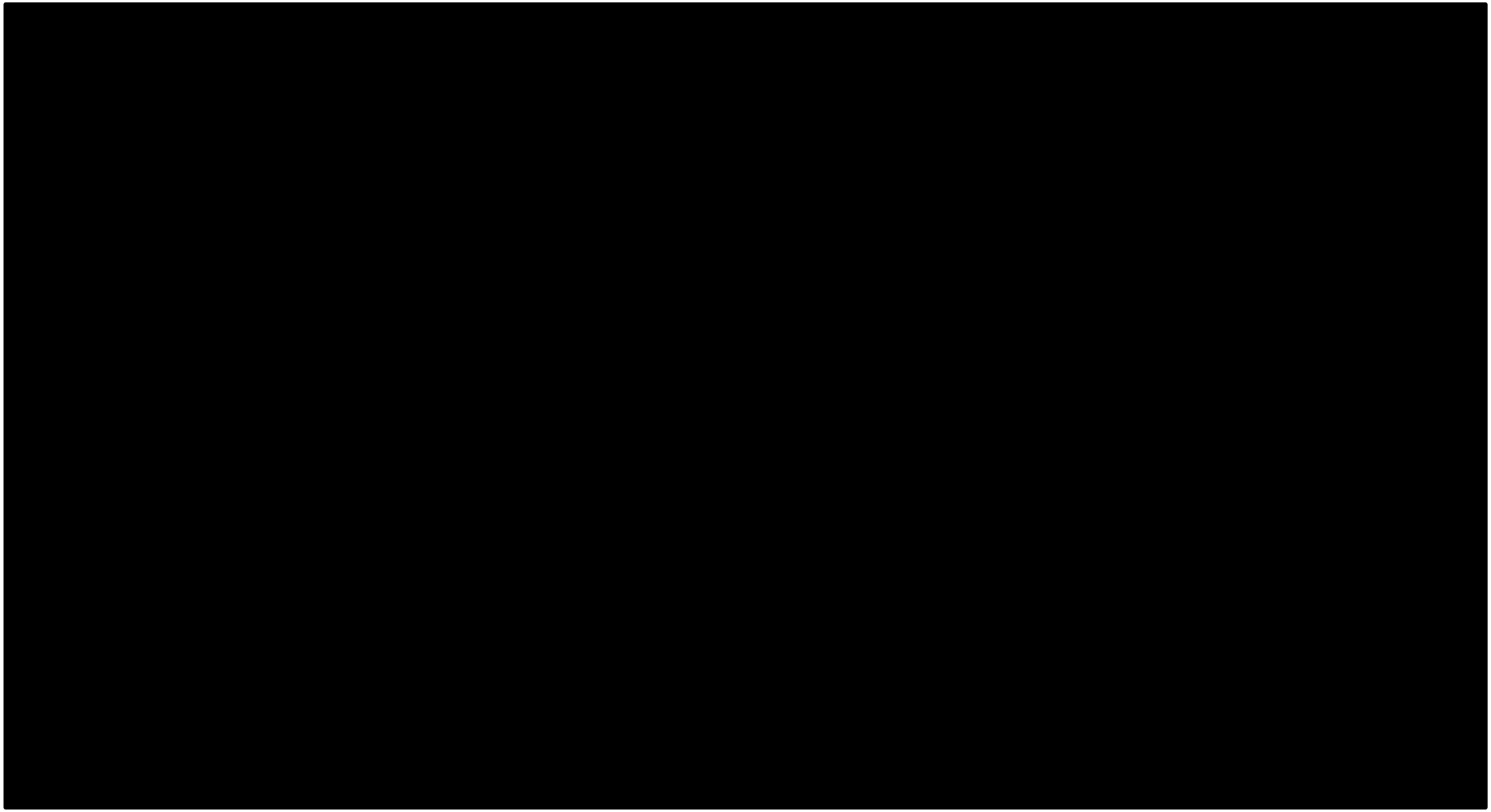


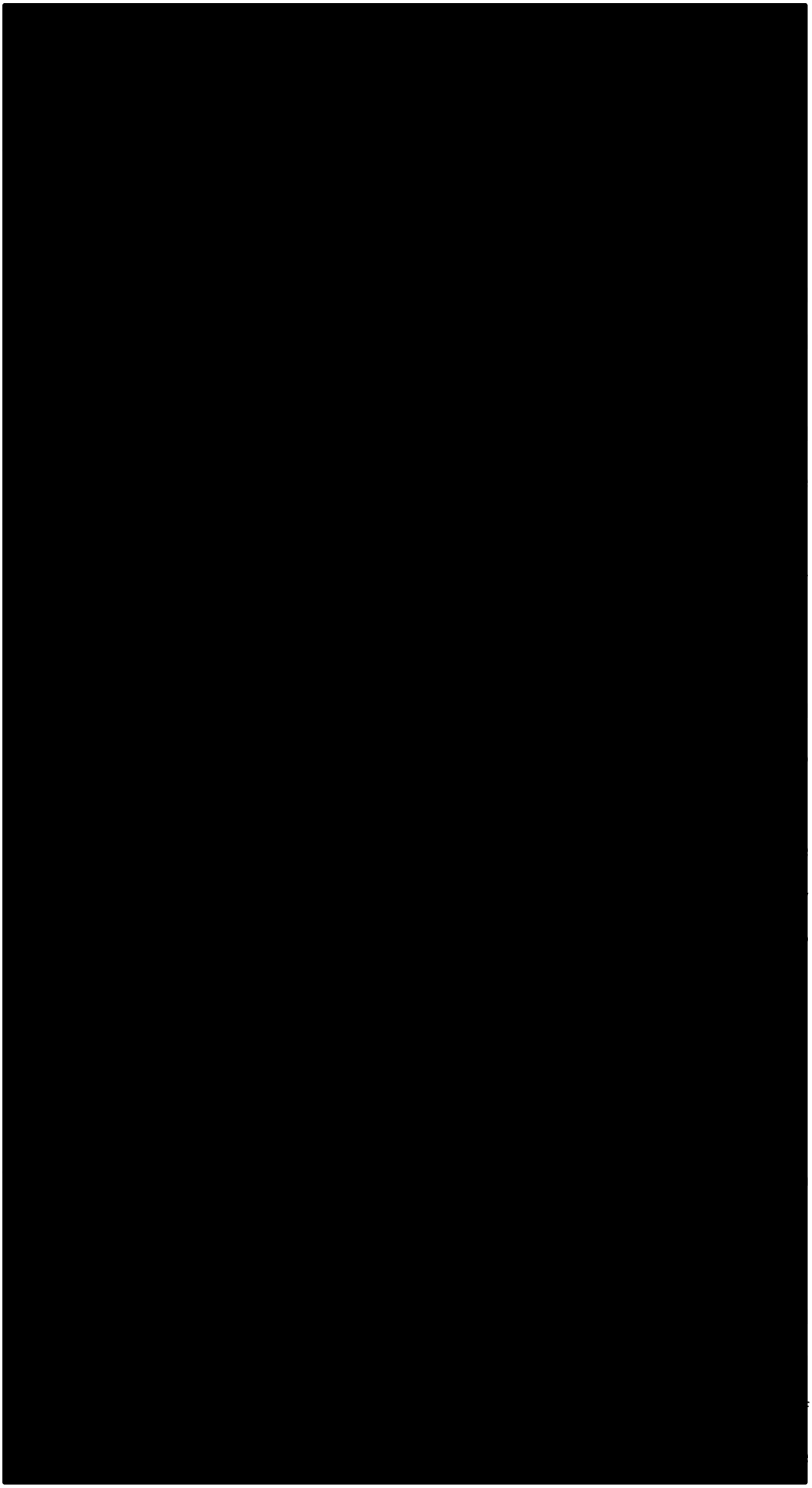




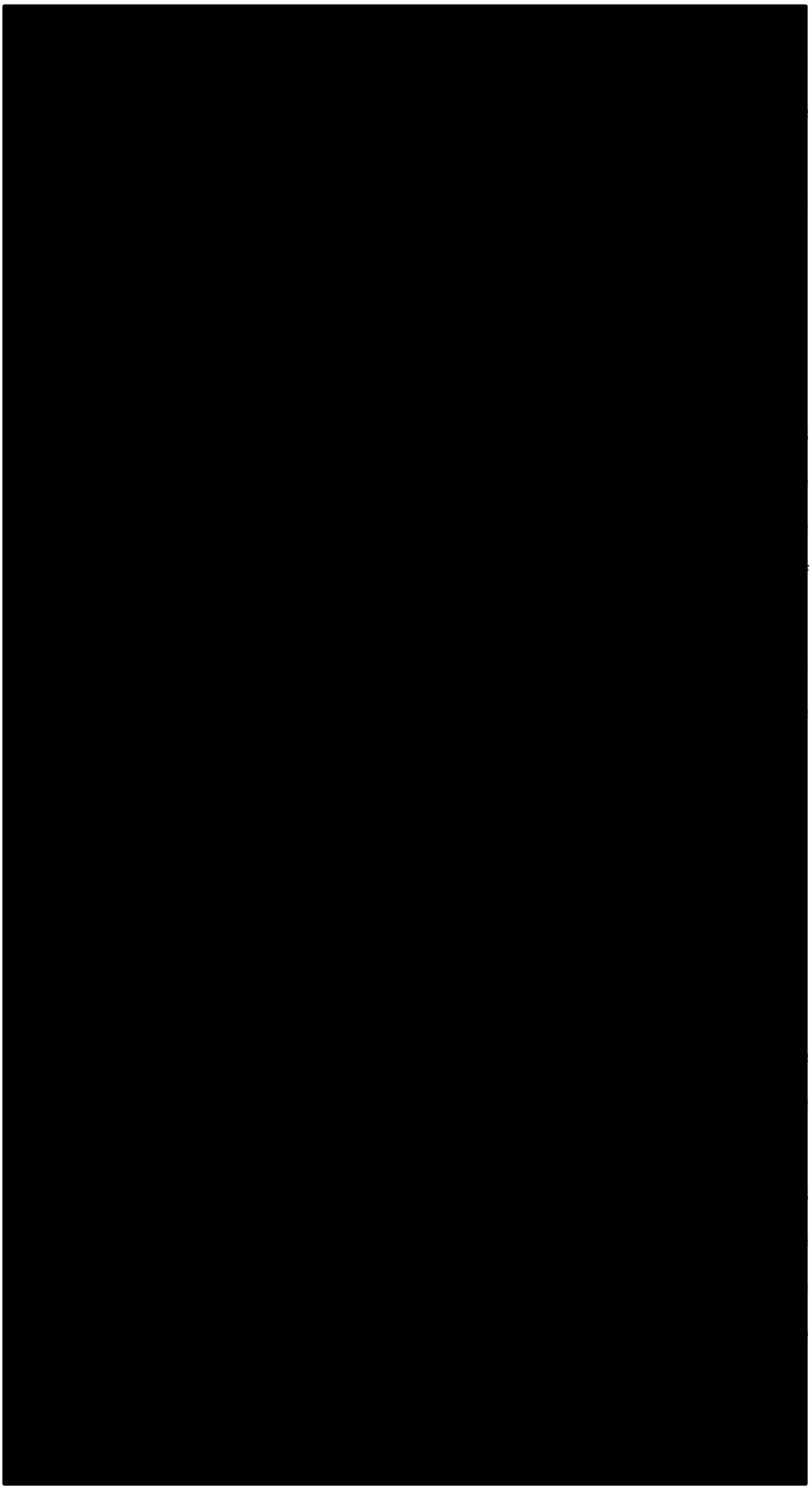


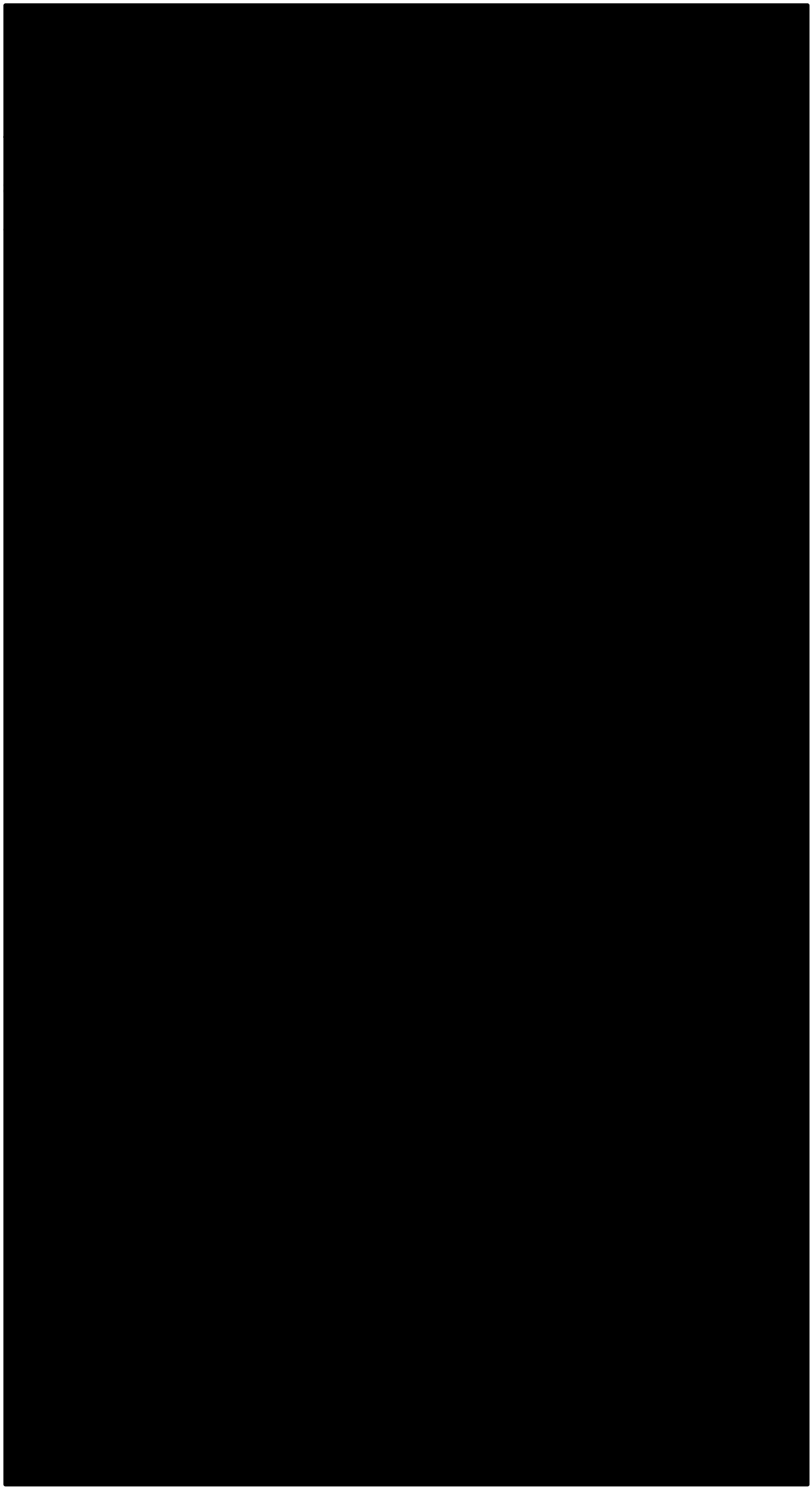




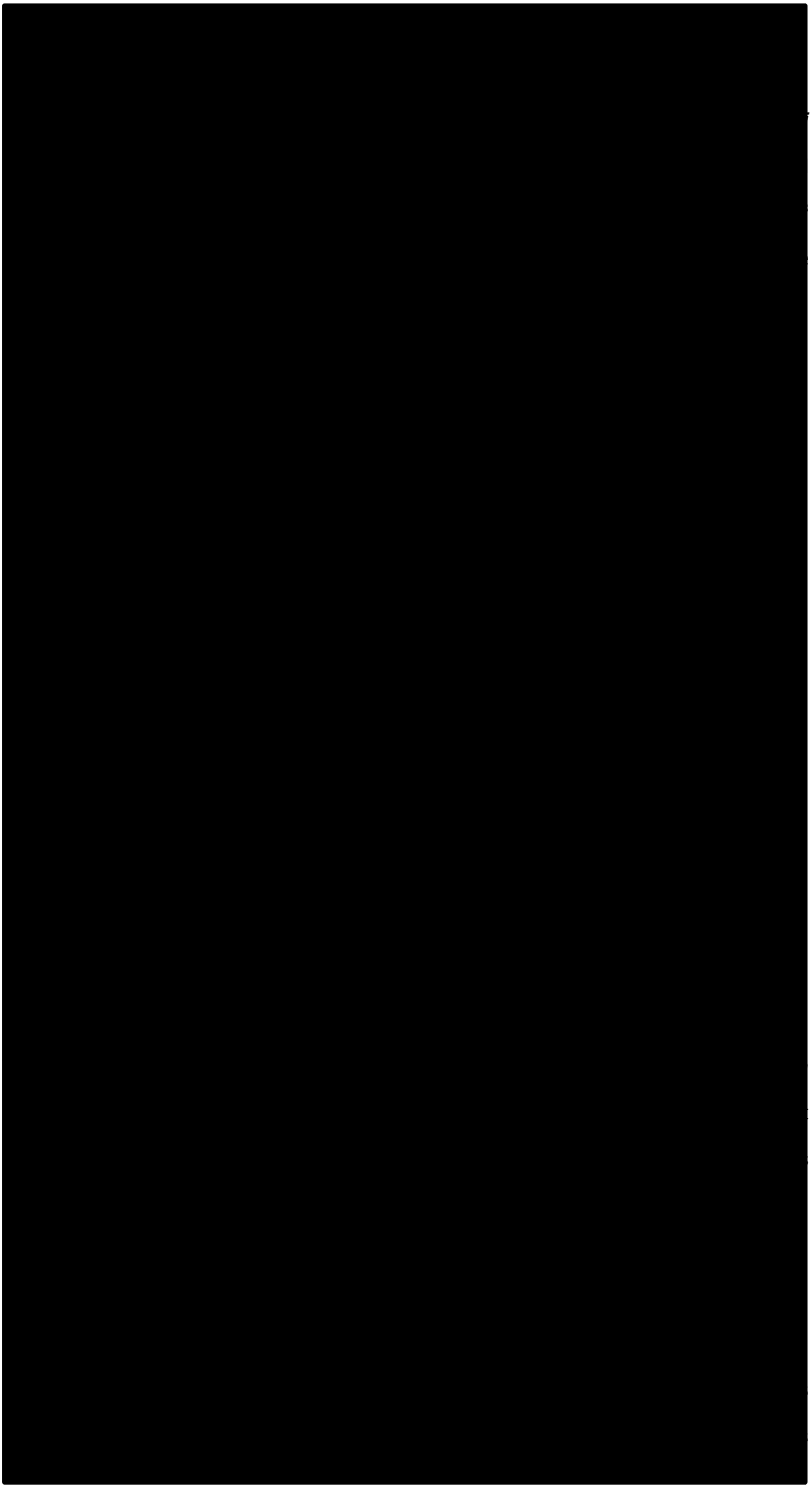


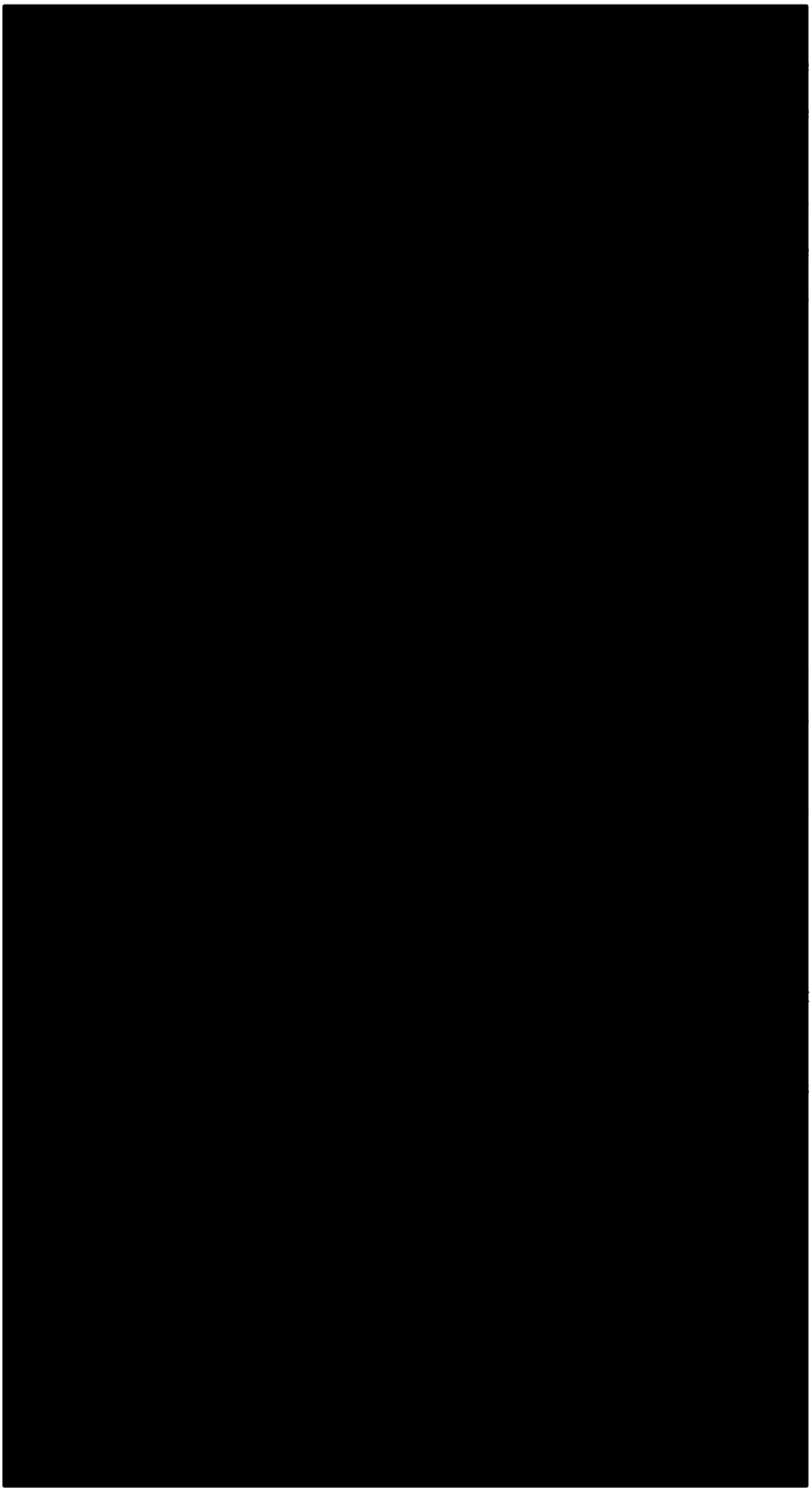
trap was positioned at a height of 1.5 meters. Samples were taken the first week in

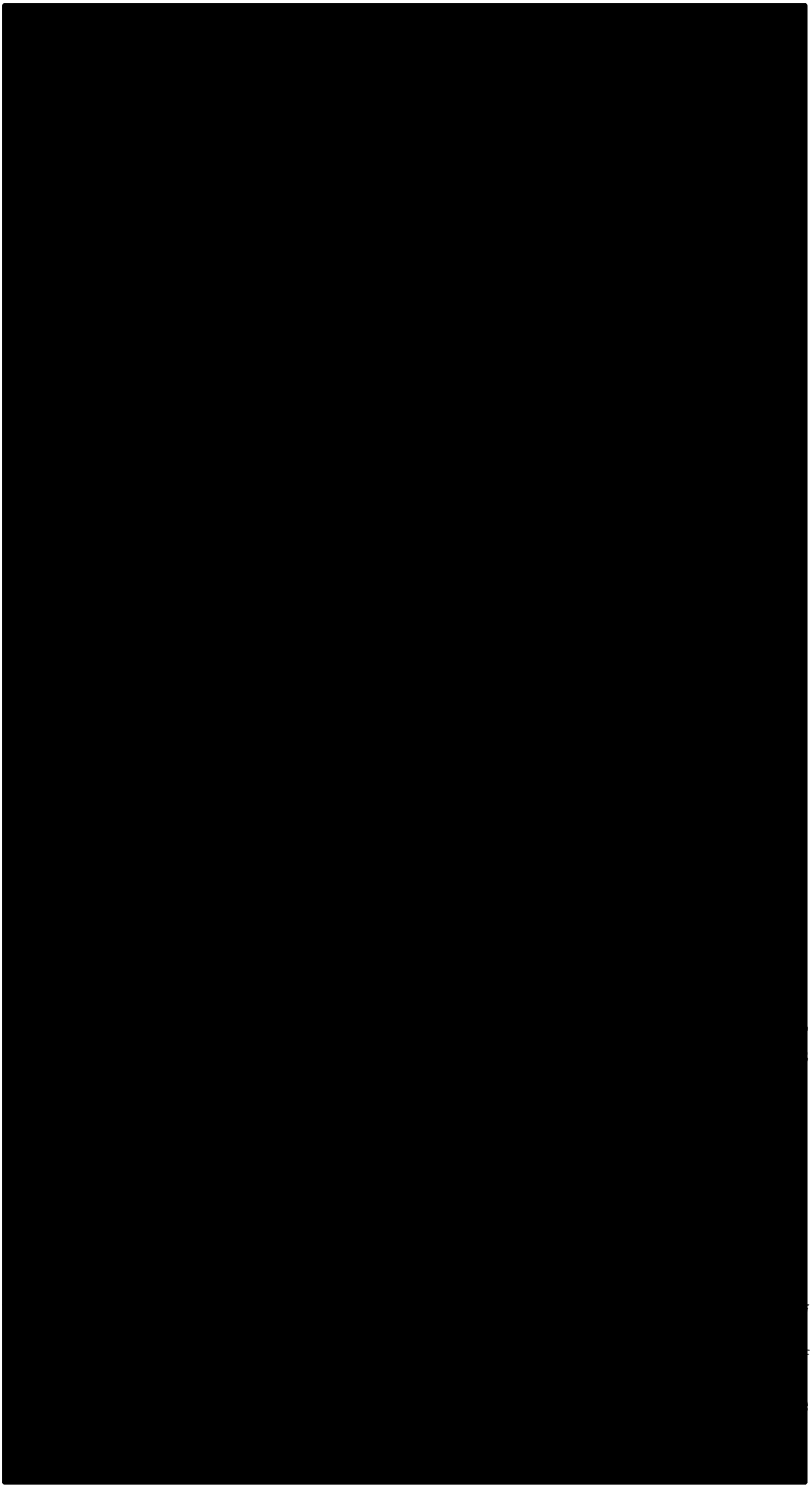


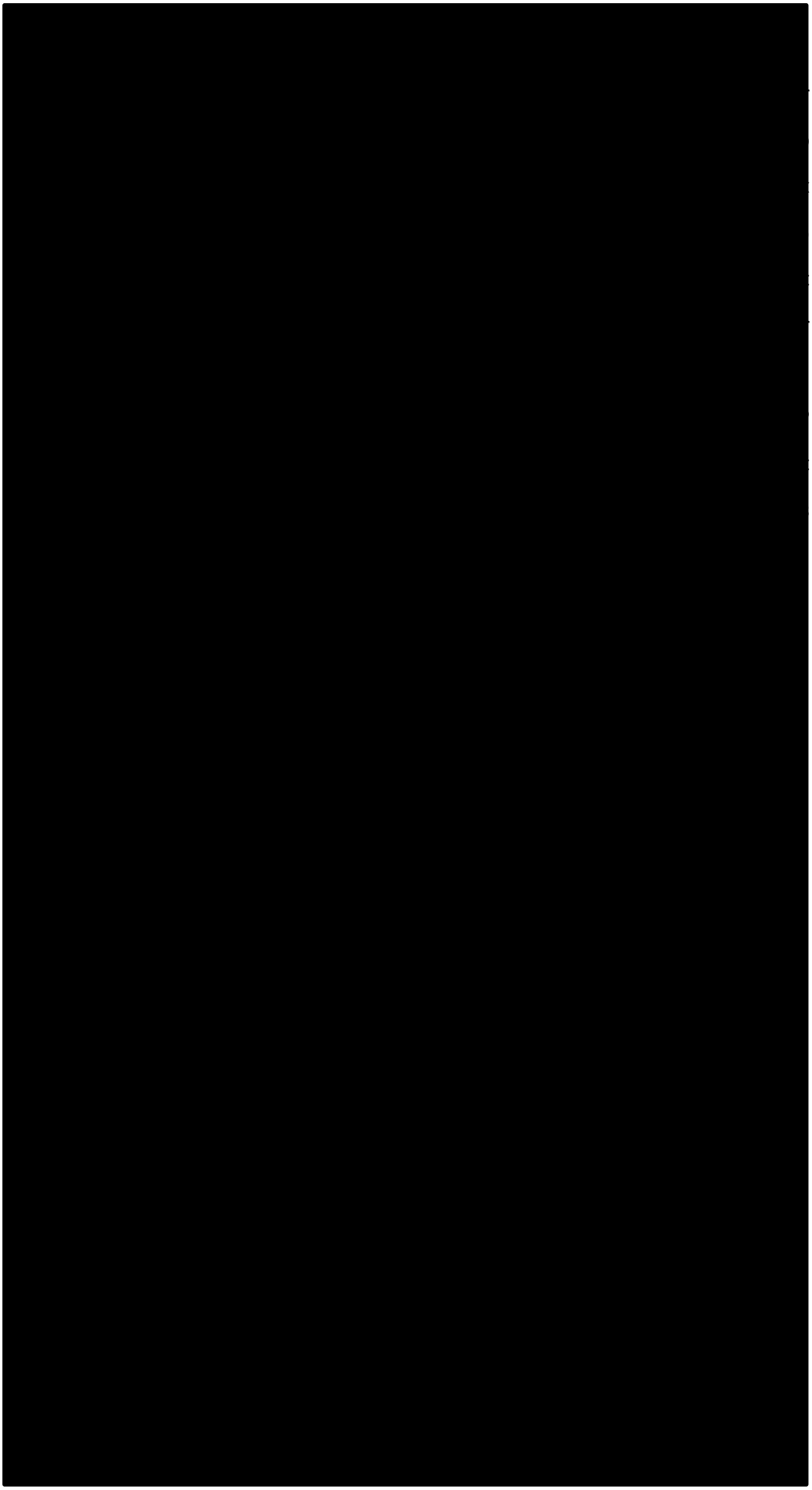




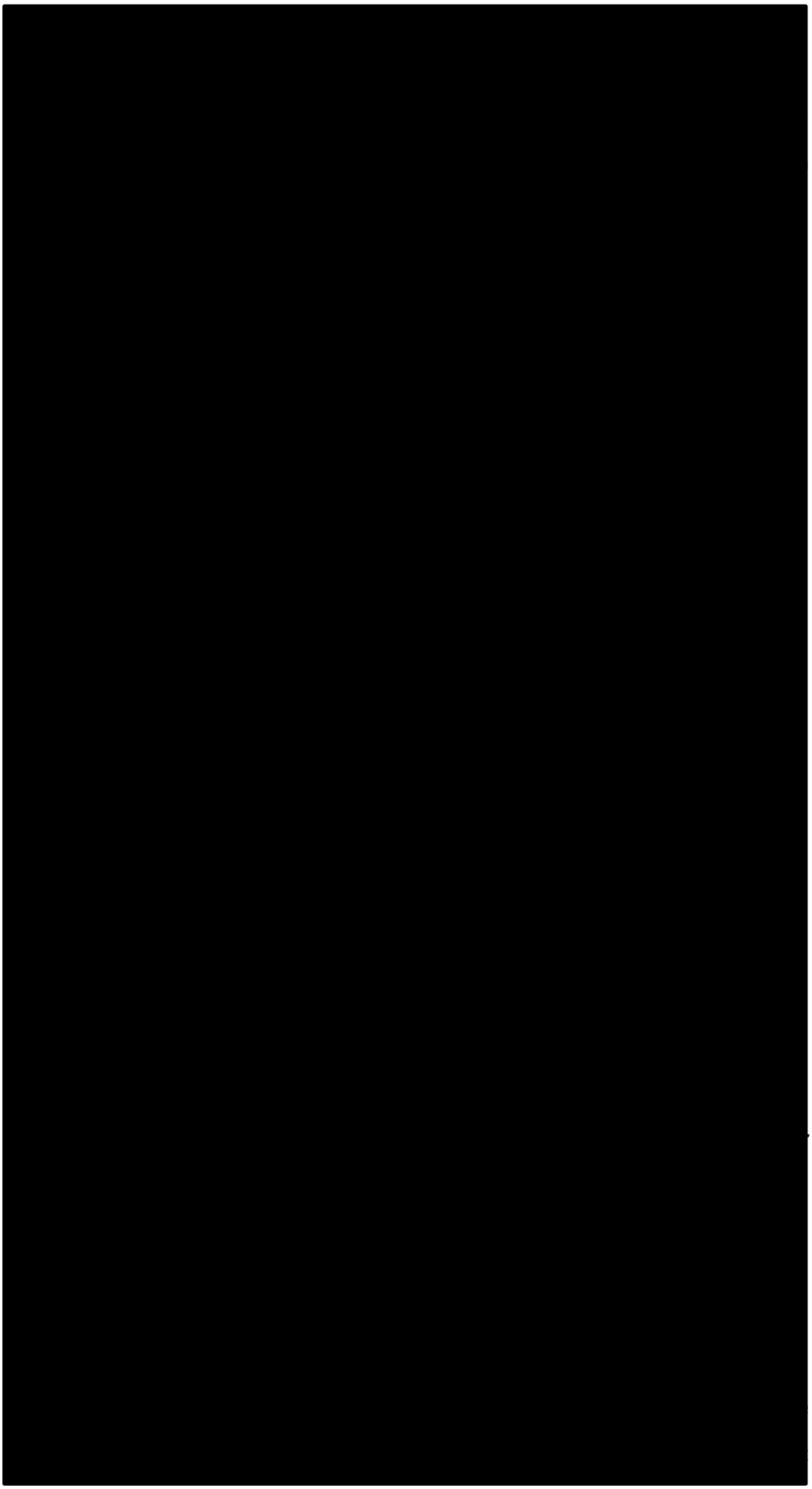


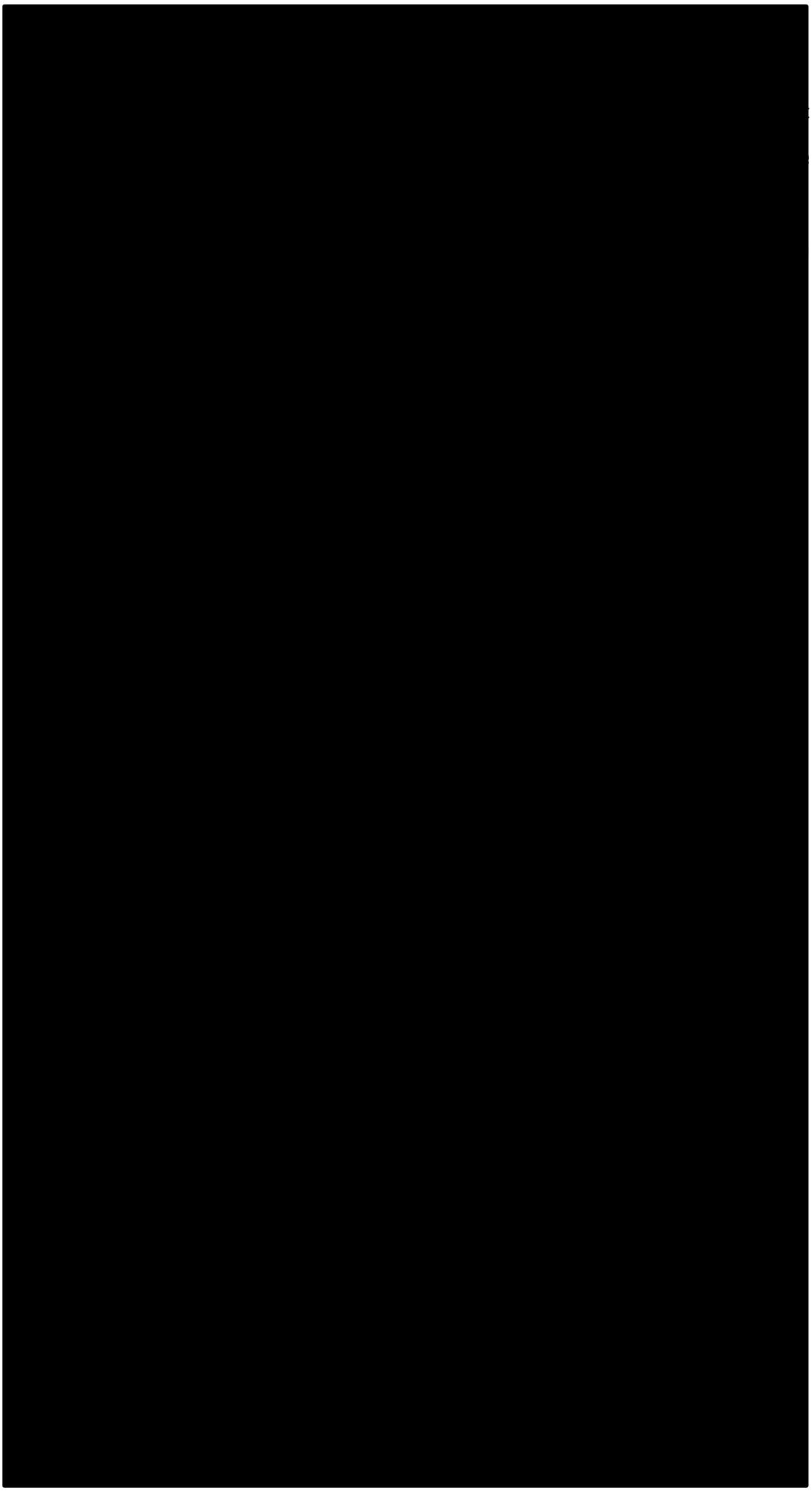


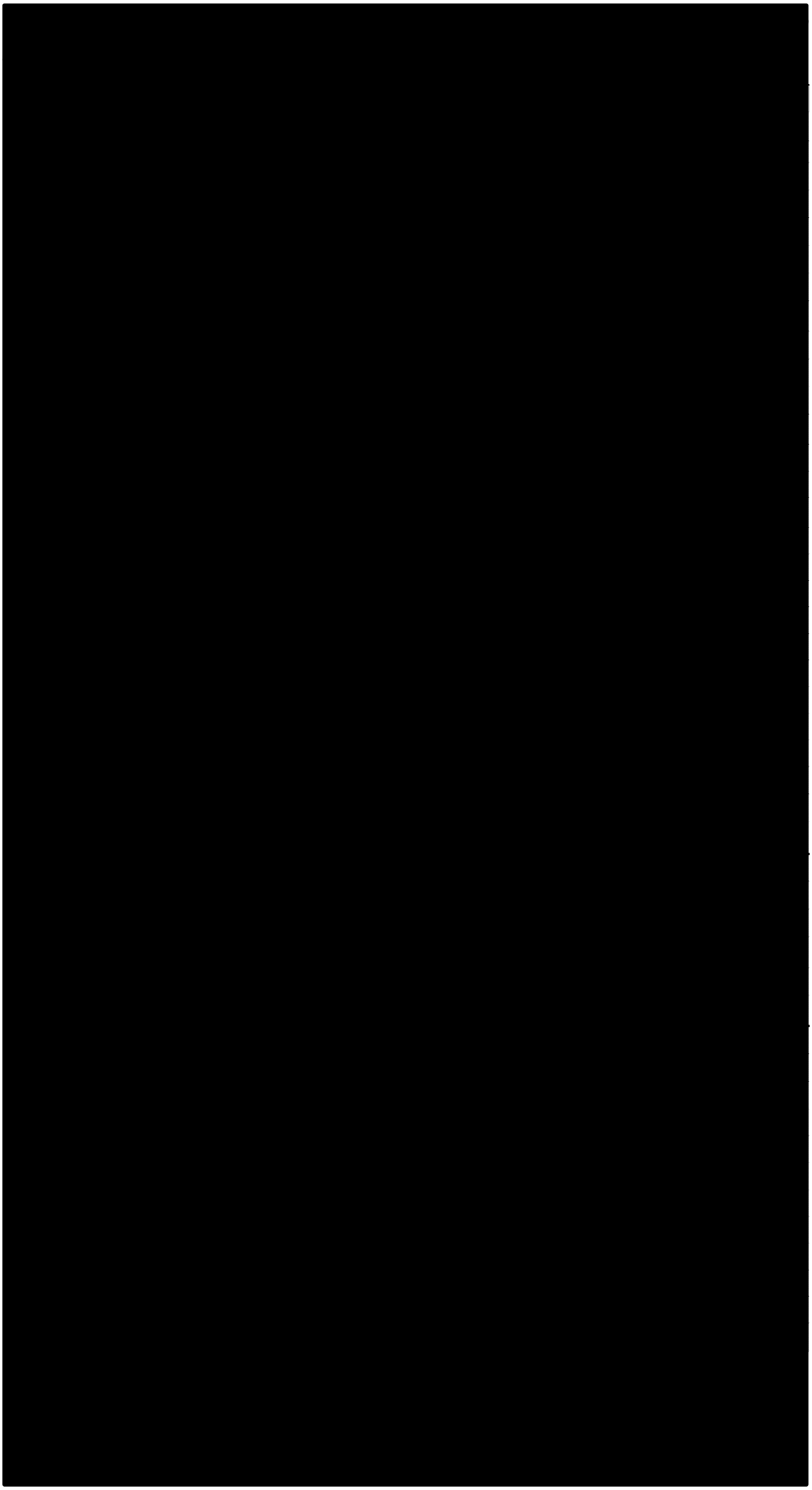


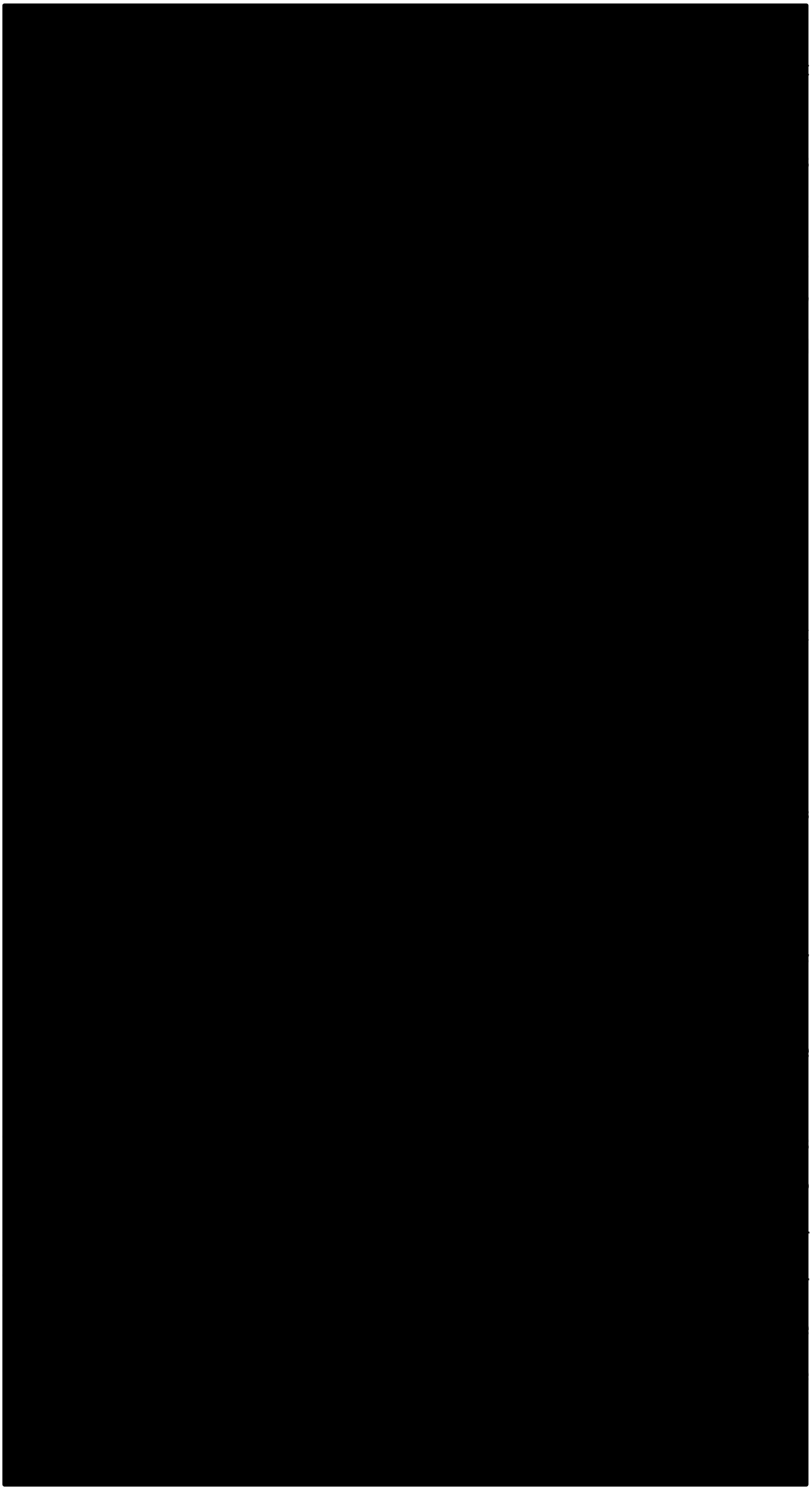


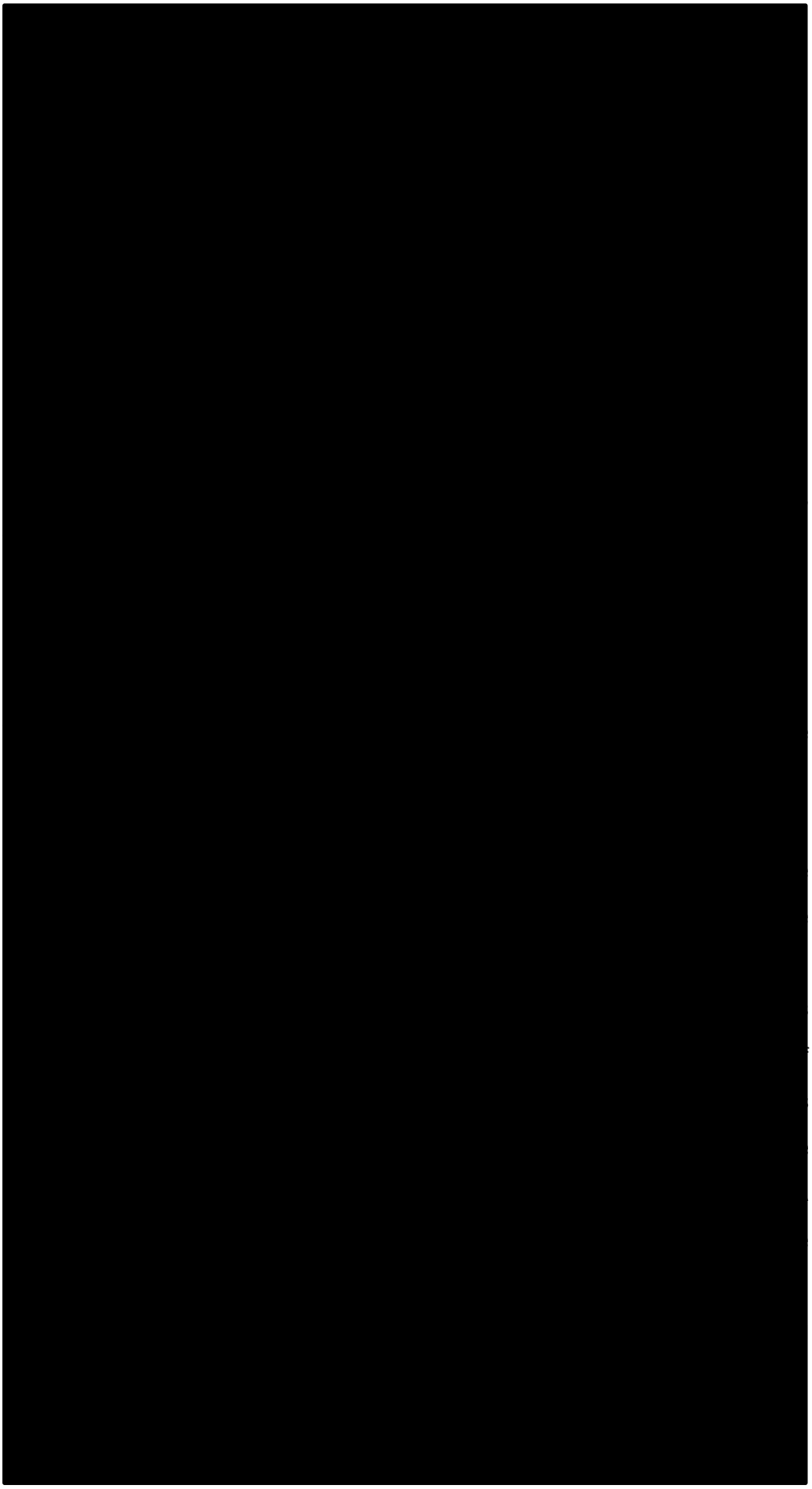


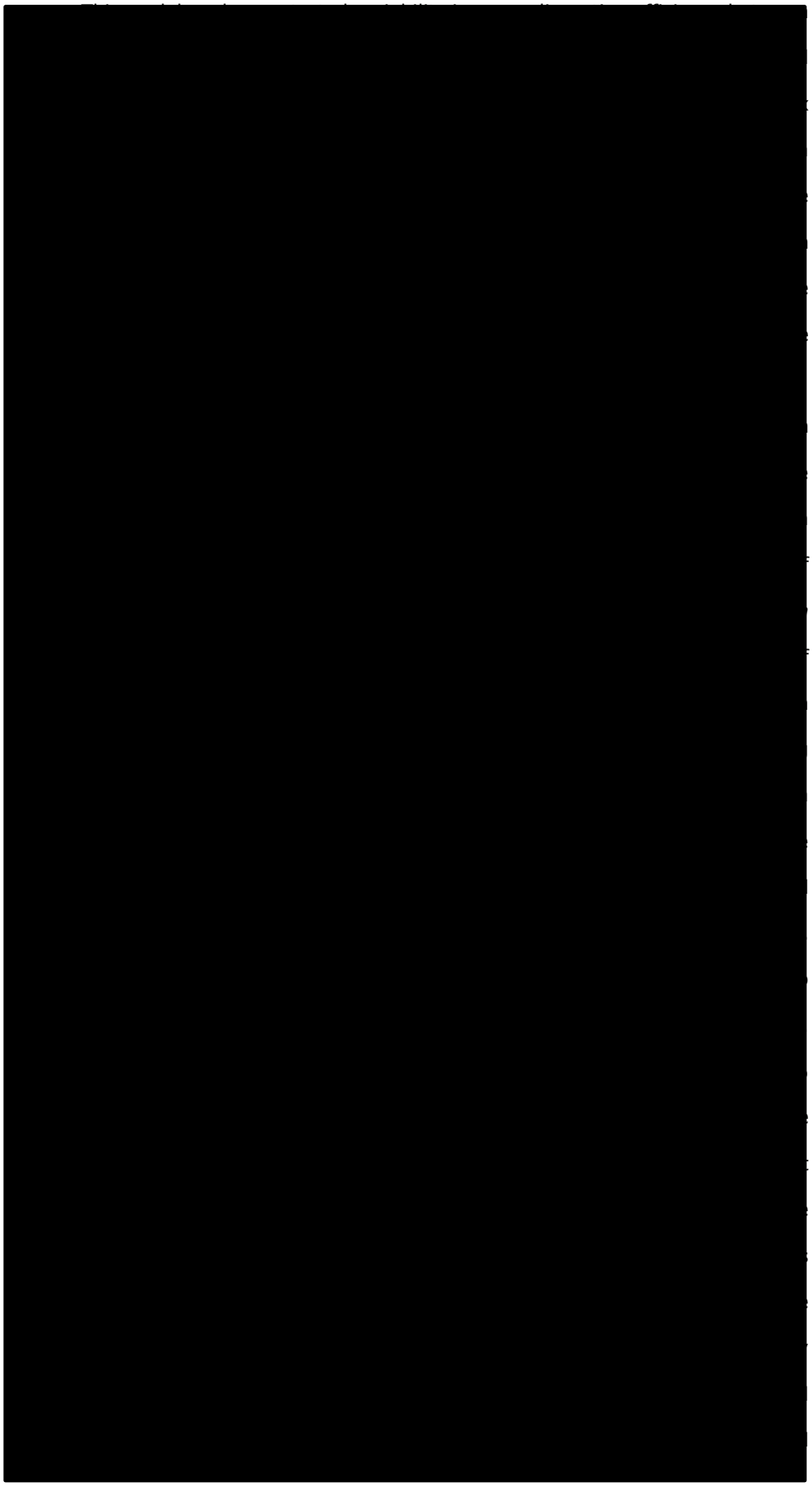


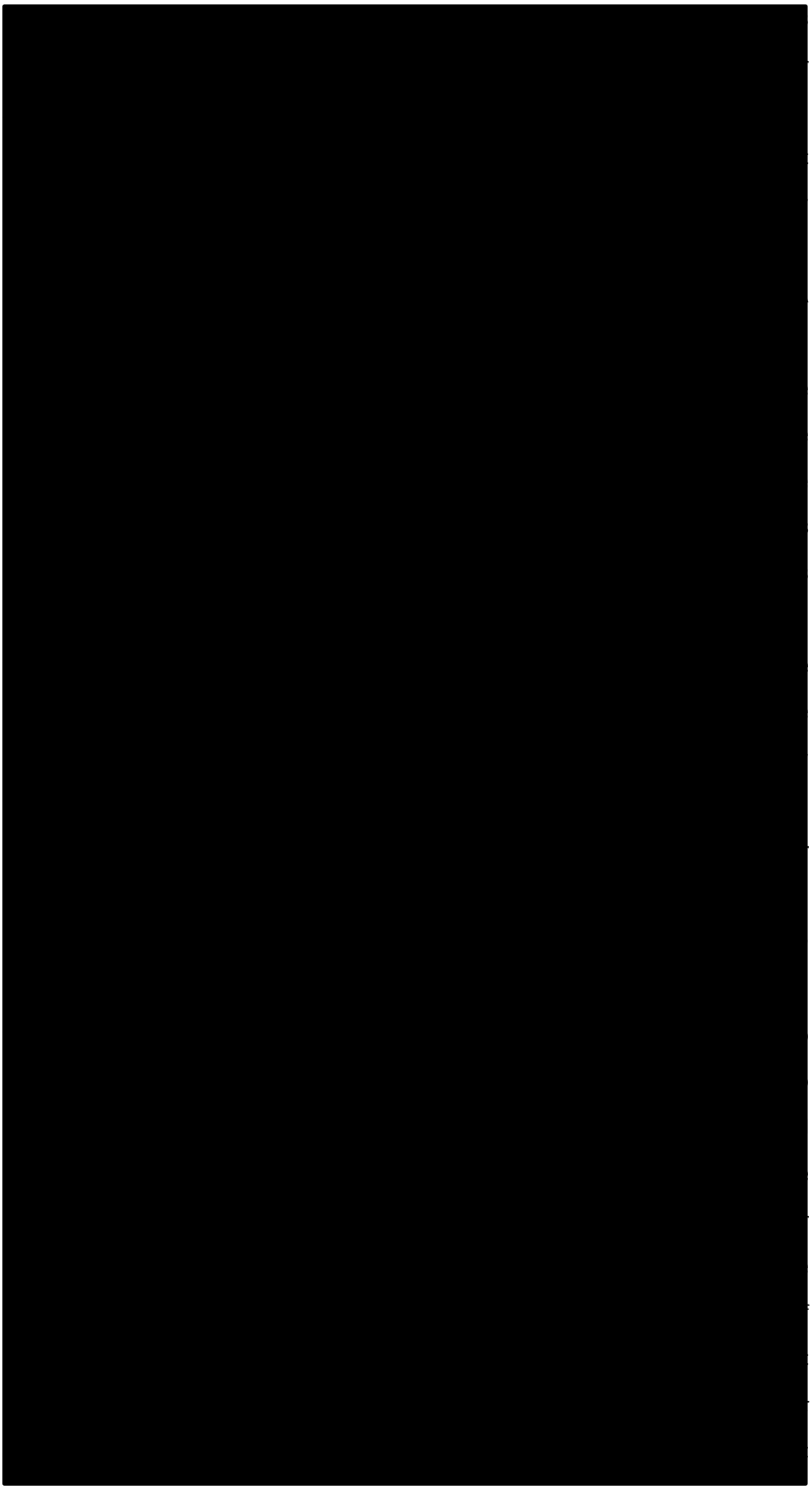


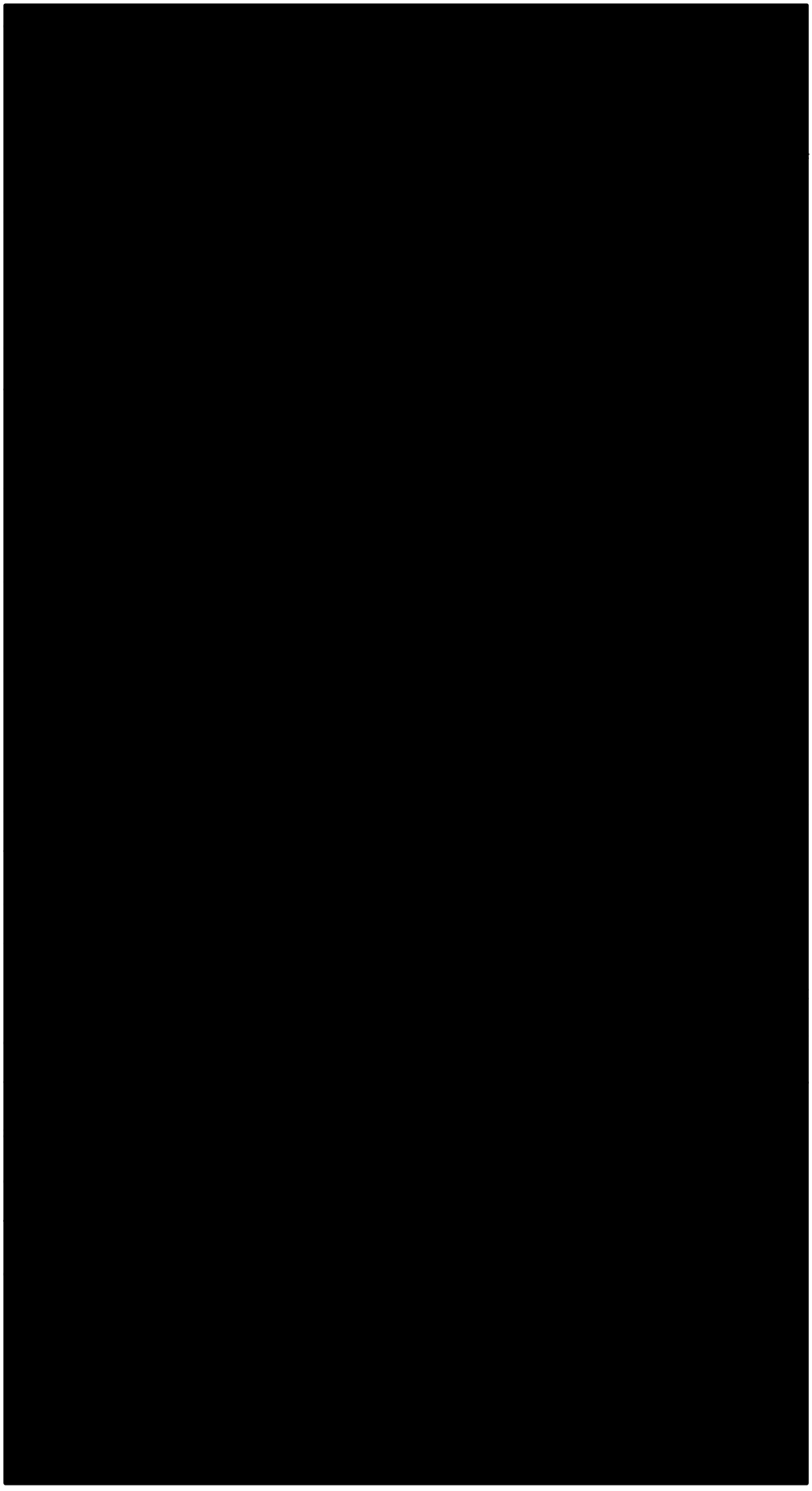


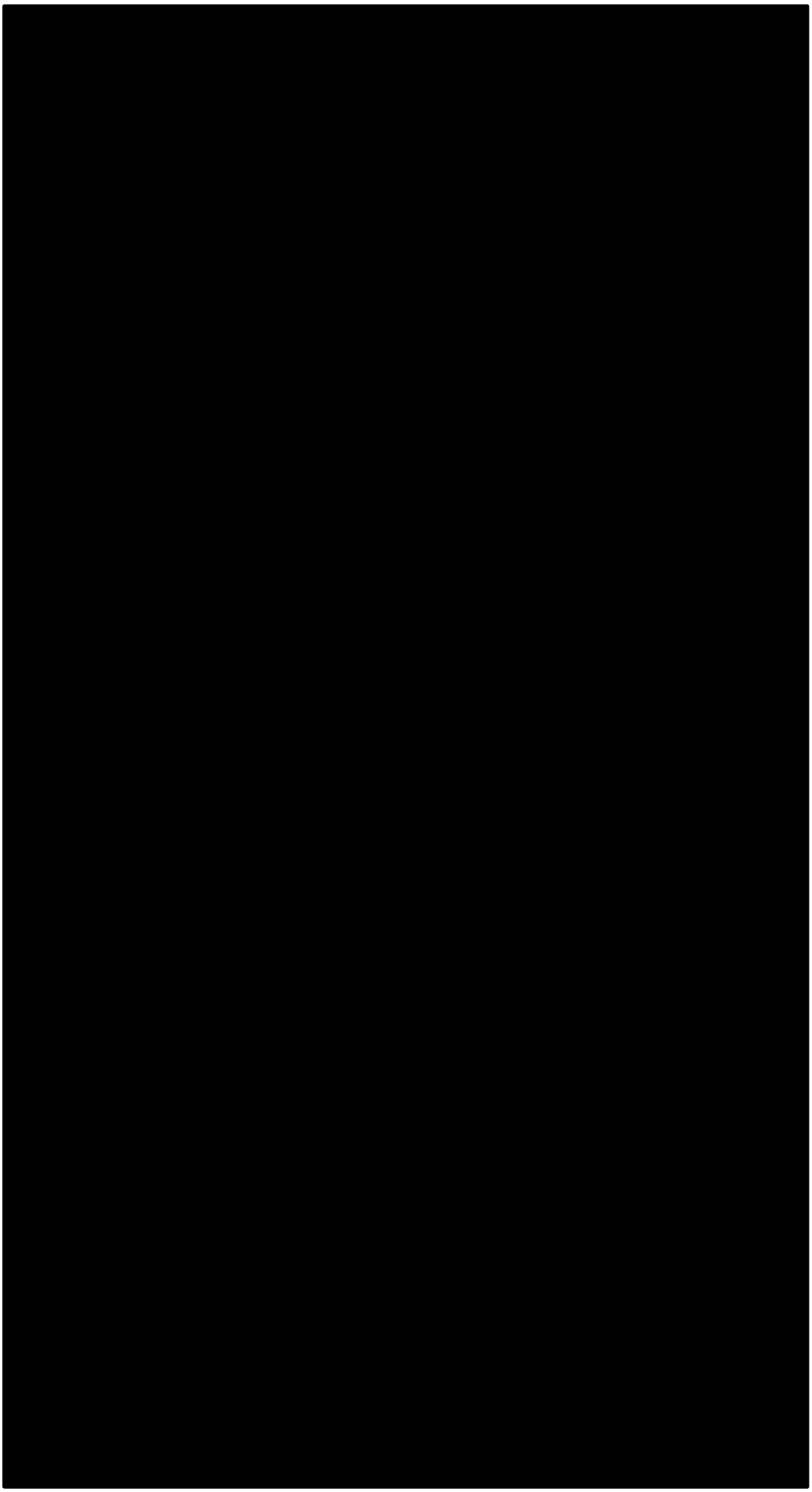












Chapter 4 . Rapid and automated in-field detection of *Puccinia striiformis, P. triticina and Zymoseptoria tritici* airborne spores

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4.1 Abstract

The suitability of LAMP as a detection method to identify the airborne inoculum of *Puccinia striiformis*, *P. triticina* and *Z. tritici* in-field in spore trap samples was evaluated following comparison with qPCR. Initially air sample extracts collected using a Burkard multi-vial cyclone previously tested by qPCR in 2015 were tested using LAMP. In 2016, air samples were collected using two Burkard multi-vial cyclones situated next to each other in an untreated plot of winter wheat. Samples from the first trap were tested on a Genie II using a crude extract by an in-field operator using premade mastermix. Samples from the second spore trap were sent back to the laboratory for conventional DNA extraction and qPCR. Levels of inhibition were monitored by adding an internal DNA reference standard prior to sample extraction. In 2017, a fully automated spore detection prototype system comprising a high volume cyclonic air intake and an internal LAMP detection module was also tested for the detection of fungal spores, and comparisons made with results from an accompanying Burkard spore trap. An influence of inhibitory compounds sampled by the spore trap was identified throughout the monitoring period in 2016 resulting in an underestimation of spore numbers by qPCR and is likely to be caused by air spora such as pollen and/or pollutants generated by human and industrial activities. LAMP was capable of providing high levels of sensitivity comparable to qPCR during

laboratory-based tests on conventionally extracted spore samples collected in 2015. A weak positive correlation was identified between the spore trap samples tested by field LAMP compared to samples collected by the additional spore trap that were tested by qPCR. Results indicate this is largely due to differences in the number of target species spores sampled by each trap. Successful automated detection of fungal spores was achieved using the fully automated spore detection prototype system and was comparable to qPCR carried out on samples collected by an accompanying Burkard spore trap. Initial tests suggest the automated spore trap can provide improved levels of detection compared to the Burkard multi-vial cyclone. Fungal spore detection using LAMP was shown to be rapid, simple and provided high levels of specificity and sensitivity both in the laboratory and in-field. This study has demonstrated its potential to be used for in-field spore detection as either a semi or fully automated detection solution for informing disease management decisions.

4.2 Introduction

Many fungal plant pathogens infect and restrict the yield potential of agricultural crops on a global scale. The presence and extent of infection by a particular species or number of species in a region is influenced by local climate and surrounding agricultural practices. It is therefore important to identify which pathogens are infecting the current growing crop to enable selection and application of the most appropriate fungicide at the correct time (McCartney *et al*., 2003).

Air dispersal is one mechanism by which plant pathogens spread and can occur on a local and continental scale (Morris *et al.,* 2011). Much of the work carried out in the study of airborne spore detection has been restricted by the range of aerobiological sampling equipment. Many studies to date have favoured the Hirst spore trap (Hirst, 1952) which collects air spora onto adhesive tapes for visual or molecular testing. This has provided valuable insights into airborne spore dispersal and provides a benchmark for validating new spore traps and detection methods. There is a need however to understand how pathogen dispersal influences disease on multiple scales and timeframes; and to help achieve this, the ability to rapidly detect airborne inoculum is essential (Mahaffee and Stoll, 2016).

It has been identified that improvements in spore trap design and function are required for the surveillance of plant pathogens particularly when trying to identify the initial ingress into a previously uninfected area (Jackson and Bayliss, 2011). Infection leading to an epidemic could potentially be triggered by an isolated low spore dispersal event originating from a neighbouring infected plant or distant source as a result of long distant dispersal (Brown and Hovmeller, 2002). Because of this variability in inoculum source, spore traps would benefit by being capable of sampling higher volumes of air which are more representative of a larger area such as an entire crop growing area within a farm. The volume of air sampled by a traditional volumetric Burkard spore trap equates to 14.4 m^3 of air per day when sampling at 10 Litres per minute which is approximately the volume of air present in an average sized office, roughly 22 m^3 (Jackson and Bayliss, 2011). The potential to not sample airborne fungal spores which have been subjected to significant dilution in the air, for example, as a result of long distance dispersal or low inoculum source is therefore high. There is a need for spore traps with more flexibility in terms of their sampling rates, sampling times, diagnostic sensitivity and reporting capabilities. Currently this sector of research is receiving heightened attention due to the innovative potential air sampling has for the detection of plant pathogens (West and Kimber, 2014).

In addition to air sampling equipment, detection methods are being explored that allow integration of sample collection, testing and result reporting. Historically laboratory based molecular techniques have been the preferred method of detection due to their high levels of sensitivity and ability to process large sample volumes. Methods that have been developed to date are able to identify spores down to species level via the DNA sequences present in air samples such as PCR and ELISA (Li *et al*., 2010; Kennedy *et al*., 2000). The main drawback to these approaches is the fact that it can take a number of days to achieve a diagnostic result. A rapid isothermal method to amplify target DNA in less than an hour known as Loop mediated isothermal amplification (LAMP) has been demonstrated to be effective for providing molecular detection at comparable levels of sensitivity to qPCR (Notomi *et al*., 2000). Methods have since been developed enabling the use of this technology for in-field detection (Tomlinson *et al*., 2010). Thiessen *et al.* (2015) successfully detected airborne

inoculum of *Erysiphe necator,* the cause of powdery mildew on grape vines, using rotor rod spore traps in conjunction with in-field LAMP tests. Currently the combined technologies of LAMP and spore traps have had limited application in the detection of airborne fungal spores.

The purpose of this research is to apply the rapid LAMP based methods of spore detection developed in chapter 3 with the detection of air borne spores of *P. striiformis, P triticina* and *Z. tritici* in air samples collected using Burkard multi-vial cyclonic spore traps during the winter wheat growing season in the UK. In addition, a fully automated prototype spore trap will be tested to determine the potential for daily automated spore detection. This will enable evaluation of the combination of technologies to provide the basis for an in-season real-time spore monitoring platform that could be used as part of an integrated pest management strategy.

4.3 Methods

4.3.1 Spore trapping

Spore trap samples were collected using Burkard multi-vial cyclones which sample air at a rate of 16.5 litres per minute and deposit air particulate into 1.5 ml microcentrifuge tubes. The spore traps were positioned at a height of 1.5 meters. In 2015, a single spore trap Burkard multi-vial was placed in an untreated plot of winter wheat between 25 April and 1 July at a site near Alford (53.2635 Latitude, 0.18653691 Longitude) in the UK. Spore trap samples obtained in 2015 were extracted using conventional methods and quantified by PCR and tested using LAMP in the laboratory. In 2016, two Burkard multi-vial cyclones were positioned adjacent to each other at the same location at another untreated winter wheat field trial site. One spore trap collected samples which were sent back to the laboratory for conventional DNA extraction and qPCR. The accompanying spore trap samples were tested by LAMP in the field. In 2017 a fully automated spore detection system was deployed alongside a Burkard multi-vial cyclone at a winter wheat field trial site near York (54.014754 Longitude, -0.972231 Latitude).

4.3.2 Conventional DNA extraction

To each 1.5 ml tube containing 24 hours of air particulate, 150 µl CTAB lysis buffer (120 mM sodium phosphate buffer pH 8, 2 % CTAB, 1.5 M NaCl, 1 % antifoam B emulsion) and 10 µl RNase 'A' were dispensed. Approximately 0.5 g 2.3 mm zirconia silica beads (approximately 20 beads) were subsequently added. In addition, an internal DNA reference standard comprising 1 ng of plasmid (pGEM-3Z Vector, Promega, USA) was aliquoted to each spore trap tube and extraction blank included in each batch (Coyne *et al.*, 2005; Hietala *et al.*, 2013). Sample disruption was carried out on a Vortex Genie for 5 minutes using a horizontal tube adapter. A Nucleospin Plant 2 extraction kit (Machenery-Nagel, Germany) was used to capture and clean the DNA present. The resulting 100 µl volume DNA samples were stored at -20 ^{o}C until required for qPCR and LAMP testing.

4.3.3 Quantitative PCR

Primers and probe sequences designed in Chapter 3 to target internal transcribed spacer regions (ITS) highly specific to *P. striiformis, P. triticina* and *Z. tritici* (Table 4-1) were used to perform qPCR. Quantification of the external reference plasmid (pGEM vector) was performed using forward primer 50-CCCAGTCACGACGTTGTAAAACG-30, reverse primer 50-TGTGTGGA ATTGTGAGCGGA-30 and the FAM-labeled TaqMan probe 50-CAC TATAGAATACTCAAGCTTGCATGCCTGCA-30 (Coyne *et al.*, 2005; Hietala *et al.*, 2013). All reactions were carried out using Environmental MasterMix (TaqMan® Environmental Master Mix 2.0) with the following cycling conditions: 50 °C for 2 min, 95 °C for 10 min, and 45 cycles of 95 °C for 15 s and 65 °C for 1 min. Reaction volumes comprised of 24 µl master mix and 1 µl sample. Each sample was tested in duplicate on a 7500 real-time PCR machine (Applied Biosystems, UK) and the average cycle threshold value obtained. Standards used to quantify spore numbers in qPCR testing were prepared from spore suspensions consisting of 10, 10^2, 10^3 and 10^4 spores. An extraction blank and a negative control comprised of water was used in each run to ensure no contamination was present throughout.

Table 4-1 qPCR primers and probes used for quantification of spore numbers in daily air samples

Pathogen	Name	Sequence (5'-3')	Product size (bp)
Puccinia striiformis	F	GAGTGTCATGAAACCCTCTCATT	
	R	CAAGTCAATCCAACTTATAGAAAAG	122
	Pe	CAATGGATGTTGAGTGCTGCTGTAATTAGC	
P. triticina	F	AAGTAGCAATACTTGCCATCTTG	
	R	TTATTGATATGCTTAAGTTCAGCG	117
	Pe	TTTCCAAAATTTAAGACCTCAAATCAGGTGGGACT	
Zymoseptoria tritici	F	GACCCTGCCGGGCGC	
	R	AAAGTTTTGTTTCGATTTACTCGTAAA	84
	Pe	CCAAAAAACACTGCATCTCTGCGTCGGA	

4.3.4 Laboratory LAMP

LAMP primers designed in Chapter 3 targeting the ITS region highly specific to *P. striiformis, P. triticina* and *Z. tritici* (Table 4-2) were used to carry out the LAMP tests. Each 25 µl LAMP reaction was comprised of 15 µl isothermal mastermix (ISO-004LNL, OptiGene), 5 µl LAMP primer mix (5 pmol F3 & B3, 10 pmol LoopF & LoopB, 20 pmol FIP & BIP), 2.5 µl sample DNA and 2.5 µl 0.6 M KOH. A Genie II (Optigene, UK) was used to carry out the LAMP reaction by incubating the samples at 65 ^{o}C for 30 min with a slow annealing step (98 ^{o}C to 80 ^{o}C ramping at 0.05 ^{o}C s^{-1}). The Genie II user interface was used to record the time to positive (TTP) and annealing temperatures.

Table 4-2 LAMP primers used for the detection of three important agricultural plant pathogens

Pathogen	Name	Sequence (5'-3')	Product size (bp)
P. striiformis	P.stri.F3	TTGAACGCACCTTGCGCC	272
	P.stri.B3	CCTGATTTGAGGTCTTAAGGT	
	P.stri.FIP	AATTACAGCAGCACTCAACATCCATTGGTATTC-CAAAAGGCACACCTG	
	P.stri.BIP	CTATAAGTTGGATTGACTTGGTGTAATAATTGGTT-TTTAGGAGTCTCCCTTAA	
	P.stri.FLP	TGAGAGGGTTTCATGACACTC	
	P.stri.BLP	TTATCATCACATCAAGGATTGTAGC	
P. triticina	P.trit.F3	GAGTGTCATGAAACCCTCTCAT	268
	P.trit.B3	CTTCACTCGCCGTTACTAGG	
	P.trit.FIP	CCTTATATTCAACAAGATGGCAAGTATTGAACTT-GTTTAGTGGATGTTGAGTG	
	P.trit.BIP	TTTAAGACCTCAAATCAGGTGGGACGAATCCTT-GTTAGTTTCTTTTCCTC	
	P.trit.FLP	TATTTAAAGTGAGCTAATGACAGCA	
	P.trit.BLP	CGCTGAACTTAAGCATATCAATAAG	
Z. tritici	Sept.F3	GATCATTACCGAGCGAGG	255
	Sept.B3	GTTCAAAGATTCGATGATTCACT	
	Sept.FIP	GCAGAGATGCAGTGTTTTTTGGTGCGACCTCCA-ACCCTTTGT	
	Sept.BIP	TACGAGTAAATCGAAACAAAACTTTCAAAATTCA-CATTACTTATCGCATTTCGC	
	Sept.FLP	CCGAAGCAACGGGATGTGT	
	Sept.FLP	TCTGGCATCGATGAAGAACG	

4.3.5 Field LAMP

The most effective rapid spore disruption method overall that was developed in Chapter 3 was used to disrupt fungal spores in air samples collected from the additional Burkard spore trap. This involved adding 0.5 g of 2.3 mm zirconia silica beads and 150 µl 0.3 M KOH to each of the 7 air samples collected per week. Tubes were then pulverized using a mini vortex for 30 seconds. The field operator then added 5 µl of each sample to the corresponding well across three pre-made LAMP reaction strips (Figure 4-1). Each strip had previously been made up in the laboratory (1 strip per pathogen per week) then sent to the spore trapping site for storage at -20 ^{o}C until required. Assays and reagents used were the same as those used for laboratory-based LAMP tests; however, due to the limited number of available wells only a single test could be performed per sample per pathogen. A control well was also included to monitor assay performance through the addition of 2.5 µl of target pathogen DNA extracted from a 10^3 spore aliquot.

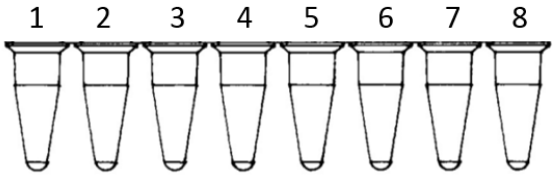


Figure 4-1 Example of a single LAMP reaction pathogen test strip used to test 7 daily air samples for a single target plus a positive control. Well 1-7 corresponds to daily air samples 1-7, well 8 = positive control

The control was tested in the laboratory prior to being sent to the site to enable a comparison to be made with the TTP obtained from the in-field test. Reagent strips were prepared and sent out monthly to cover a testing period of approximately four weeks. On the day of sample change-over, test strips were defrosted and 5 µl of the crude 0.3 M KOH sample DNA extract from the Burkard spore trap tube added to each test strip. A Genie II was used to carry out the LAMP test and the TTP and annealing temperature recorded.

4.3.6 Automated spore detection

A fully automated cyclonic spore detection system named 'Tantei' (Optisense, University of Hertfordshire, FERA, Bayer, Innovate UK) developed under an Agritech catalyst project funded by Innovate UK was positioned at York in an untreated plot of winter wheat in 2017 (Figure 4-2).



Figure 4-2 The automated spore detection system 'Tantei'.

The system comprised a high volume cyclonic air intake, drawing air in at a rate of 100 litres per minute and an internal LAMP detection module. The air intake is approximately 1 meter off the ground and samples at a full 360 degrees. The trap was programmed to sample air throughout the day from 10:00 am until 16:30 pm with the cyclone switching on repeatedly for 4 minutes then pausing for 6 minutes. The air sample was subsequently subjected to an automated spore disrupted step followed by a LAMP test. The device was configured to screen for a single pathogen per day and required attending daily to collect the test results and set the next run going. LAMP reactions and assays used are as previously described for conducting the laboratory and field based LAMP tests. Mastermix was made up in the laboratory and placed in the automated spore trap to be used on consecutive days to assess assay performance over time, as the trap was capable of operating unattended for 4 days. Tests were carried out in 8-well Genie strips placed within the system. Of these wells, 6 were used for pathogen testing and single wells for the negative and positive control. A positive detection was recorded when at least one of the 6 wells provided amplification. LAMP was also carried out in the laboratory for all three-fungal species under study using the crude automated spore trap sample after the in-field test had completed. To validate the efficiency of the automated spore trap, a Burkard multi

vial cyclone was sited next to it and programmed to sample continuously for 24 hours on the same days as the automated spore trap tests were conducted. qPCR was then used to quantify spore numbers of *P. striiformis, P. triticina* and *Z. tritici.*

4.4 Results

4.4.1 Sensitivity and specificity of LAMP carried out in the laboratory

A selection of previously quantified daily spore trap samples collected in 2015 with spores of *P. striiformis, P. triticina* and *Z. tritici* ranging from 0 to 10^4 using qPCR, were selected for comparative testing using LAMP. Tests were carried out in the laboratory with fresh reagents. Overall, LAMP tests were highly sensitive and specific (Table 4-3). *P. striiformis* DNA was detected in 33 out of the 34 air samples which had previously tested positive using qPCR. LAMP maintained high levels of detection with 30 out of 31 and 24 out of 29 for samples which had tested positive previously for *P. triticina* and *Z. tritici* DNA respectively. In addition, all extracts which previously tested negative using qPCR were also negative when tested using LAMP. Therefore, there was a zero rate of discrepancy in detecting absence of spores (potential false negatives) and a very low number of discrepancies in detecting presence (potential false positives), with the main issues being with detection of *Z. tritici* spores.

Table 4-3 Diagnostic sensitivity and specificity of laboratory based LAMP when testing spore trap samples quantified by qPCR collected in 2015 and extracted conventionally using an extraction kit

			qPCR				
			+ (A)	- (B)	Total	sensitivity	specificity
Lab LAMP	Y.rust	+ (C)	33	0	33	97	100
		- (D)	1	6	7		
		Total	34	6	40		
	B.rust	+ (C)	30	0	30	97	100
		- (D)	1	6	7		
		Total	31	6	37		
	Z.trit	+ (C)	24	0	24	83	100
		- (D)	5	4	9		
		Total	29	4	33		

[a]Number of samples tested positive (+) and negative (-) by qPCR for spore numbers quantified above 1 spore per daily air sample and LAMP used to determine diagnostic sensitivity [AC/(AC+AD)] and specificity [BD/(BD+BC)].

Positive correlations were observed between spore sample aliquots at spore numbers from 10 to 10^4 extracted conventionally and tested by qPCR and LAMP (R^2=0.753 *P. striiformis*, R^2 = 0.877 *P. triticina*, R^2 = 0.954 *Z. tritici*) (Figure 4-3).

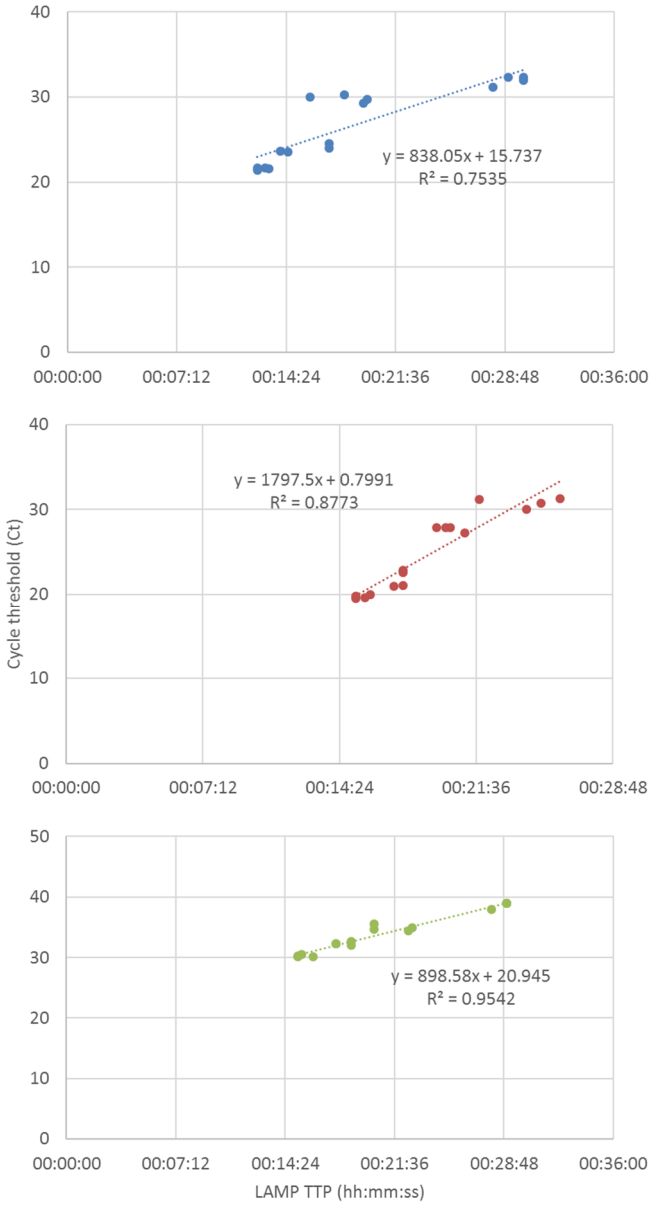


Figure 4-3 Relationship between spore numbers from 10 to 10^4 extracted conventionally and tested by qPCR and LAMP, P. striiformis (top), P. triticina (middle) and Z. tritici (bottom)

Positive correlations were also observed between spore numbers quantified by qPCR and TTP using LAMP (R^2=0.362 *P. striiformis*, R^2 = 0.403 *P. triticina*, R^2 = 0.785 *Z. tritici*) (Figure 4-4).

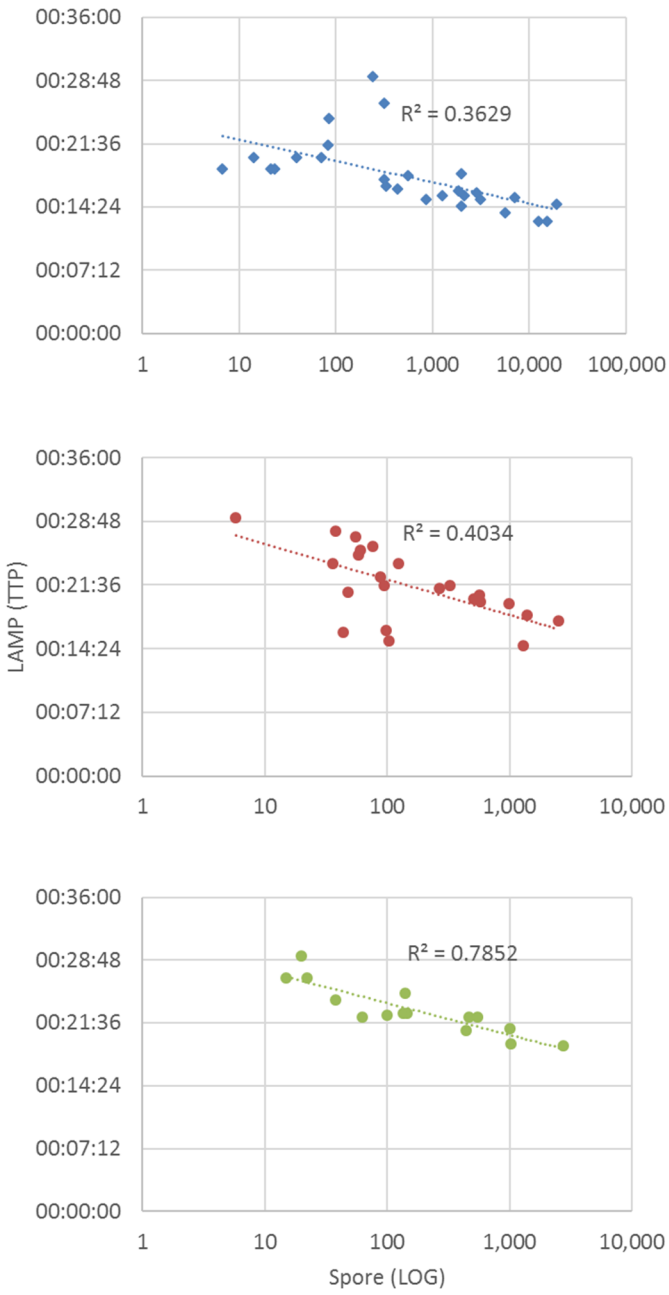


Figure 4-4 Relationship between spore trap samples tested by qPCR and quantified in terms of daily spore numbers sampled and comparative testing using laboratory-based LAMP, P. striiformis (top), P. triticina (middle) and Z. tritici (bottom)

4.4.2 Quantification and inhibition of spore numbers collected in daily spore trap samples using qPCR

In 2016, two spore traps were positioned adjacent each other in a winter wheat field trial site near Alford to collect daily air samples and the number of *P. striiformis, P*

tritici and *Z. tritici* spores quantified. The performance of the sample disruption, DNA extraction, PCR steps and an indirect measure of the effect of sample inhibitory compounds was monitored by adding a known concentration of plasmid (external DNA reference standard) to the spore trap samples prior to DNA extraction. The concentration of plasmid in each sample following molecular testing was quantified by qPCR targeting a known region of the plasmid DNA. The difference in the concentration of plasmid detected in the extraction blank compared to each sample in terms of the Ct values was then applied to the Ct originally obtained from the qPCR when quantifying the number of *P. striiformis*, *P. triticina* and *Z. tritici* spores sampled. This provided a revised spore number based on the performance of the molecular test (Figure 4-5).

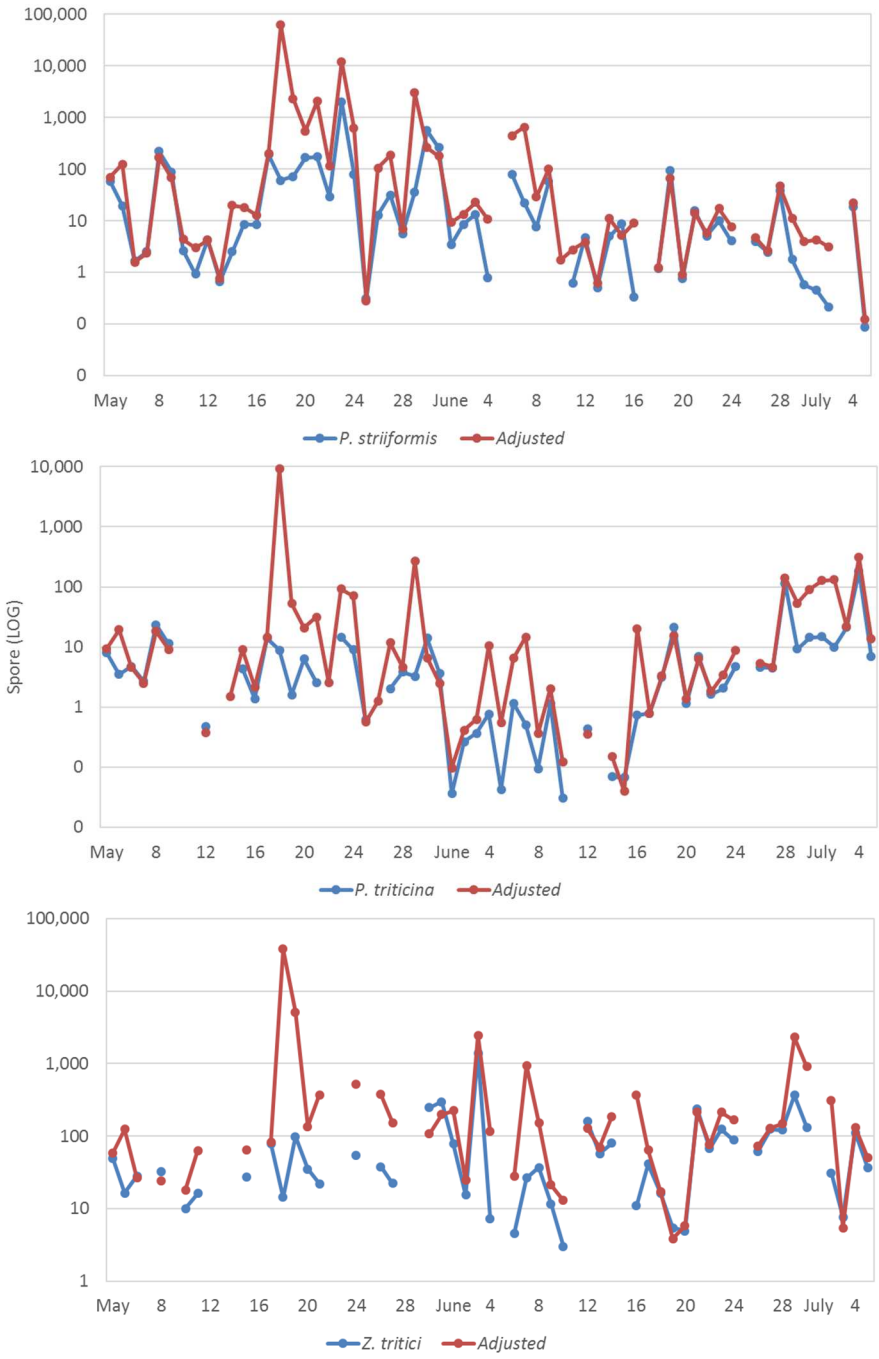


Figure 4-5 Comparison of* P. striiformis *(top),* P. triticina *(middle) and* Z. tritici *(bottom) spore numbers quantified in daily air samples using qPCR and spore numbers adjusted based on reaction performance using an external DNA reference standard (pGEM plasmid)

Plasmid Cts in the extraction blanks were highly reproducible suggesting an efficient DNA extraction. As a result, most of the variation can be attributed to co-extracted air particulates such as pollen, dust, air spora and/or pollutants causing inhibition. The concentration of plasmid extracted in air samples compared to the extraction

blanks was reduced on average by 1.3 Cts. This identified that direct quantification of *P. striiformis, P. triticina and Z. tritici* spore numbers were in general underestimated. To account for the variation in terms of spore number the percentage of days the difference in spore numbers reached pre-determined levels between the original quantification and the adjusted is presented in Figure 4-6. This highlights that 73 %, 75 % and 51 % of samples tested for *P. striiformis, P. triticina* and *Z. tritici* varied by less than 10 spores respectively. The majority of the remaining variation occurred at spore numbers between 10^2 and 10^3.

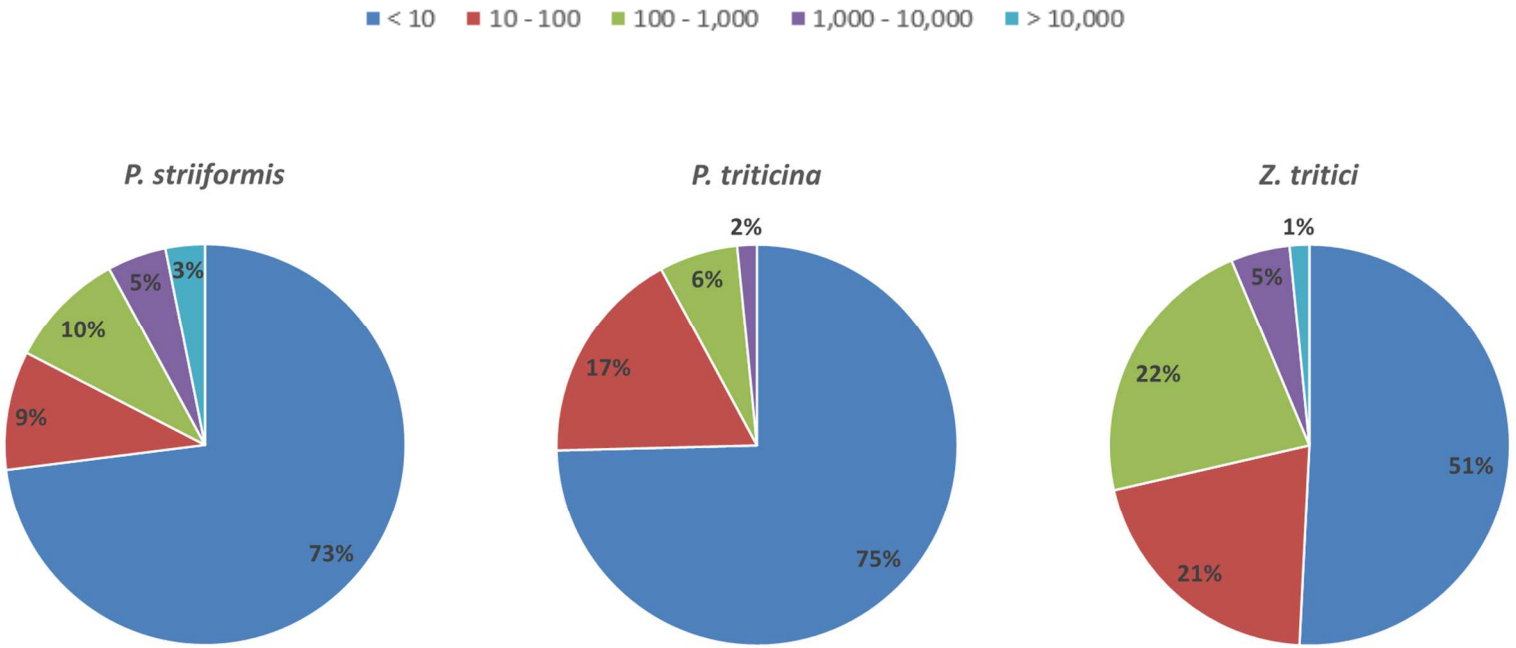


Figure 4-6 Percentage of daily spore samples underestimated by qPCR categorised by the estimated number of spores by which each sample is underestimated

The greatest deviation in spore numbers and therefore inhibitory compounds sampled occurred on the 18th May and was sustained over a period of 4 consecutive days. Further variation was observed intermittently throughout May and June. From 16 to 28 June the concentration of plasmid quantified in spore trap samples was more comparable to the extraction blanks suggesting fewer co-extracted inhibitory compounds present in the air sampled over that time period.

4.4.3 Sensitivity and specificity of LAMP carried out in the field

In total there were 48, 31 and 43 daily detections of *P. striiformis*, *P. triticina* and *Z. tritici* spores respectively made over the 56-day study period using qPCR. Positive detections using LAMP carried out on samples obtained from a separate sampler over the same sampling period in-field was made on 20, 19, and 18 of these days respectively. If both spore traps sampled the same number of target species fungal spores then overall a reduction in the sensitivity of LAMP for the detection of *P. striiformis* (39 %), *P. triticina* (36 %) and *Z. tritici* (41 %) spores was observed. (Table 4-4).

Table 4-4 Diagnostic sensitivity and specificity of field-based LAMP for the detection of* P. striiformis*,* P. triticina *and* Z. tritici *in daily spore trap samples compared to samples collected by an accompanying spore trap extracted conventionally and quantified by qPCR

			qPCR				
			+ (A)	- (B)	Total	sensitivity	specificity
Field LAMP	Y.rust	+ (C)	28	1	29	58	87.5
		- (D)	20	7	27		
		Total	48	8	56		
	B.rust	+ (C)	19	3	22	61	88
		- (D)	12	22	34		
		Total	31	25	56		
	Z.trit	+ (C)	18	6	24	42	45
		- (D)	25	5	30		
		Total	43	11	54		

[a]Number of samples tested positive (+) and negative (-) by qPCR for spore numbers quantified above 1 and LAMP used to determine diagnostic sensitivity [AC/(AC+AD)] and specificity [BD/(BD+BC)]

No correlations (Figure 4-7) were made between daily air sample spore numbers quantified using qPCR and LAMP TTP (R^2=0.023 *P. striiformis*, R^2 = 0.085 *Z. tritici*) and a weak positive correlation identified for *P. triticina* (R^2=0.2294). This suggests that the level of spores quantified by qPCR in one trap is not necessarily reflected by the LAMP TTP produced when testing the sample in the adjacent spore trap. Evidence suggests the variation of detection between the traps and associated molecular test is likely a result of actual spores sampled as there was a total of 18 daily spore trap samples (3 *P. striiformis,* 7 *P. triticina,* 8 *Z.* tritici) that resulted in a positive detection using LAMP and no detection made using qPCR on the same day (Figure 4-8). Furthermore, LAMP provided detection of target species DNA in spore trap samples on days when less than 10 spores of each target fungal pathogen were detected in the accompanying spore trap sample by qPCR.

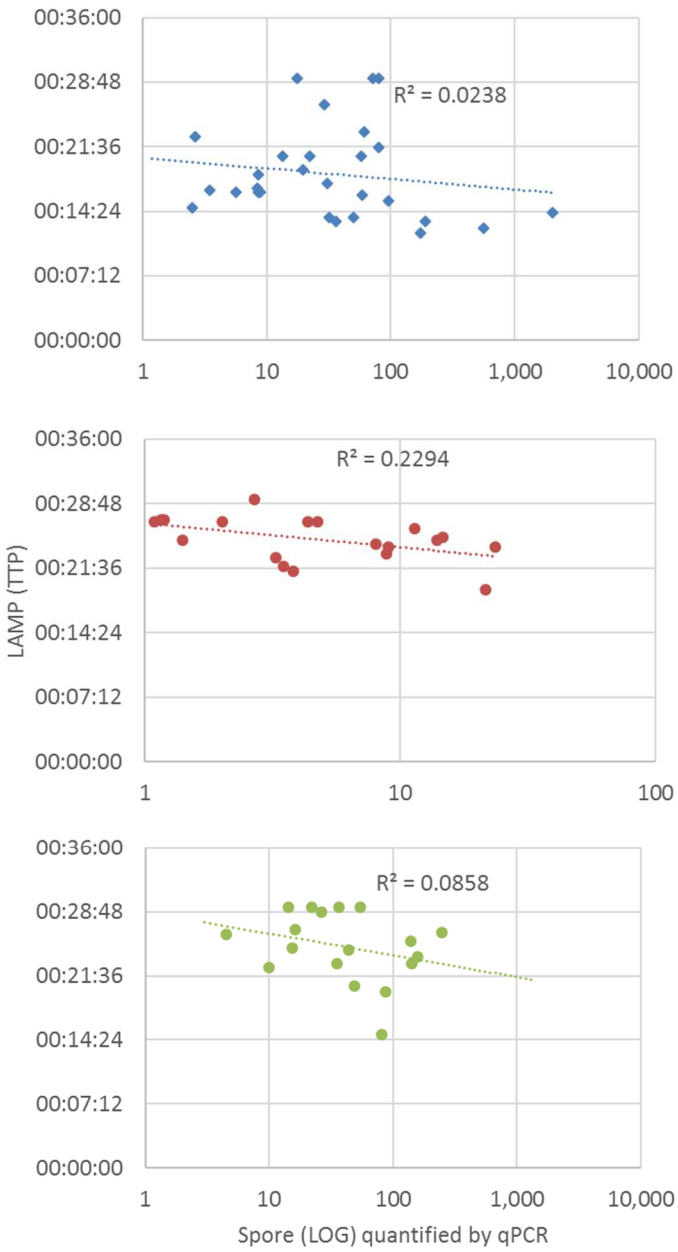


Figure 4-7 Relationship between TTP using field-based LAMP and spore numbers quantified using qPCR on samples collected by the accompanying spore trap For* P. striiformis *(top),* P. triticina *(middle) and* Z. tritici *(bottom)

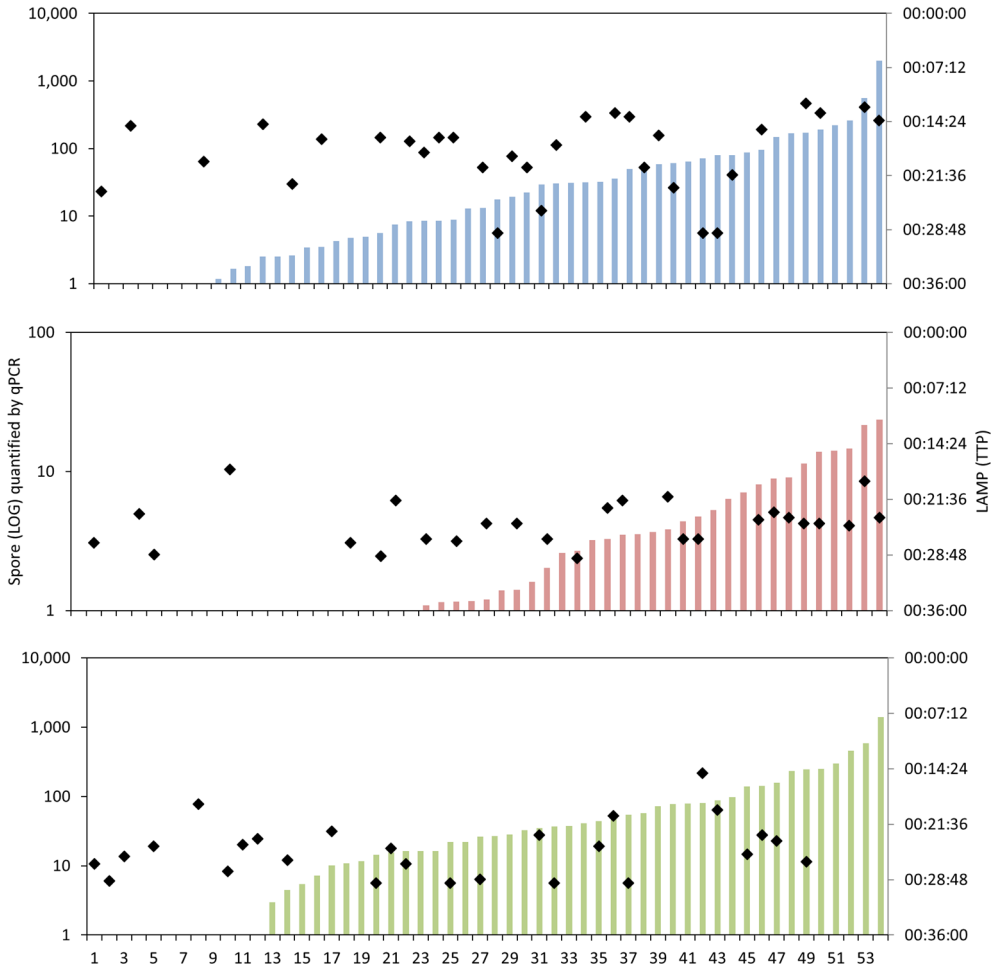


Figure 4-8 Quantification of daily spore numbers of* P. striiformis *(top),* P. triticina *(middle) and* Z. tritici *(bottom) in spore trap samples using qPCR sampled in 2016 (bar) and TTP achieved using LAMP tests carried out on samples collected by an accompanying spore trap of the exact same make and specification (diamond)

4.4.4 Re-testing in-field crude extracts

To investigate in more detail the effect of the in-field test on sensitivity, a selection of crude extracts tested in-field were retested in the laboratory using freshly made-up reagents. Re-tests on samples which previously tested positive for *P. striiformis* correlated closely with results obtained in the field (Table 4-5). This was not the case for *P. triticina* as many samples previously tested negative by field LAMP came up positive following re-testing and were more closely correlated with results obtained from the accompanying Burkard samples tested by qPCR. As the number of *P. triticina* spores quantified were primarily below 10 it is possible the LAMP assay was

not sensitive enough to detect the low levels of DNA present at the time of the test. However, the discrepancies could be related to the efficiency of the spore disruption as each sample re-tested will have been subjected to a much longer lysis compared to when the sample was tested in the field. This would equate approximately to the time taken to send the sample back in the post to Fera. One of the conditions to achieve improved detection of *P. triticina* spores identified in Chapter 2 was a longer lysis and proved more effective than bead disruption.

Table 4-5 Re-testing in-field crude lamp extracts in the laboratory

P. striiformis			*P. triticina*		
In-field LAMP	reLAMP	qPCR Spore No.	In-field LAMP	reLAMP	qPCR Spore No.
00:22:45	00:17:00	10	-	00:24:30	1
-	-	16	-	00:26:15	1
-	-	-	-	-	-
-	-	-	-	00:29:15	1
00:14:45	00:16:00	-	-	-	-
00:16:30	00:28:15	27	-	00:20:45	-
00:17:00	00:16:15	-	-	-	-
00:13:15	00:25:30	79	-	00:18:30	-
00:23:15	00:18:15	14	-	-	-
00:29:15	-	98	00:17:45	00:22:45	-
-	00:23:15	35	00:29:00	00:21:15	1
00:12:00	00:14:15	22	-	-	1
00:26:15	00:17:45	-	-	-	3
00:14:15	00:14:45	-	00:19:15	00:19:15	22
00:21:30	00:21:45	55	00:27:00	00:29:00	1
00:23:45	00:20:30	-	-	00:25:45	7
-	-	38	-	00:22:45	2
00:13:45	00:13:45	22	-	-	2
00:16:30	00:15:00	-	-	00:22:15	5
00:13:15	00:24:45	-	-	00:29:15	-
00:12:30	00:13:00	247	-	00:18:45	5

4.4.5 Stability of LAMP reagents

To characterise variations in LAMP TTP between samples tested in the laboratory to those tested in field, a control comprising DNA extracted from 10^3 spores was added to well 8 in each Genie test strip. Each control was tested in the laboratory prior to being shipped to enable a comparison of TTP.

There was little between-batch variation in the TTP obtained when testing only the control prepared in the laboratory (Figure 4-9). The most variation was

observed when the same control was subsequently tested in the field. The *P. triticina* assay control proved to be relatively stable with a standard deviation of 00:01:05 (hh:mm:ss) in TTP out of the 4 batches made and the most stable over the 34 LAMP tests carried out by the in-field operator with a standard deviation of 00:01:15. The *P. striiformis* assay performed the most consistently in the laboratory with a standard deviation of 00:00:24; however, across the 34 in-field tests a standard deviation of 00:02:54 was recorded. The *Z. tritici* LAMP assay showed more variation in the TTP recorded in the control batches in the laboratory largely due to the third batch producing a much faster TTP around 00:12:45 compared to the remaining batches which averaged 00:18:30. A standard deviation of 00:02:44 was recorded across the 34 tests carried out in-field.

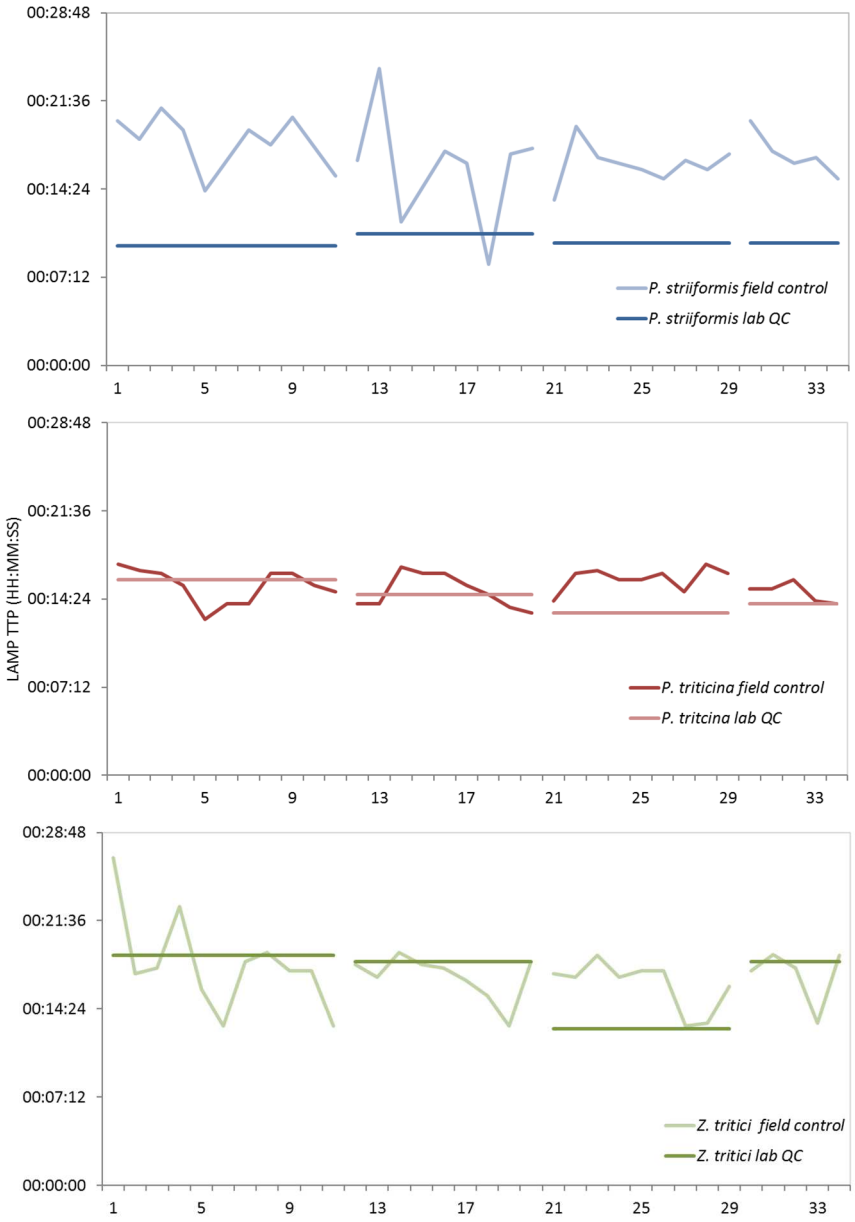


Figure 4-9 Comparison of the variation in TTP obtained from mastermix controls tested in the laboratory to when the same mastermix control was subsequently tested in the field alongside the daily spore trap samples

4.4.6 Automated spore detection

The automated spore trap was operated on a total of 7 days to test for *P. triticina* and 6 days to test for *P. striiformis* spores in air samples between March and May 2017 (Table 4-6). Each automated spore trap sample was also tested using LAMP in the laboratory. A Burkard multi-vial cyclone spore trap accompanied the automated spore trap to provide samples for comparative qPCR testing.

P. striiformis was detected in 1 of the 6 air samples collected and tested using the automated spore trap. An error was encountered during the LAMP test on 10 May and as a result no detection data was obtained for that day. Laboratory LAMP produced detection on 2 out of 6 days, one of which included the sample that failed to be tested using the automated spore trap. The detection of *P. striiformis* spores using the Burkard multi-vial cyclone and qPCR was achieved on 5 out of the 6 samples tested and was therefore less comparable to the LAMP based tests using the automated spore trap sample. Both automated LAMP and laboratory LAMP tests did not detect *P. striiformis* DNA when 11, 6 and 11 spores were quantified using qPCR on 11, 12 and 13 of April. Detection was achieved however when 30 and 55 spores were quantified on 9 and 10 May respectively. As a result, the discrepancy between the detection methods could be related to variation in actual spore numbers sampled, possibly influenced as a result of the automated spore trap only operating during daylight hours whereas the Burkard was sampling for a full 24 hours.

P. triticina DNA was detected in 6 out of the 7 air samples collected and tested using the automated trap. The same level of detection was achieved when testing the crude samples using LAMP in the laboratory. *P. triticina* DNA was detected in a total of 5 out of 7 samples collected using the Burkard multi-vial cyclone and tested using qPCR. This indicates the automated spore trap was able to capture and detect *P. triticina* spores on a day when the more traditional technique was not. The sensitivity of the automated spore detection system was highlighted further following the detection of *P. triticina* on a day when as few as 2 spores were detected in the accompanying Burkard spore trap sample using qPCR.

Although the automated spore trap was not specifically set up to test for *Z. tritici* spores, tests using LAMP in the laboratory were carried out on the lysed samples collected throughout the 13 days the system was run. Detection of *Z. tritici* spores was highly comparable between laboratory LAMP and qPCR with both methods resulting in the detection of *Z. tritici* spores on the same 12 out of the 13 days samples were collected. Detection of *Z. tritici* using the automated spore trap sample was possible when as few as three *Z. tritici* spores were quantified by qPCR in the corresponding Burkard spore trap sample.

The positive control failed to produce a TTP on 5 out of the 12 tests performed by the automated spore trap when completed successfully. These were generally observed after the third day of testing which may indicate a degree of reagent degradation. However, some amplification was observed after the third day of testing in some of the 6 remaining wells containing only LAMP mastermix and recently added air sample lysate, highlighting its ability to still provide amplification after 3 days of being made up in the laboratory and placed within the automated spore trap.

Across each automated spore trap pathogen test strip, only a few wells amplified out of a possible 6 when target species DNA was present, even when new fresh reagents were installed in the device. This could be explained by the fact that target species spore numbers quantified by qPCR carried out on the Burkard spore trap were low on the days the automated spore trap was tested. As a result, multiple tests, and in the case of *P. triticina* a longer lysis, would be preferred to detect low spore numbers.

Table 4-6 Comparison of air samples collected and tested by the automated spore trap and laboratory LAMP for* P. striiformis*,* P. triticina *and* Z. tritici *with samples collected by an accompanying Burkard multi-vial cyclone and tested with qPCR

		Auto spore trap							Laboratory LAMP					Burkard (qPCR spore numbers)					
Sampling Date	Mastermix made	Air sampled (litres)	*P.stri*	*P. trit*	*Z. trit*	No. of LAMP reactions positive (6)	Positive control	Negative control	*P.stri*	*P. trit*	*Z. trit*	Positive control	Negative control	Air sampled (litres)	*P.stri*	*P. trit*	*Z. trit*	Positive control	Negative control
27/03/2017	27/03/2017	31680		-		0	-	-	-	-	+	+	-	23760	0	0	6	+	-
28/03/2017		31680		+		4	+	-	-	+	+	+	-	23760	0	0	3	+	-
29/03/2017		31680		+		3	-	-	-	+	+	+	-	23760	2	2	52	+	-
30/03/2017		11520		+		1	-	-	-	+	+	+	-	23760	2	32	308	+	-
20/04/2017	20/04/2017	79200		+		2	+	-	-	+	+	+	-	23760	0	56	41	+	-
21/04/2017		25920		+		1	+	-	-	+	+	+	-	23760	29	117	50	+	-
26/04/2017		79200		+		1	+	-	-	+	+	+	-	23760	15	53	13	+	-
10/04/2017	10/04/2017	50400	-			0	+	-	-	+	-	+	-	23760	0	398	0	+	-
11/04/2017		28800	-			0	+	-	-	+	+	+	-	23760	11	148	46	+	-
12/04/2017		14400	-			0	+	-	-	+	+	+	-	23760	6	29	11	+	-
18/04/2017		28800	-			0	-	-	-	+	+	+	-	23760	11	49	7	+	-
09/05/2017	09/05/2017	79200	+			5	-	-	+	+	+	+	-	23760	55	67	27	+	-
10/05/2017		79200	Failed			0	-	-	+	+	+	+	-	23760	30	100	35	+	-

*Grey shading indicates no test was carried out

4.5 Discussion

This study has for the first time successfully evaluated the detection of airborne fungal plant pathogen spores in spore trap samples collected in the field using both LAMP carried out by a field trial specialist and LAMP integrated into a fully automated spore detection system. Successful detection of *P. striiformis, P. triticina* and *Z. tritici* spores using a crude DNA extraction method in conjunction with LAMP carried out by the field operator was achieved. Furthermore, automated detection of *P. striiformis* and *P. triticina* was demonstrated by the automated spore detection system with additional laboratory testing confirming the cyclone had successfully sampled *Z. tritici* spores.

One area of spore monitoring that is poorly understood is how the effect of co-sampled particulates effects the sensitivity of detection. Monitoring an external DNA reference standard identified that an element of inhibition was present in the majority of spore trap samples extracted conventionally and tested by qPCR. If dust was suspected, there may have been an indirect correlation with wind speed. Daily wind speed data was acquired from a local meteorological station; however, no significant relationship could be identified suggesting dust was an unlikely factor contributing to the inhibition. The time of year when the most inhibition was noted from the end of May through to the middle of June is when pollen levels from trees such as oak, pine and grass are high (National Pollen and Aerobiology Research Unit, University of Worchester). Pollen therefore may have been a significant contributing factor for influencing the reduced performance of the real-time PCR.

A further consideration influencing detection efficiency is the air particulates generated by human and industrial activity. For example, air close to roads and motorways will contain larger quantities of particulates produced by car exhaust fumes (Ma *et al*., 2008) which could lead to reductions in sensitivity. Furthermore, agricultural activities such as spray applications and spray drift could also result in a reduction in the detection efficiency on key dates. Although a measure of the effect of inhibition on LAMP was not determined in this study, reports of LAMP being able to maintain high levels of sensitivity in light of inhibitory compounds has been documented previously (Tomlinson *et al.,* 2010). As seasonal fluctuations in air

particulates have been routinely documented (Sreeramulu, 1959; Mitakakis and Guest, 2001) a quantitative measure of their effect on detection sensitivity would be required to provide a highly accurate spore trapping and detection capability. However, to achieve this additional testing, analysis and therefore time and expense would be required. A better understanding of inhibitory compounds on LAMP performance carried out in-field would be beneficial and may identify that extra steps to elucidate inhibitory effects when using LAMP are unnecessary.

Throughout this study the performance of LAMP with the current gold standard molecular method (qPCR) for the detection of *P. striiformis*, *P. triticina* and *Z. tritici* airborne spores in spore trap samples has been monitored in order to determine if this simple and rapid in-field method can provide comparable levels of detection. LAMP has proved to be a suitable alternative detection method due to its ability to detect target species DNA in conventionally extracted spore trap samples highly comparable to qPCR. In-field testing of LAMP resulted in successful detection of low numbers of target species spores on days when no detection was made using the accompanying trap tested by qPCR. A number of days when spores were detected in the Burkard using qPCR produced no detection by LAMP on samples collected at the same time using the accompanying spore trap. This therefore highlights discrepancies in sampling performance between the two traps rather than sensitivity of the detection techniques as the number of spores quantified were within LAMPs limit of detection. To assist in validating the in-field LAMP result further, additional tests of the spore trap sample tested using LAMP by qPCR would be required.

A direct comparison between the two molecular techniques on the LAMP sample tested in- field was not possible due to the pH of the lysis buffer (0.3 M KOH) being too alkaline for the qPCR reaction to perform effectively. In addition, the time taken between transferal of the in-field sample to the laboratory for clean-up and qPCR would not provide a comparable result due to the extended periods of lysis and potential DNA degradation the sample would have experienced. Experiments should be conducted to discover if the crude DNA extract can be extracted by conventional methods and tested using qPCR. This would need to be carried out immediately following the LAMP testing in a location nearby the spore trap to reduce the influence of additional lysis time. Other solutions which could help provide a more comparable

test would be to source a buffer which is able to provide the high levels of sensitivity comparable to Lyse and LAMP that is also capable of being tested by qPCR or a qPCR mastermix capable of accepting 0.3 M KOH.

In relation to the LAMP reaction performance, a reduction in the TTP produced by the positive controls were observed between the laboratory and the in-field test. It might be assumed this would influence the sensitivity of the test. However, comparative tests carried out using the in-field extract suggests the sensitivity of the LAMP reaction was not significantly impacted due to comparable levels of *P. striiformis* detections made. It is possible the positive control reaction could be compromised when 5 µl 0.3 M KOH containing target DNA is added and present within the reaction over extended periods of time at ambient temperatures.

The apparent differences in target species spores sampled by both spore traps poses significant challenges when considering risk predictions using Burkard multi-vial spore traps in conjunction with molecular testing if variability in detection occurs between spore traps positioned next to each other. A more efficient spore trap is therefore required capable of sampling larger quantities of air to ensure target spore species are not missed.

Interestingly, the automated spore trap tests carried out in 2017 provided good correlation with samples collected by an accompanying Burkard spore trap that were tested by qPCR over the 13 samples collected, than when in-field LAMP was compared to qPCR using two Burkard spore traps in 2016. Although the automated spore trap failed to detect *P. striiformis* spores on days when detection was made by the Burkard using qPCR, evidence suggests *P. striiformis* spores were dispersing at a time the automated spore trap was not switched on because the automated spore trap sampled and detected the highly similar shape and size *P. triticina* spores on days the Burkard did not. The improved sampling and detection capability of the automated spore trap may be a result of the its ability to sample six times more air per minute in addition to having a 360-degree air intake and a cyclone specifically optimised for the sampling and recovery of fungal spores across multiple size ranges. An added benefit to the automated spore trap will be its ability to switch the air intake on, to sample at any given point during the day for pre-determined durations of time. This will reduce the amount of power required to sample large volumes of

air and increase the chances of sampling target spore species specifically if hourly patterns of dispersal can be pre-determined.

One aspect of the automated test which requires further attention is that although detection of target spore DNA was achieved in a number of air samples collected, few of the six test wells per pathogen strip used produced amplification. The mastermix is an expensive component of the molecular test and traditionally testing samples in duplicate is usually sufficient for providing a reliable diagnosis. As a result, to test for a single pathogen using 6 reactions would be expensive at current reagent costings. Work to provide reproducible amplification using LAMP is required to reduce the number of tests needed to determine if a sample is positive or negative. To help address this issue the spore disruption method and the preparation and storage of reagents prior to testing could be investigated further. The potential for LAMP reagents 'shelf life' to be extended over longer periods of time and therefore reduce the number of visits a field operator would need to make in order to replace consumables is evident. For example, the mastermix used for the detection of *P. triticina* maintains a high degree of sensitivity as it was still able to amplify *P. triticina* DNA in the automated spore trap air sample on day four. This adds evidence to suggest the reduced number of *P. triticina* detections made in the in-field test compared to the re-test in the laboratory was influenced by an insufficient spore disruption, benefitting from a longer lysis as a result of the transportation time between the field and the laboratory.

This study has demonstrated the potential of an automated spore trap using LAMP as the primary molecular method to carry out detection in the field in real-time to a standard comparable with a conventional spore trap and PCR test, with early evidence to suggest the automated spore trap is more efficient at sampling fungal spores compared to the Burkard multi-vial cyclone.

One drawback to using spore trapping as a tool for detection of airborne pathogens has historically been the extended periods of time required to process samples and a high degree of uncertainty associated with their ability to detect low spore numbers from what may be an isolated spore release event. This study provides evidence to suggest sampling air at higher volumes over shorter periods of time can achieve comparable levels of sensitivity to current spore trapping

methodologies. Furthermore, LAMP provides a viable alternative to achieve credible detection at point of source rather than facing the limitations associated with transferal of samples to the laboratory. With the ability to sample larger volumes of air, carry out a diagnostic test and report a result remotely, the potential for reliable, early real-time pathogen detection prior to disease development is becoming more realistic.

Author contributions

Conceptualization: IB NB JT

Data curation: IB

Formal analysis: IB

Funding acquisition: NB JT MA WC DM

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Validation: IB

Visualization: IB

Writing – original draft: IB

Writing – review & editing: IB JT NB MD

Chapter 5 . Spore detection and disease forecasting for improved risk assessment of *P. striiformis*, *P. triticina* and *Z. tritici* in winter wheat

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5.1 Abstract

Timing of fungicide applications for control of air-dispersed fungal plant pathogens of winter wheat is traditionally based on calendar day, crop growth stage, weather and/or disease incidence. Effective treatment requires accurate knowledge of disease risk in order to target the pathogen prior to symptom development whilst avoiding the use of unnecessary sprays. Due to the lack of information regarding spore presence, the potential for fungicides to be applied in the absence of infectious inoculum is high, particularly in the early part of the season.

To investigate the relationship between spore dispersal, infection and subsequent disease incidence, a network of UK winter wheat field trial sites was studied in 2015. Burkard multi-vial cyclonic spore traps were positioned at four untreated winter wheat field trial sites and real-time PCR used to detect DNA of *Puccinia striiformis, P. triticina* and *Zymoseptoria tritici* in daily spore samples. Potential risk periods for infection were identified using an infection risk model in conjunction with daily spore counts, estimated pathogen latency duration and disease incidence data. Results were evaluated to measure the contribution of spore load to accuracy of existing risk models. In total, 76.6 % of leaf layers infected by either *P. striiformis, P. triticina* or *Z. tritici* could be associated with an infection risk alert and spore detection during the 7-day infection windows identified. The remaining 23.3 % of leaf layers expressing disease symptoms could only be associated with spore detections. Correlations between spore numbers detected at each site and severity of disease observed on leaf two were the highest in March for all three pathogens, suggesting early season spore activity had an influence on the

epidemic. This study provides evidence to suggest in-season spore detection could be used to improve current risk forecasts for foliar diseases by increasing or decreasing risk alerts based on spore presence, with more attention being directed to early season spore monitoring. As a result of an improved disease risk forecasting system, farmers and agronomists can make more informed decisions regarding the timing of fungicide applications.

5.2 Introduction

Winter wheat yields are beginning to decline on a global scale as yield ceilings are reached on an increasing proportion of agricultural land. Despite this the demand for the commodity is high and predicted to increase (FFF report, 2011). Between 1940 and 1990 the UK national average yields of winter wheat rose from 2.7 t/ha to 7.6 t/ha because of the green revolution, but have remained stable ever since (Food Security Report, DEFRA 2014). The total area of wheat in the UK is valued at £2 billion p/a grown over approximately 1.9 million hectares.

Control of fungal plant pathogens in commercial winter wheat crops relies heavily on the application of fungicide sprays applied throughout the growing season (Waggoner and Berger, 1987). This study showed a correlation between healthy area duration (HAD) loss on the top 3 leaves and yield loss for wheat. As a result, particular attention needs to be directed towards protecting the main yield contributing leaf layers which include the top 3 leaves. The degree of inoculum pressure around the time of year these leaf layers emerge in April and May is influenced by the survival of fungal pathogens over winter and control of early infectious airborne inoculum. Despite all the efforts used to date to time applications appropriately, severe epidemics still frequently occur (DEFRA surveys, http://www.cropmonitor.co.uk).

Incidence and severity of the most common fungal pathogens of wheat are routinely controlled by the application of fungicides. The average number of sprays applied to a crop of winter wheat has increased steadily over the last decade, yet epidemics still persist (DEFRA surveys, http://www.cropmonitor.co.uk). There is a need to understand the interaction between the presence of inoculum in the air within

a wheat field and the subsequent development of symptoms following successful spore deposition, infection and establishment. Understanding this is crucial in order to avoid unnecessary use of fungicides through better selection and optimal timing of application. Three of the most economically significant air dispersed fungal plant pathogens of winter wheat are *Puccinia striiformis* (yellow rust), *P. triticina* (brown rust) and *Z. tritici* (septoria leaf blotch). The most prolific of these within the UK winter wheat crop is *Z. tritici* and can cause yield losses of up to 50 % in susceptible cultivars (Ziv and Eyal, 1978).

Initial infection of a newly emerged crop of winter wheat by *Z. tritici* is thought to primarily be caused by wind dispersed sexual ascospores resulting from sexual reproduction of the fungus on winter wheat debris (Shaw and Royle, 1989). The most significant reductions in winter wheat yield caused by *Z. tritici* are understood to be a result of the splash dispersed asexual pycnidiospores produced on the lower leaves. It has been estimated that polycyclic asexual pycnidiospores can cycle 6 times on a maturing crop. As a result, large quantities of inoculum can develop and has been estimated at $10^{10\text{-}11}$ spores per hectare (Fones and Gurr, 2015).

P. striiformis (yellow rust) and *P. triticina* (brown rust) are obligate pathogens and can cause common and widespread disease of winter wheat grown around the world. In the UK, survival of the pathogens is reliant on volunteer plants and crop debris to bridge the gap between harvest and the following crop. This phenomenon, known as the 'Green Bridge', is supported by evidence obtained from a spore trapping study on the airborne inoculum of *P. triticina* in Belgium (Duvivier *et al*., 2016). Spread by windblown uredospores, the pathogen can travel for thousands of kilometres from source (Brown and Hovmoller, 2002). Rust epidemics tend to be more sporadic in nature compared to *Z. tritici* due to more specific conditions required for sporulation and infection. A good example of this is in 2007 when a severe unpredicted *P. triticina* epidemic developed in the UK resulting in substantial yield loss. Prediction of such an epidemic may have been made possible through sampling airborne spores escaping from the crop earlier in spring (West *et al*., 2008).

The presence of air-borne plant pathogens can be determined using spore traps and DNA based detection systems (Bayliss and Jackson, 2011). In addition, understanding pathogen dispersal characteristics in relation to weather conditions can be studied in detail over a prolonged time period (Duvivier *et al*, 2016; Parker *et al*., 2014; Jones and Harrison, 2004). Molecular diagnostics are making it more feasible to carry out long term spore trapping studies by providing the ability to process large sample numbers in a relatively short amount of time using automated laboratory equipment. Detection of air borne spores of *P. striiformis* using spore traps and real-time PCR has successfully been related to the development of *P. striiformis* observed about 15 days post detection (Dedeurwaerder *et al*., 2011). Such data are important for assisting in validating infection risk forecast models.

Accurate prediction of epidemics is crucial to enable the application of fungicides at the optimum time. Currently infection risk forecast models are a useful resource to assist growers with the control of fungal plant pathogens. Such models combine detailed information on pathogen epidemiology gathered both in the laboratory and in the field, alongside meteorological data to determine when conditions are conducive for infection. However, information regarding inoculum presence has potential to substantially improve disease forecast models. Research has shown PCR assays used for the detection of inoculum in spore trap samples can enhance disease management through more targeted fungicide applications (West *et al*., 2008, Gent *et al* 2009). Many models however lack actual spore data for either validating historic predictions or for assisting with current in season forecasting. CropMonitor™ (www.cropmonitor.com) is currently the only UK decision support system that provides 'live' infection risk estimates for crop diseases. A recent analysis shows that the risk alerts of CropMonitor™ are seldom too late, but often too early (unpublished data). This might be at least partly due to limited inoculum availability. Here we investigate the potential to improve infection risk models through the incorporation of data on *P. striiformis, P. triticina and Z. tritici* spore presence.

5.3 Methods

5.3.1 Field trial sites

Four winter wheat field trial sites (Stokesley, Latitude 54.483294°N; Longitude 1.1604585°W, Haywold, Latitude 53.986022°N; Longitude 0.59551084°W, Alford Latitude 53.2635 °N; Longitude 0.18653691 °W and Wansford Latitude 52.547627 °N; Longitude 0.43445349 °W) were selected for study (Figure 5-1) from a wider network which provides national and regional coverage of the main wheat growing areas in England. In 2014, fifteen plots measuring 1.5 m x 12 m were sown with 5 varieties of winter wheat replicated 3 times, with the individual varieties having differing levels of resistance to the main wheat pathogens (Table 5-1). An Oyjørd drill was used at a rate of 350 seeds/m^2 to achieve a target plant population of 200-250 plants/m^2. Buffer plots were incorporated at the end of replicate blocks to ensure no overspray from commercial field fungicide applications. Crops were managed to obtain peak canopy sizes with a green area index (GAI) in the range of 6-7 and received no fungicide application. Other treatments (plant growth regulators, fertiliser, micronutrients, herbicides, insecticides and molluscicides) followed standard farm practice. Detailed examination of epidemic progress on the five wheat varieties was recorded on a weekly basis from GS31 (The Decimal Code system for wheat growth stages using the prefix GS is described in Zadoks *et al*., (1974)). Ten shoots from each plot were sampled and assessed for disease and green leaf area on each fully emerged leaf layer.

Table 5-1 Varieties used and their resistance to disease, derived from the HGCA Recommended List 2015/16. The resistance rating ranges between 1 and 9, whereby ratings of ≤ 4 refer to susceptible, ratings of 5 to 7 refer to moderately susceptible and ratings ≥ 8 refer to resistant cultivars, respectively

Cultivar	*P. striiformis*	*P. triticina*	*Z. tritici*
Cordiale	5	4	5
Cougar	8	9	7
KWS Gator	9	3	5
KWS Kielder	4	8	5
KWS Santiago	6	6	4

5.3.2 Spore trapping

A single Burkard multi vial cyclone spore trap was located at each trial site. Air was sampled at a rate of 16.5 litres per minute into 1.5 ml microcentrifuge tubes. The spore trap was positioned at a height of 1.5 meters. Automated sample tube changeover was programmed to occur every 24 hours at midnight. Operators attended the device on a weekly basis to replace the sample tubes and battery. Samples where then sent to the laboratory for analysis by qPCR.

5.3.3 DNA extraction of air samples

To each sample a mixture of different sized zirconia silica beads was added (approximately 0.2 g). Subsequently 150 µl of CTAB lysis buffer (120 mM sodium phosphate buffer pH 8, 2 % CTAB, 1.5 M NaCl, 1 % antifoam B emulsion) and 10 µl RNase 'A' were then dispensed over the sample followed by homogenisation at full speed on a Vortex Genie. An extraction blank was included in each batch of samples extracted to monitor for any evidence of cross contamination. Sample tubes were then placed in a hot block at 65 °C for 15 minutes. The resulting supernatant was then passed through a Nucleospin Plant 2 Kit (Machenery-Nagel, Germany).

5.3.4 Quantitative PCR

All qPCR primers and probes for *P. striiformis, P. triticina* and *Z. tritici* used were as described in Chapter 2 (Table 5-2).

Table 5-2 qPCR primers and probes used for quantification of spore numbers in daily air samples

Pathogen	Name	Sequence (5'-3')	Product size (bp)
Puccinia striiformis	F	GAGTGTCATGAAACCCTCTCATT	
	R	CAAGTCAATCCAACTTATAGAAAAG	122
	Pe	CAATGGATGTTGAGTGCTGCTGTAATTAGC	
P. triticina	F	AAGTAGCAATACTTGCCATCTTG	
	R	TTATTGATATGCTTAAGTTCAGCG	117
	Pe	TTTCCAAAATTTAAGACCTCAAATCAGGTGGGACT	
Zymoseptoria tritici	F	GACCCTGCCGGGCGC	
	R	AAAGTTTTGTTTCGATTTACTCGTAAA	84
	Pe	CCAAAAAACACTGCATCTCTGCGTCGGA	

An Applied Biosystems 7500 real-time PCR machine was used to test each sample in duplicate using Environmental MasterMix (TaqMan® Environmental Master Mix 2.0) with the following cycling conditions: 50 °C for 2 min, 95 °C for 10 min, and 45 cycles of 95 °C for 15 s and 65 °C for 1 min. Reaction volumes comprised of 20 µl master mix and 5 µl sample. Each sample was tested in duplicate and the average cycle threshold obtained.

5.3.5 Production of standards

Uredospores of *P. striiformis* and *P. triticina* were supplied by the University of Nottingham. *Z. tritici* spores were isolated from infected wheat leaves then cultured on PDA to bulk up the inoculum. Spore suspensions were then created through the addition of 0.1 % Tween and the number of spores in suspension calculated and adjusted using a haemocytometer. Spore numbers were aliquoted into 1.5 ml microcentrifuge tubes at numbers 10, 10^2, 10^3 and 10^4 and stored at -12 °C until required.

5.3.6 Meteorological data and risk forecast modelling

Meteorological data for modelling was obtained from the UK MetOffice's DataPoint service (http://www.metoffice.gov.uk/datapoint) which provides free access to a range of weather information services. Fera Science Ltd. makes extensive use of the service which provides hourly site-specific observations from approximately 140 locations across the UK. The records are accessed by a daily automated web service which feeds data into a dedicated database maintained at Fera and therewith builds into a continuous hourly record for all locations. The parameters included in the DataPoint service are temperature (degrees Celsius), wind direction (16 point compass), wind speed (mph), wind gust (mph), dew point (degrees Celsius), screen relative humidity (%), weather type, visibility (m), pressure (hPa) and pressure tendency (Pa/s). Hourly weather data can therefore be obtained for any point in the growing season from locations both close to and remote from the spore monitoring sites.

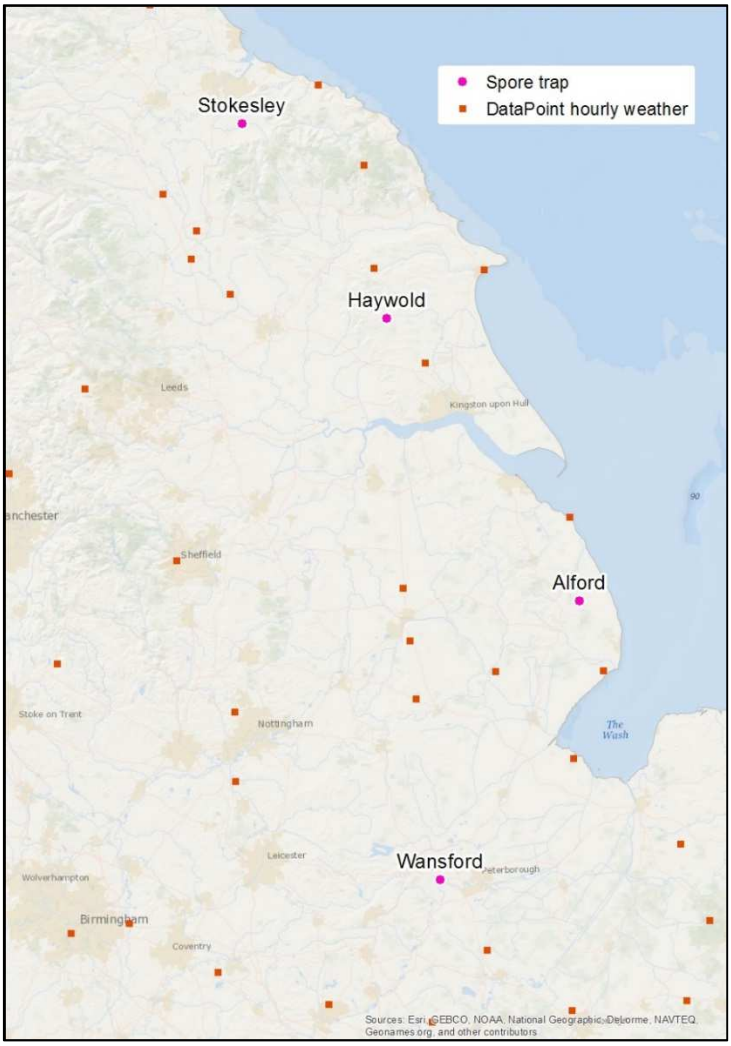


Figure 5-1 The locations of the hourly site-specific weather observation sites across eastern and north-eastern England in 2015 are shown in relation to the spore trap locations. The distance to the nearest weather observation site to each spore trap location was 20 km (Alford), 16 km (Haywold), 24 km (Stokesley) and 4 km (Wansford)

CropMonitor™ (www.cropmonitor.co.uk) is a web-based information service hosted by Fera which provides daily estimates for infection risk, whereby risks are classed as either low, medium or high.

The underlying risk estimates are determined according to decision tree algorithms, which are based on specific thresholds for air temperature, relative humidity and rainfall. The exact algorithms used are subject to IP and cannot be disclosed. However, the thresholds used were derived and applied using methods like those described by Jarroudi *et al* (2017). These algorithms have been used to

identify infection risk periods for the four sites for use in analysis of the role of spore data in interpreting and improving disease risk predictions.

In this chapter analyses focus on risk alerts for a highly susceptible cultivar for each of the three pathogens, namely KWS Santiago (*Z. tritici*), KWS Kielder (*P. striiformis*) and KWS Gator (*P. triticina*). The performance of the infection risk model was analysed according to whether the inferred date of infection was strongly correlated with the presence of high (and medium) infection risk alerts. The inferred infection time was determined by tracing back a latent period in degree days from the date of first symptom appearance (160, 120 and 280 degree days for *P. striiformis*, *P. triticina* and *Z. tritici*, respectively). Data analyses were then extended to assess to what extent the model performance could be improved by the inclusion of information on spore presence. The model performance was assumed to have improved if there was a closer match between inferred infection periods and the simultaneous presence of a high (or medium) risk alert and spore presence versus the inferred infection period and the presence of a high (or medium) risk alert alone. As observations were carried out weekly and disease symptoms may have been expressed up to one week prior to the assessment, a single infection period of up to 7 days had to be considered.

5.4 Results

5.4.1 Quantification of pathogen DNA

The concentration of target species DNA in daily spore trap samples identified using qPCR was quantified in terms of spore numbers using a set of standards comprised of DNA extracted from spore numbers consisting of 10, 10^2, 10^3 and 10^4 spores. Spore suspensions produced for *Z. tritici* were comprised of pycnidiospores. As pycnidiospores contain between four and eight nuclei and ascospores are binucleated cells, whereas the spore trap is expected to sample predominantly ascospores due to its height above the crop, the estimated number of spores per sample was multiplied by three (Shaner, 2010, Duvivier *et al.,* 2013, Morais *et al.,* 2015). A

strong linear relationship was identified between spore number and cycle threshold values output by the real-time PCR software throughout (Figure 5-2).

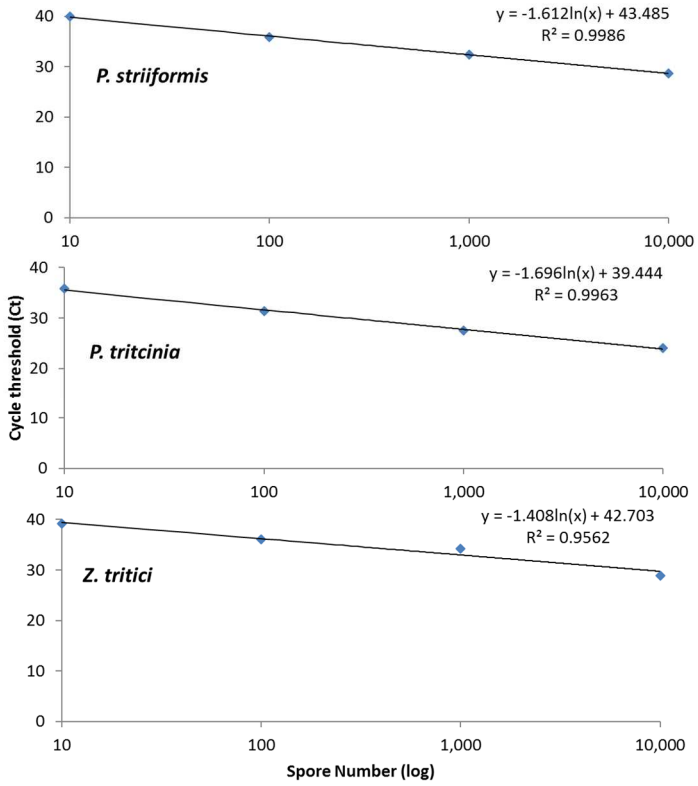


Figure 5-2 Example of relationships between spore number and cycle threshold for the three target pathogens

5.4.2 Site-specific detection of spore loads and relationship with disease observed

P. striiformis

In total approximately 156,479 uredospores of *P. striiformis* were detected across the 4 spore trapping sites with 87.3 % of these detections made at Alford, 10.9 % at Wansford, 1.1 % at Haywold and 0.2 % at Stokesley (Table 5-3). The highest amount of disease observed on leaf 2 was at Wansford, however, much larger quantities of inoculum were detected at Alford. A total of 0.2 % of *P. striiformis* spores were detected in March at Wansford which equated to approximately 378 spores. No spore detections were made at Alford in March. No relationship between total number of spores detected at each site and the disease observed on leaf 2 was

identified (R^2 = 0.03, Figure 5-3). There was however a positive correlation between monthly percentage of *P. striiformis* spores detected in March and final percentage of disease observed on leaf 2 at the end of June (R^2 = 0.1). A positive relationship between the total monthly number of risk alerts forecast and disease observed on leaf 2 was identified in March and May (R^2 = 0.8).

P. triticina

Approximately 128,929 uredospores of *P. triticina* were detected over the spore trap network with 62.6 % of detections made at Haywold, 28.5 % at Wansford, 8.6 % at Alford and 0.2 % at Stokesley (Table 5-3). *P. triticina* spores were detected as early as March at Wansford and Alford, April at Haywold and June at Stokesley. Disease was only observed at Wansford and Alford at trace levels. A weak relationship between total number of spores detected at each site and the disease observed on leaf 2 was identified (R^2 =0.13, Figure 5-3). The strongest correlation between monthly percentage of *P. triticina* spores detected at each site between March and June and final percentage of disease observed on leaf 2 at the end of June was in March (R^2 = 0.6). A positive relationship between the total monthly number of risk alerts forecast and disease observed on leaf 2 was identified in May (R^2 = 0.8)

Z. tritici

In total approximately 33,624 spores of *Z. tritici* were trapped across the 4 sites. The largest number of spores detected occurred at Wansford during June at 20 % followed by 13 % at Stokesley in April, 9.7 % at Alford in April and 6.3 % at Haywold in June (Table 5-3). *Z. tritici* symptoms were identified below 10 % on leaf two at all sites. There was no correlation between total number of spores detected at each site and the disease observed on leaf 2 (R^2 =0.08, Figure 5-3). The strongest correlation between monthly percentage of *P. triticina* spores detected at each site between 16 March and 30 June and final percentage of disease observed on leaf 2 at the end of June was in March (R^2 = 0.91). A positive relationship between the total monthly number of risk alerts forecast and disease observed on leaf 2 in March (R^2 = 0.8), May (R^2 = 0.4) and June (R^2 = 0.6) was identified.

Table 5-3 Monthly site-specific percentage of spores detected across the trapping season from 15 March 30 June 2015

		% Spores released			
	Month	Wansford	Alford	Haywold	Stocksley
P. striiformis	March	0.2	0.0	0.0	0.2
	April	0.7	0.4	0.0	0.0
	May	1.3	40.3	0.0	0.0
	June	8.6	47.0	1.1	0.0
Final % leaf area 2 affected		33.3	17.6	47.1	0.1
P. triticina	March	0.1	0.1	0.0	0.0
	April	1.2	0.4	0.2	0.0
	May	7.8	1.2	2.8	0.0
	June	19.4	6.9	59.5	0.2
Final % leaf area 2 affected		0.3	0.6	0	0
Z. tritici	March	3.1	3.9	2.5	1.3
	April	5.9	9.7	3.7	13.2
	May	8.5	6.9	3.2	0.5
	June	20.0	6.4	6.3	4.8
Final % leaf area 2 affected		6.6	9	7	2

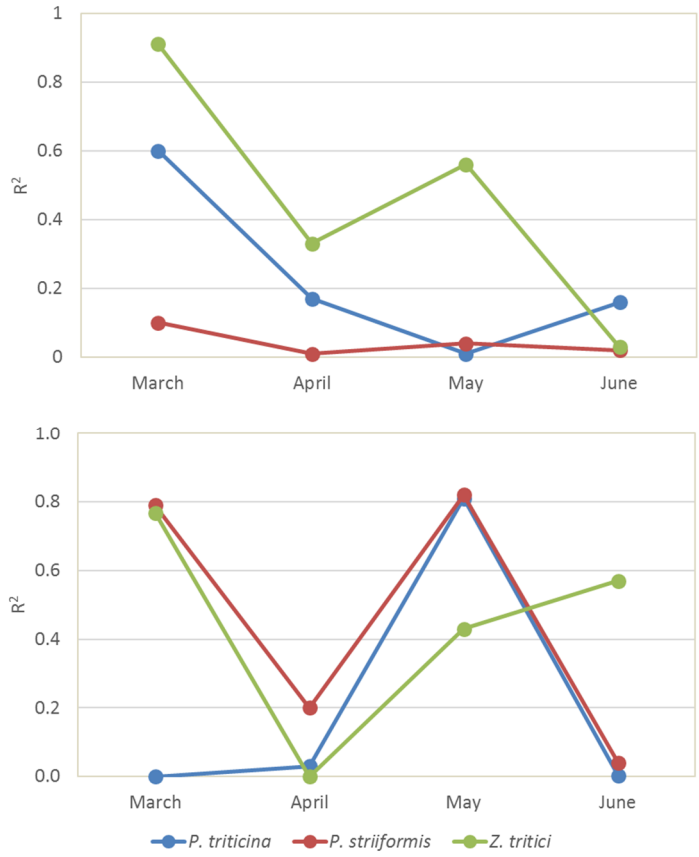


Figure 5-3 Relationship between final percent disease observed on leaf 2 with monthly percentage of spores detected (top) and number of days risk alerts forecast (bottom)

5.4.3 Evaluation of combined infection risk and spore loads for disease prediction

Data from two sites (Wansford, Alford) were analysed to examine the potential correlation between combined risk periods and spore presence data and disease development for the three target pathogens. Graphs in Figures 5-4 to 5-6 show the relationship between risk alerts (medium/high), the inferred risk period (a 7-day window adjusted according to latent period relative to first symptom) and spore load. When analysing the data for the two remaining sites (Haywold, Stokesley) it became clear that technical problems reported in the field has influenced the daily sampling duration resulting in a potential reduction in the number of spores sampled per day. As a result, spores primarily released in the early hours of the morning were potentially missed and could explain the low number of *P. striiformis* spores detected

at Haywold despite the large epidemic which emerged. Therefore, a more detailed analysis was not carried out.

P. striiformis

Wansford - The first symptom of *P. striiformis* was observed at Wansford on the 13th of April on eventual leaves 5 and 6 (Figure 5-4). Taking into consideration a latent period of 160 degree days for *P. striiformis,* infection of leaf 5 and 6 would have occurred between the 18th and 25th of March. Within this infection period spores were detected on 6 days and two medium and one high risk alert was output by the infection risk model. Spores were detected on all 7 days during the infection period identified for leaf 4 however no infection risk alerts were forecast. However, additional infection periods were identified for leaves 3, 2 and 1 when spores were also trapped on those days. Over the total spore trapping period 25 of the 34 medium-risk and 6 of the 11 high-risk alerts coincided with spore detections. Symptoms were identified on all 6 leaf layers but whereas the epidemic on leaf 4 was relatively small and late, the epidemics on leaf 3 and 2 were very severe, with up to 40 % leaf area expressing symptoms on leaf 3.

Alford - Initial *P. striiformis* pustules were observed on leaf 6 at Alford on the 20th of April (Figure 5-4). An infection period was identified between the 25th and the 31st of March corresponding with 4 medium and 2 high infection risk alerts. Few spore detections were made during March, however low numbers of *P. striiformis* spores were detected on the two high risk days falling within the infection window. Similar relationships can be discerned for leaves 5 to 2 due to frequent spore detections corresponding with infection risk alerts. Over the total spore trapping period, 28 of the 39 medium-risk and 9 of the 16 high-risk alerts output by the model had corresponding spore detections made. *P. striiformis* symptoms were observed on all 6 leaf layers, with 20 % of the leaf area expressing symptoms on leaf 3.

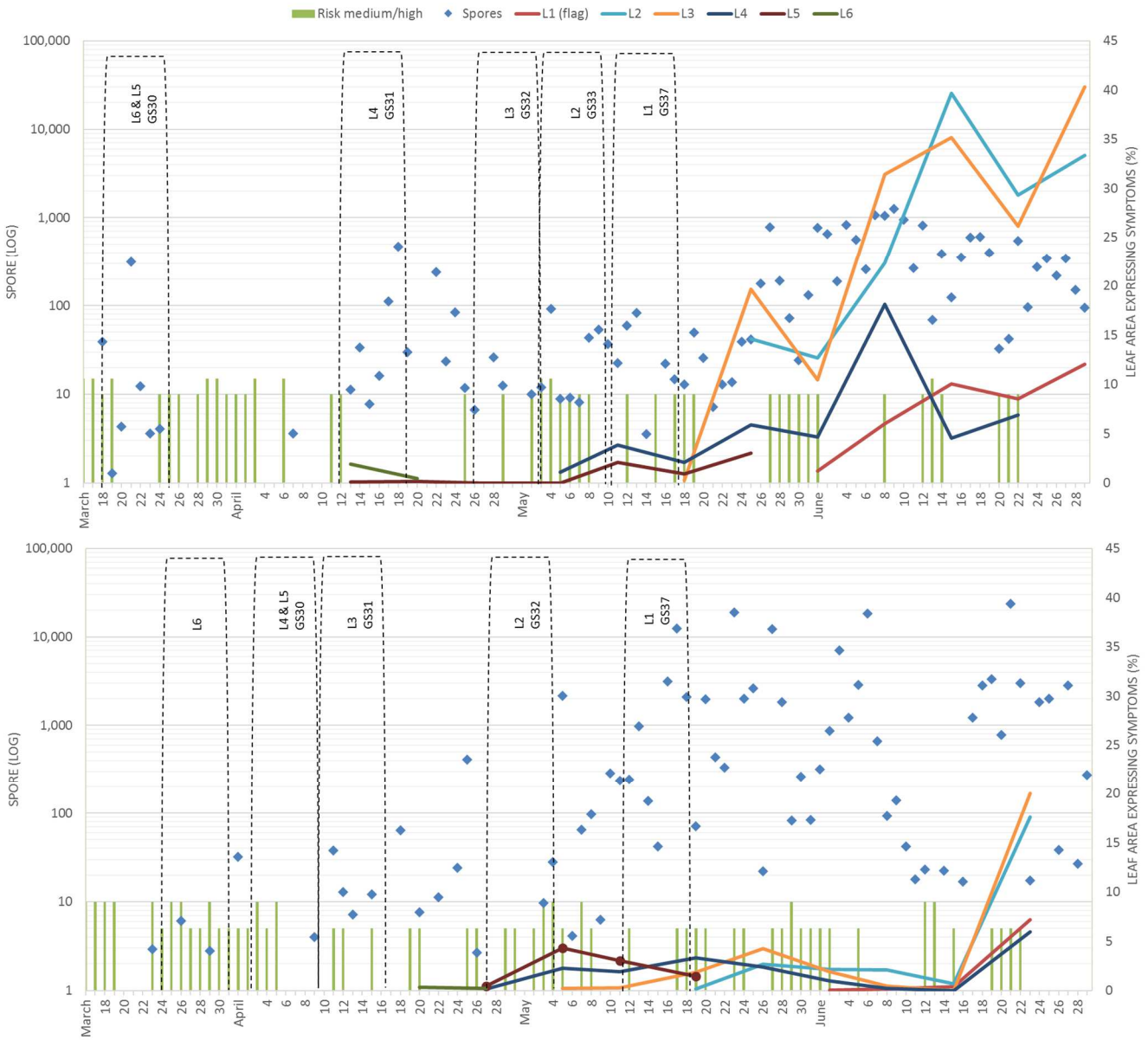


Figure 5-4 Relationship between the detection of* P. striiformis *spores from 16 March to 30 June at Wansford (top) and Alford (bottom) in 2015 to risk alerts forecast and disease symptoms observed in winter wheat field trials over the same monitoring period. The expected periods of infection are highlighted for each leaf layer that disease symptoms were observed

P. triticina

Wansford - *P. triticina* symptoms were first identified on the 11th of May on eventual leaf 3 (Figure 5-5). Many detections of *P. triticina* spores were made prior to the first symptoms, however, the estimated infection risk during this period (April and May) was low. Over the total spore trapping period 6 of the 8 medium and the single high-risk alert output by the model had corresponding spore detections. Disease was recorded on leaves 1, 2, 3 and 4, but always at trace levels, with no significant epidemics developing on any of the leaf layers.

Alford - Trace levels of *P. triticina* symptoms were identified at on the 23rd of June on leaves 4 to 2 (Figure 5-5). Only 2 medium risk periods were recorded prior to symptoms occurring on the 12th and 13th of June however no spore detections were made on either of these days. *P. triticina* spores were however detected on 4 consecutive days prior to the two medium risk alerts during an infection period that resulted in *P. triticina* symptoms at trace levels being observed on leaves 2, 3 and 4. Note also that the only two risk alerts given by the model prior to symptom expression fell within the inferred infection period. During the last week of spore trapping, 5 medium risk alerts were forecast each with corresponding spore detections.

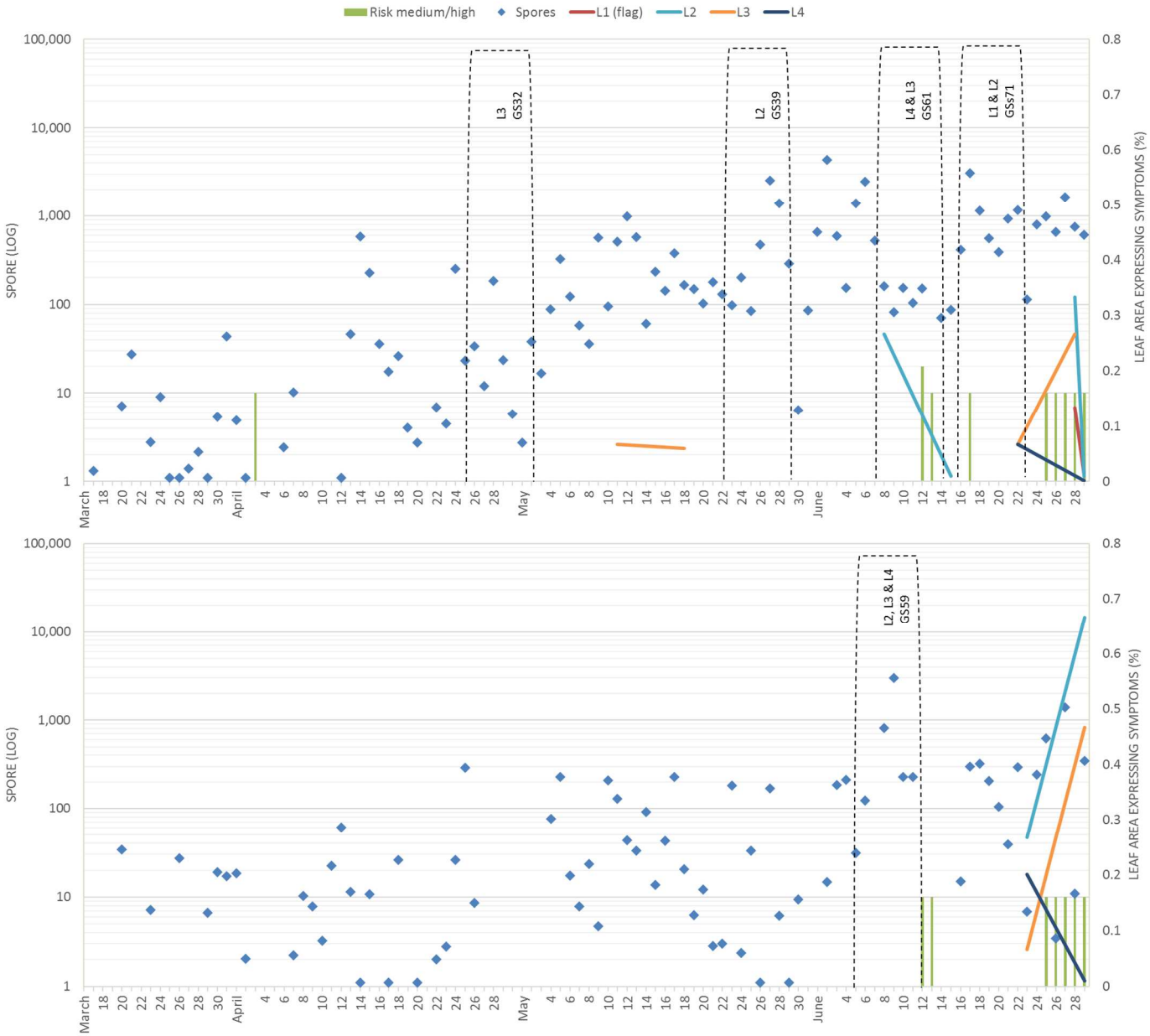


Figure 5-5 Relationship between the detection of* P. triticina *spores from 16 March to 30 June at Wansford (top) and Alford (bottom) in 2015 to risk alerts forecast and disease symptoms observed in winter wheat field trials over the same monitoring period. The expect periods of infection are highlighted for each leaf layer that disease symptoms were observed on

Z. tritici

Wansford *Z. tritici* was first observed on the 13th of April on leaf 6 and the 20th of April on leaf 5 (Figure 5-6). For leaf 6 the corresponding period of infection could not be identified; infection could have occurred prior to sampling. Disease symptoms identified on L4, L3 and L1 were however associated with an infection period during which spores were detected and conditions for infection were estimated to be favourable. Over the total spore trapping period 19 of the 21 medium-risk and 9 of the 16 high-risk alerts output by the model coincided with spore detections. *Z. tritici* symptoms were recorded on all leaf layers with 36 % of the leaf area expressing symptoms on leaves 4 and 3.

Initial incidence of *Z. tritici* at Alford was observed on the 20th of April on leaves 6 and 5. No infection period could be identified for these leaves suggesting that infection occurred prior to the start of sampling. Incidence was later recorded on leaf 4 on the 27th of April and an infection period was identified during which spores were detected and a medium and high-risk alert provided on the 26th and 28th of March, respectively. Spores were detected frequently throughout the season in conjunction with infection risk alerts. Incidence on L2 and L1 can be associated with an infection period however no risk periods were identified for the inferred infection period for L3. *Z. tritici* symptoms were identified on all leaf layers reaching 27 % leaf area infected on leaf 4.

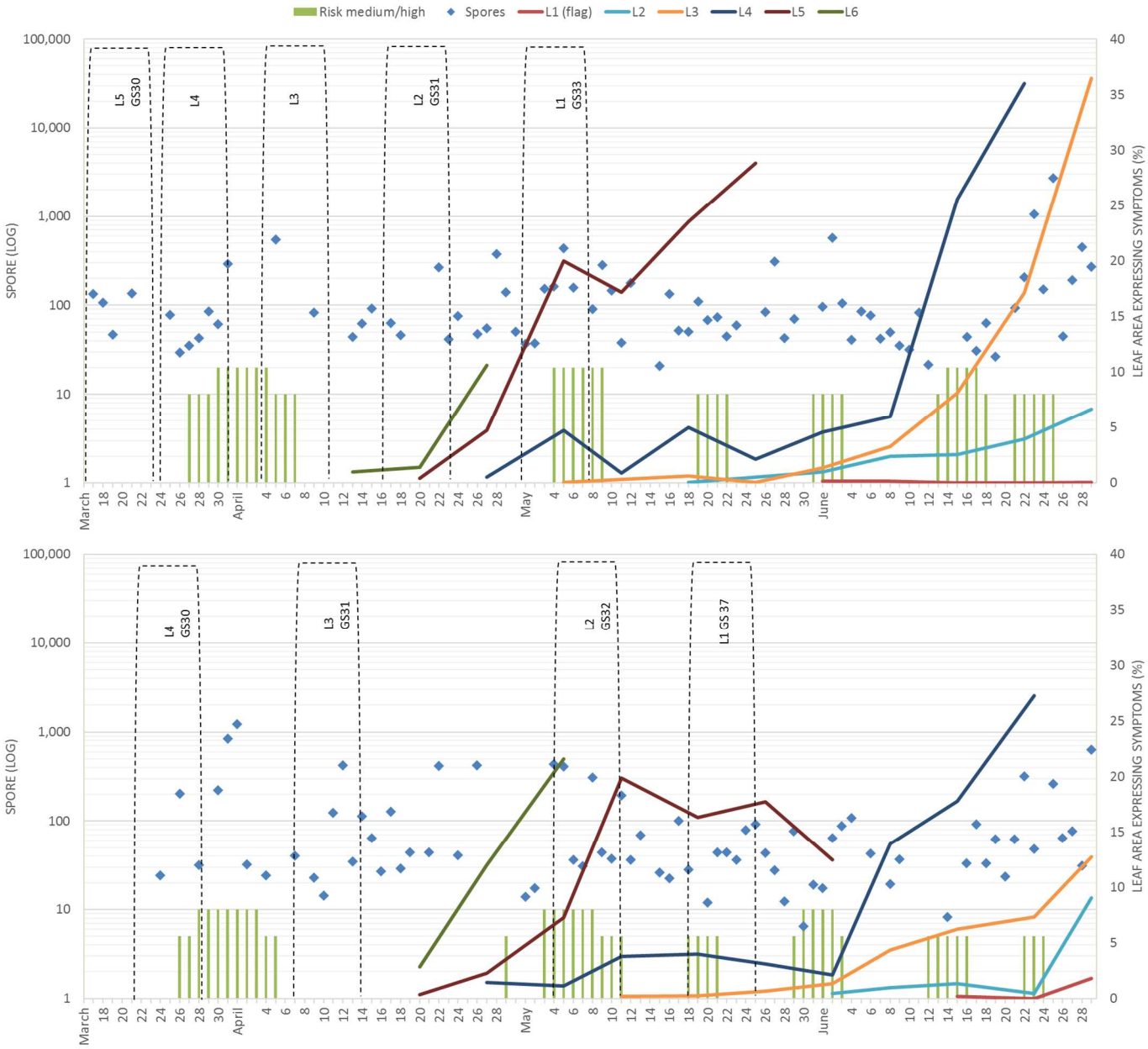


Figure 5-6 Relationship between the detection of* Z. tritici *ascospores from 16 March to 30 June at Wansford (top) and Alford (bottom) in 2015 to risk alerts forecast and disease symptoms observed in winter wheat field trials over the same monitoring period. The expected periods of infection are highlighted for each leaf layer that disease symptoms were observed

5.4.4 Relevance of coincidence of spore detection and risk alerts for identification of disease risk

P. striiformis

A total of 70 days over 10 infection periods (comprising 7 days each) were identified when infection could have occurred resulting in the *P. striiformis* disease symptoms observed on all 12 leaf layers assessed at Wansford and Alford (6 per site). Over the infection periods, 48 daily spore detections were made, and 38 infection risk alerts forecast. A total of 8 periods of potential infection (9 leaf layers) were successfully identified when risk alerts and spore detection occurred on the same day within the 7-day infection window (Figure 5-7). There was one infection period when large quantities of *P. striiformis* spores were detected but no infection risk alert was forecast (1 leaf layer) and one infection period when risk was forecast prior to spore detection (2 leaf layers). No infection periods were identified when risk alerts alone were forecast that resulted in infection.

P. triticina

A total of 35 days were identified when infection could have resulted in the *P. triticina* disease symptoms observed on 9 of the 12 leaf layers assessed between Wansford and Alford. Of these, 31 daily spore detections were made, and 5 infection risk alerts forecast. Only 2 infection periods could be identified resulting in disease (on 4 leaf layers) when spores and risk occurred on the same day. Infection on a further three leaf layers can be associated with infection periods when risk alerts occur after spore detection and two when only spores are detected within the same 7-day infection window.

Z. tritici

A total of 63 days were identified when infection could have occurred resulting in *Z. tritici* symptoms on 9 of the 12 leaf layers assessed at the two sites. Of these, 46 daily spore detections were made, and 24 infection risk alerts forecast. Six infection periods were identified when spores and risk occurred on the same day resulting in disease (on 6 leaf layers). There were no instances when risk alerts occurred on days

after spore detection within the 7-day infection window. There were no infection risk alerts forecast during the further two infection periods associated with the remaining three leaf layers however daily *Z. tritici* spore detections were made.

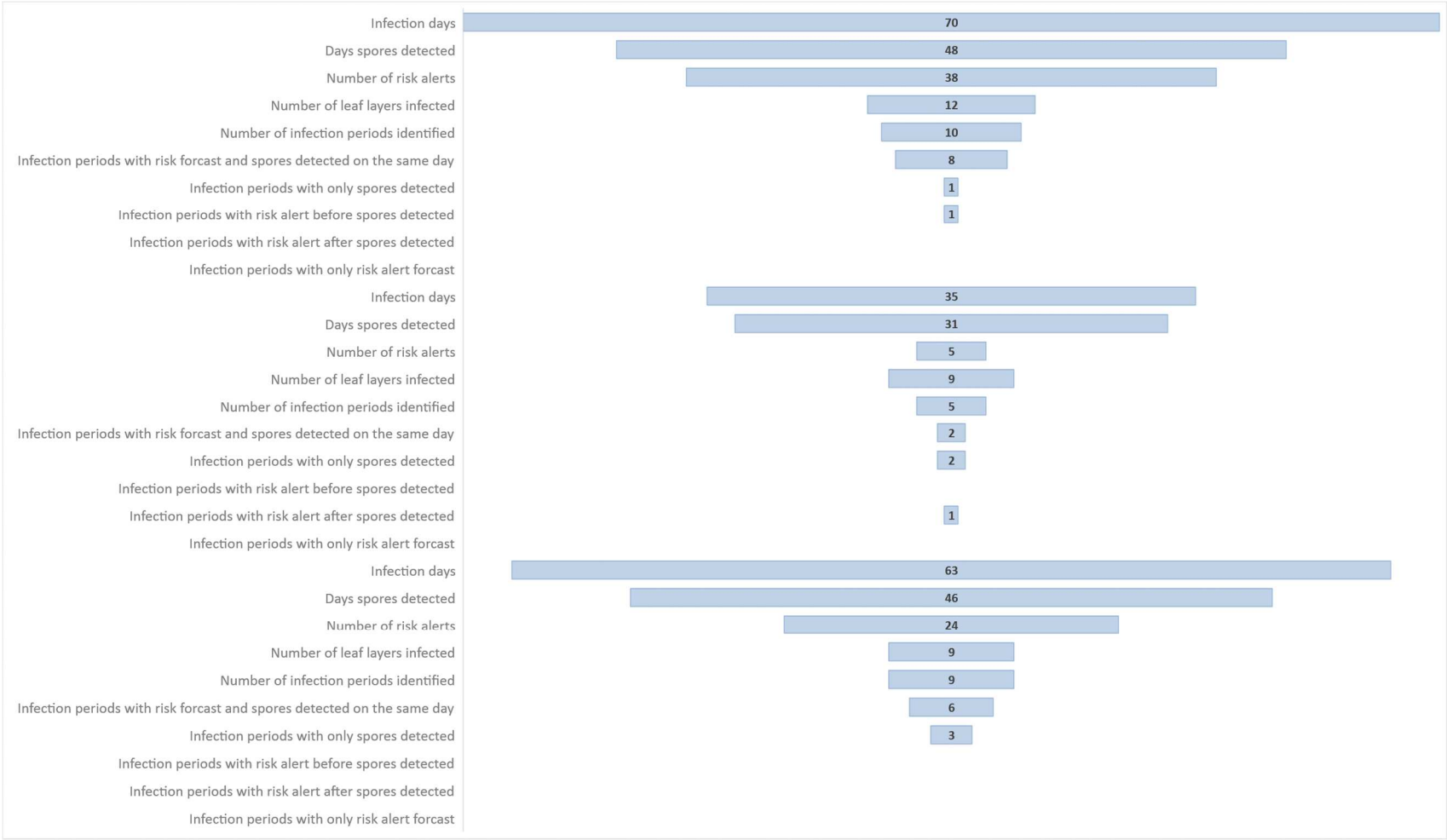


Figure 5-7 Comparison of the relationship between days spores detected and risk forecast during the infection periods identified across the monitoring period associated with infection of* P. striiformis *(top),* P. triticina *(middle) and* Z. tritici *(bottom) identified on different leaf layers. Data combined from Wansford and Alford only

5.5 Discussion

This study implements spore detection using Burkard spore traps and laboratory based qPCR with the data analysed in conjunction with an infection risk forecast model and in-field disease observations to identify if the technology can improve current risk forecasts. Four Burkard spore traps were programmed to sample air continuously across 4 separate areas in the UK between 16 March and 30 June 2015. Periods of infection of winter wheat by *P. striiformis, P. triticina* and *Z. tritici* spores were identified in the data by back-tracking a latent period from when disease symptoms appeared on each leaf layer. These inferred infection periods were then assessed for simultaneous spore presence and risk alerts as predicted by the infection risk model. The majority of cases for which the inferred infection period coincided with spore presence and an infection risk alert occurred was at Alford and Wansford. Fewer successfully predicted infection periods could be discerned at the two most northern sites of Stokesley and Haywold and was caused by technical issues experienced with the spore traps sampling duration. As a result, only the data from Wansford and Alford was used for a more detailed analysis.

The quantitative PCR methods developed in this study proved to be highly sensitive for the detection of *P. striiformis, P. triticina* and *Z. tritici* spores. Cycle threshold values from standards comprised of spore numbers of 10^4, 10^3, 10^2 and 10 were highly repeatable and reproducible.

Spore detections were made on 73.1, 79.2 and 70.2 % and risk alerts forecast on 47.2, 7.5 and 35.8 % of the days monitoring was carried out between 16 March and 30 June for *P. striiformis, P. triticina* and *Z. tritici* respectively. *P. striiformis* achieved high levels of infection in the untreated winter wheat plots at all but the most northerly site of Stokesley. *P. triticina* was identified at trace levels at Wansford and Alford only. *Z. tritici* achieved high levels of infection on earlier leaf layers however a relatively low severity was recorded on leaf two at the end of the monitoring period. The high number of *P. striiformis* spores and infection risk forecasts supports the high severity of *P. striiformis* observed in the crop. National disease survey data on *P. striiformis* showed 2015 as only the 3rd year when no incidence of the disease was identified in commercial samples surveyed since 1970.

Reports early in the season were frequent suggesting control in commercial crops must have been effective at supressing symptoms prior to the time of surveying in late July. To support this theory *P. striiformis* inoculum and risk days appeared to coincide with routine standard spray application windows (T1, T2 and T3), determined by crop growth stage. The low severity of *P. triticina* recorded reflects the small number of suitable days for infection forecast rather than the high inoculum presence detected. Disease incidence in 2015 across the UK for *P. triticina* was lower than the long-term average (2001 – 2010) as identified by the national winter wheat disease survey (CropMonitor, FERA). A relatively low number of *Z. tritici* spores were detected over the season in comparison to *P. striiformis* and *P. triticina* spores. In general severity of *Z. tritici* in commercial crops was low in 2015. Mean infection recorded on the flag leaf was 0.3 % and 1.3 % on leaf 2 compared to the long-term average of 1.0 % and 3.7 % respectively (DEFRA surveys, http://www.cropmonitor.co.uk). *Z. tritici* infection relies heavily on the splash dispersed pycnidiospores re-infecting newly emerged leaf layers to sustain itself within a crop. It is likely the dry spring in conjunction with correctly timed fungicide applications assisted in maintaining low spore levels.

There is early indication that the time of year spores are dispersing may influence the extent of the epidemic. Spores detected in March were found to have the greatest influence on the disease subsequently observed in June for all three pathogens across the four sites monitored. Duvivier *et al.,* (2013) identified a correlation between *Z. tritici* spores trapped early on in the growing season with disease pressure in the field. This highlights further the importance of early season spore ingress, as successful early infection in the winter wheat crop enables the pathogen to cycle more often, if weather conditions are suitable within the crop. To understand this in more detail it would be necessary to monitor spore dispersal throughout the entire winter wheat growing season.

As mentioned in the introduction, a recent analysis of the performance of current CropMonitor™ models shows that the forecasts generated were seldom too late, but often too early. A potential explanation put forward for the earliness of the alerts was limited inoculum availability. *P. striiformis*, *P. triticina* or *Z. tritici* disease symptoms were observed on a total of 30 leaf layers. The majority of these disease

symptoms can be associated with an infection period comprising days when spores were detected and a risk forecast (76.6 %). A total of 8 leaf layers infected were associated with spore detections only (23.3 %). This information clearly highlights the significant contribution of inoculum limitation to the degree of infection risk and stresses the potential for forecast improvements when spore presence data is taken into consideration.

There are few cases where spores and alerts are present simultaneously and not associated with an infection event. In some instances, this is simply because the matches occur very late in the season and therefore the infection event is likely to lie beyond the disease assessment window. However, in some cases there were matches early in the season, which should have led to disease symptoms, but did not. This indicates that some biological components are potentially still missing from the models, which results in the alert being given whereas conditions were in fact not conducive. In other words, the alert criteria may be too strict. To get a fairer comparison a further analysis should be performed comparing the first spore and risk alert combination arising after leaf appearance (for each leaf under consideration) with the inferred time of infection. If the model performs well then the time difference should be small.

Initial *P. striiformis* infection at Wansford and Alford can be traced back to March, suggesting the pathogen entered the crop early in the season following favourable winter survival conditions. Further infection periods were identified for disease incidence on newly emerged leaf layers. The level of epidemic at the end of the season confirms the high risk from *P. striiformis* in these crops which was identified early in the season by the combined metrics of spore detection and infection risk. These data support the hypothesis that spore detection can make a significant contribution to risk forecasting for *P. striiformis* in wheat.

Very few infection risk alerts were forecast for *P. triticina* across the sites and study period, however a large amount of inoculum was detected throughout. This highlights the importance of incorporation of an infection risk model alongside spore trapping data for accurate forecasting. The lack of suitable conditions for infection identified by the infection risk model supports the trace levels of *P. triticina* observed. Again, the use of spore detection combined with infection risk alerts performed well

in predicting risks of *P. triticina* in these crops. The model criteria requires recalibration based on weather conditions experienced over these infection periods in light of the inoculum pressure. As a result of large spore numbers being detected a risk could be forecast on days when conditions did not quite meet the minimum criteria for a medium risk alert.

Correlations of risk alerts and spore presence with infection events were weakest for *Z. tritici*. Potential *Z. tritici* infection periods were identified in this study, however the 7-day infection window may not be extensive enough considering variation in reported latent periods. Measurement of infection events for leaf layers which were emerging or emerged prior to the start of this study was not possible as the latent period of approximately 280 degree-days (Lovell *et al*., 2004, Morais *et al*.,2015) meant infection occurred before sampling commenced. This indicates that for this disease the monitoring work needs to start earlier in the season, probably when ascospores are entering the crop before the winter period. A number of factors could have influenced situations when infection events were observed despite there being no relationship between infection risk and spore detection being made. The spore trap used in this study is expected to only sample ascospores due to the height of the sampling orifice above the wheat crop. The latent period of ascospores has been noted to exceed that of pycnidiospores by approximately 60 degree-days when carried out under controlled environmental conditions (Morais *et al*., 2015). As a result, the symptoms observed may originate from a separate infection event caused by splash dispersed pycnidiospores which were unlikely to be sampled by the spore trap. For 6 out of the 9 leaf layers, infection periods could be identified correlating with ascospores' latent period; it could be that the infection of the additional three leaf layers was predominantly initiated by pycnidiospores. Visual examination of spore samples could be used in the future to investigate this further.

The number of successfully identified infection events increases for *P. triticina* only when an infection risk alert occurs post spore detection within the same 7-day infection window but not on the same day. *P. triticina* uredospores have proven to be considerably robust in comparison to other *Puccinia spp.* and capable of surviving a number of weeks on plant material at high temperatures (Imbaby *et al*., 2005). This suggests the potential for *P. tritcinia* spores deposited on wheat leaves to be

able to initiate infection a number of days after the dispersal event. In addition, *P. striiformis* has much narrower criteria for infection which will reduce success in establishment of disease in comparison to *P. triticina*. Whilst both rusts have very robust spores, the spores of *Z. tritici* are much more fragile and unlikely to survive long on the leaf surface in the absence of favourable conditions for infection.

This study has focused on disease observations taken on the most susceptible winter wheat varieties and has therefore not taken into consideration varietal resistance and spore concentration. An understanding of spore concentrations required to initiate infection on winter wheat varieties with varying resistance would be useful to incorporate into a risk forecast model to either up or down-grade a risk forecast. As real-time PCR is highly sensitive, detection of low numbers of spores was achievable, however it's likely that more resistant varieties will require higher spore numbers before successful infection can occur. Evidence from this study suggests that as few as 10 *P. striiformis* spores can initiate infection in the relatively susceptible variety KWS Kielder. The models tend to have been developed using data on susceptible varieties resulting in them being highly risk averse. It is more plausible that this detection and forecasting approach can benefit more resistant varieties (Audsley *et al*., 2005).

Many infection models use a combination of growth studies in controlled environments and correlation of disease observations made in the field alongside environmental variables (Magarey *et al.,* 2005). One limiting factor in forecast models is inoculum data which ultimately determines the likelihood of infection. In this study a considerable number of risk alerts are made in absence of spore detection inevitably resulting in the potential for growers to mistime fungicide sprays or use them when not necessary. This work has highlighted points in time when spore detection could have been used more accurately to identify *P. striiformis, P, triticina and Z. tritici* true infection risk days and as a result support more sustainable fungicide use. This study focused on spore detection and disease observations being made at a local field scale with a site-specific risk forecast model. The area across which a spore trap can provide accurate risk forecasts will inevitably decrease with distance from the trap due to complex microclimates and variability in the pathogen population's response to environmental cues. The extent of a spore traps useful

range has not been studied in detail, but previous work has highlighted their effectiveness for providing reliable detection for informing management decisions across a single field (Carisse *et al.,* 2009) or vineyard (Thiessen *et al.,* 2015). Positioning a spore trap on a rooftop at height, sampling dilute air avoiding the air currents containing highly mixed air spora can also provide useful local and regional data on pathogen presence (West *et al*., 2012, Brittain *et al.,* 2013).

The risk alerts would benefit from meteorological data taken at the same location as the spore traps rather than the distant met sites as this would be more representative of the environmental conditions experienced by the host and pathogen from which this study focusses. A record of leaf wetness would also be benefit the risk modelling as this has a large influence on infection success (Magarey *et* al., 2005).

The complex variability of inoculum availability and the factors which influence both presence and number has led to few forecasting schemes incorporating spore release predictions. To improve and develop such models may take many years involving replication on a large scale. Understanding spore data as part of an in-season forecast tool has ultimately been limited by the time taken to sample the air, carry out a molecular test and provide a result. As automated in-field detection becomes as sensitive and specific as laboratory based testing, the incorporation of spore detection leading to improved risk forecast models is likely to evolve.

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Chapter 6 . Detection of latent septoria in non-symptomatic wheat leaves for improved measurement of disease risk

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6.1 Abstract

The effective treatment of *Zymoseptoria tritici* infections requires accurate knowledge of risk in order to target the pathogen prior to symptom development whilst avoiding unnecessary use of sprays. This is becoming an increasingly difficult challenge due to the reduction in available fungicide products and increase in frequency of resistant strains. One approach to improve disease management is to use inoculum monitoring combined with infection risk forecasting, but there is also potential for such decisions to be guided by real-time measurement of the amount of latent infection within the growing crop at key decision points. A quick 'field extraction' test to determine the amount of latent septoria within wheat leaves prior to symptom expression was assessed using loop mediated isothermal amplification (LAMP). Wheat seedlings and detached wheat leaves were inoculated with septoria spores and duplicate wheat leaf samples taken daily over a period of 28 days. Increases in the amount of septoria DNA throughout the pathogen's latent period following inoculation were detected and measurements were comparable using both qPCR and LAMP carried out on the same conventional leaf extract and by the quick 'field extraction' method carried out on duplicate leaf samples. Symptom expression was observed when DNA concentrations increased significantly from 10 pg per leaf to over 100 pg. This occurred over a period of 4 to 5 days in inoculated seedlings resulting in a LAMP time to positive (TTP) occurring within 20 minutes of the test being initiated. LAMP and qPCR carried out on conventional DNA extracts from wheat leaves taken from naturally infected field trial seedlings also correlated well. The

LAMP methods described provide a rapid and accurate estimation of latent infection by *Z. tritici* in wheat leaf samples.

6.2 Introduction

Zymoseptoria tritici (formerly known as *Mycosphaerella graminicola*, anamorph *Septoria tritici*) causal agent of leaf blotch is an economically important pathogen of wheat (*Triticum aestivum*). Annual average yield loss in the UK as a result of *Z. tritici* between 1985 and 1989 was approximately £35 million (Cook *et al*., 1991). The pathogen remains a significant burden on wheat yields globally, capable of reducing yield by 50 % in susceptible winter wheat varieties and efforts to limit its impacts are considerable (Fones and Gurr, 2015). Approximately 70 % of fungicide use in Europe is targeted towards control of this persistent pathogen (Fones and Gurr, 2015). The range of options for agronomists to control septoria in wheat has narrowed in recent years due to withdrawal of active ingredients under EU regulations and progressive development of fungicide resistance in the pathogen. The occurrence of strobilurin resistance in the pathogen in 2001 (Fraaije *et al*., 2003) and reduction in azole curative activity over the last decade has meant that only succinate dehydrogenase inhibitors (SDHIs) can be relied upon to effectively treat established infections in the leaf. Most recently, resistance to SDHIs has been reported in *Z. tritici* at low frequency (Dooley *et al*., 2016) and judicial use of this fungicide group will be necessary if its efficacy is to be maintained. In addition, the desire to develop effective anti-resistance strategies to protect the SDHI mode of action has led to use protective chemistry with multisite activity throughout a fungicide programme. Whilst in principle this seems a logical solution to a serious issue, the physiological properties of multisites mean they interfere with the uptake of SDHIs in the leaf and reduce their curative activity.

Early detection of *Z. tritici* is crucial to enable efficient management through more accurate timing of fungicide application prior to visible leaf blotch symptoms in the field. One method to achieve early detection is through the use of spore traps to detect the movement of infectious inoculum (Jackson and Bayliss 2011). Infection of wheat by *Z. tritici* during the growing season can occur by either ascospores or

pycnidisopores (Suffert *et al*., 2011). Currently it is believed that many spore traps only sample windblown ascospores due to their height above the crop. Infection as a result of pycnidiospores occurs lower down within the crop and is driven by splash dispersal (Holmes and Colhoun 1975; Shaw 1987). As a result, pre-symptomatic activity and therefore extent of risks from *Z. tritici* becomes difficult to quantify.

Like many fungal plant pathogens, *Z. tritici* can spend considerable periods of time in a symptomless state (latent period) during its infection cycle within wheat tissue. During this period it is understood the fungus is acquiring sufficient nutrients from its host in a biotrophic state. Necrotrophic feeding proceeds in conjunction with programmed cell death in the host plant resulting in leaf blotch symptom expression (Luttrell 1974; Parbery 1996; Keon *et al* 2007). Highly sensitive DNA detection methods can be used to detect changes in levels of DNA in plant material. Polymerase chain reaction (PCR) has been used to detect and quantify *Z. tritici* infection levels in wheat leaves in glasshouse and field conditions prior to symptom development (Fraaije *et al*., 1999). Furthermore, control of *Z. tritici* through better timing, choice and dose-rate of fungicide application was improved using ELISA to accurately detect presympromatic *Z. tritici* DNA (Kendall *et al*., 1998). A LAMP method has previously been developed to detect the latent DNA of *Puccinia striiformis* in wheat leaves (Huang *et al*., 2001). Currently no in-field rapid test exists for the detection of latent *Z. tritici* in wheat leaves.

There is therefore potential for a test which would allow agronomists to analyse the amount of latent septoria DNA, primarily in leaf 2 ahead of the key T2 timing and to a lesser extent on leaf three ahead of the T1 timing. This would enable fungicide choice to be tailored according to the disease pressure in the leaf and the amount of curative activity required. In this study LAMP methods will be developed and evaluated for the detection of *Z. tritici* DNA in wheat leaves in the field and results validated alongside samples tested by qPCR.

6.3 Methods

6.3.1 Inoculated time trial

Seedlings of the cultivar KWS Kielder, eight seeds per pot replicated 30 times, were grown to GS 11 and inoculated by pipetting a 4 µl droplet of a spore suspension containing approximately 400 pycnidiospores μl^{-1} of *Z. tritici* onto each leaf. Following inoculation, pots were placed in water-saturated humidity chambers for a period of 24 hours at room temperature. The seedlings were then separated into two batches. In the first batch the seedlings were grown in a controlled environment chamber set at 20°C and a 12h/12h light/dark cycle for up to 28 days and destructive samples taken daily. Inoculated wheat leaves from the second batch were excised into equal lengths and placed in agar plates containing 2 % tap water agar with a Whatmann filter-41 to separate the leaves from the agar. Plates were stored at room temperature at approximately 20°C. Leaf samples were taken every day over a time course of 28 days.

During the time course the plants and leaves were observed for the emergence of symptoms and duplicate leaf samples collected, one for conventional extraction and the other for the 'field extraction' method.

6.3.2 Field samples

Eighteen individual non-symptomatic leaf samples taken from plants with septoria symptoms expressing on older leaf layers in May at growth stage 39 were collected from wheat trials at Long Sutton (52.793823, 0.152729) and sixty-three from trials at Callow (51.999797, -2.750488). Samples were taken across 4 varieties of winter wheat (KSW Santiago, Evolution, KWS Siskin and Reflection) with equal numbers from both untreated and treated plots. Each sample was weighed to ensure 0.5 g of leaf material was maintained throughout and extracted using conventional techniques then tested using LAMP and qPCR.

6.3.3 DNA extraction

Conventional DNA extraction for qPCR. Wheat leaf samples were weighed to ensure 0.5 g of leaf material was tested throughout. They were then washed by shaking them in sealed bags with 200 ml distilled water for 1 minute to remove surface particulates. The water was then poured away and the wheat leaf transferred to 5 ml transport tubes containing a 1/4" ball bearing. Wheat leaves were pulverised by shaking the sample for 1 minute by hand. A total of 3 ml CTAB lysis buffer (120 mM sodium phosphate buffer pH 8, 2 % CTAB, 1.5 M NaCl, 1 % antifoam B emulsion) was then added and the tubes were placed on a Kleco tissue pulveriser (Garacia machines, California) for 4 minutes at full speed. The subsequent lysate was then clarified and cleaned using a Nucleopsin Plant 2 (Machenery-Nagel, Germany) DNA extraction kit. A negative control was included to ensure no cross contamination occurred during the extraction.

Crude DNA extraction for LAMP. Individual wheat leaves washed in sealed bags containing 200 ml distilled water for 1 minute were subsequently ground by shaking them rigorously in a 5 ml tube for 1 minute with a single 1/4 " ball bearing. To each sample 5 ml of 0.3 M KOH was then added and the tube shaken for 10 seconds. A total of 5 µl of the crude lysate was then added directly into the LAMP reaction. Further dilution was carried out by adding an additional 7 ml lysis buffer which improved detection performance. A negative control was included to monitor cross contamination of septoria DNA during the crude extraction.

6.3.4 Molecular detection of *Z. tritici* DNA

Real-time PCR. All reactions were carried out using Environmental MasterMix (TaqMan® Environmental Master Mix 2.0) with the following cycling conditions: 50 °C for 2 min, 95 °C for 10 min, and 45 cycles of 95 °C for 15 s and 65 °C for 1 min. Reaction volumes comprised of 24 µl master mix and 1 µl sample. Each sample including an extraction blank and negative control was tested in duplicate on a 7500 real-time PCR machine and the average cycle threshold value obtained. qPCR assays

for the specific detection of *Z. tritici* (Table 6-1) were designed and validated to the Internal transcribed spacer region as previously described (Chapter 3).

***Table 6-1 qPCR primer sequences for the detection of* Z. tritici**

Pathogen	Name	Sequence (5'-3')	Product size (bp)
Z. tritici	F	GACCCTGCCGGGCGC	
	R	AAAGTTTTGTTTCGATTTACTCGTAAA	84
	Pe	CCAAAAAACACTGCATCTCTGCGTCGGA	

LAMP. The assay was carried out in 25 µl reactions consisting of 15 µl isothermal mastermix (ISO-004LNL, OptiGene), 5 µl assay (5 pmol F3 & B3, 10 pmol LoopF & LoopB, 20 pmol FIP & BIP) and 2.5 µl sample DNA and 2.5 µl 0.6 M potassium hydroxide (KOH). A Genie II (Optigene, UK) was used to carry out the LAMP reaction by incubating the samples at 65 ^{o}C for 30 minutes with a slow annealing step (98 ^{o}C to 80 ^{o}C ramping at 0.05 ^{o}C s^{-1}). The Genie user interface was used to record time to positive (TTP) and annealing temperature. LAMP assays for the specific detection of *Z. tritici* (Table 6-2) were as previously described in Chapter 3.

***Table 6-2 LAMP primer sequences for the detection of* Z. tritici**

Pathogen	**Name**	**Sequence (5'-3')**	**Product size (bp)**
Z. tritici	Sept.F3	GATCATTACCGAGCGAGG	255
	Sept.B3	GTTCAAAGATTCGATGATTCACT	
	Sept.FIP	GCAGAGATGCAGTGTTTTTTGGTGCGACCTCCA-ACCCTTTGT	
	Sept.BIP	TACGAGTAAATCGAAACAAAACTTTCAAAATTCA-CATTACTTATCGCATTTCGC	
	Sept.FLP	CCGAAGCAACGGGATGTGT	
	Sept.FLP	TCTGGCATCGATGAAGAACG	

6.3.5 Construction of calibration standards for the quantification of septoria DNA

Cultures of *Z. tritici* grown on PDA containing septoria spores previously used for development of inoculum were allowed to incubate for a further 8 days to allow the development of mycelial. Mycelium was used as it would more accurately represent the target for detection as a latent mycelial infection in the plant material. Using a scalpel blade mycelium was scraped off and placed into a 5 ml transport tube containing a 1/4 ” ball bearing. Samples were then pulverised on a bead-beating machine in a lysis buffer and the subsequent lysate cleaned and purified using a QiAamp DNA extraction kit (Qiagen, Germany)**.** Samples were then tested by real-time PCR and LAMP as described above.

6.4 Results

6.4.1 Calibration of a quantitative septoria LAMP assay

DNA extracted from cultured septoria was quantified using a nanodrop spectrophotometer (Thermo Scientific, USA) and yielded a concentration of 2.5^5 pg of DNA. A ten-fold serial dilution down to 0.25 pg was then carried out to provide the standards for qPCR (Figure 6-1) and LAMP (Figure 6-2).

qPCR results are recorded as a cycle threshold value (Ct) when amplification of target DNA occurs. The lower the number the higher the concentration of DNA. Using the DNA standards enables the sample results to be converted to an estimation of the quantify of septoria DNA present. LAMP results are expressed as time to positive (TTP), the earlier amplification is observed the higher the concentration of septoria DNA present. qPCR was able to detect 0.25 pg of *Z. tritici* DNA and LAMP 0.625 pg.

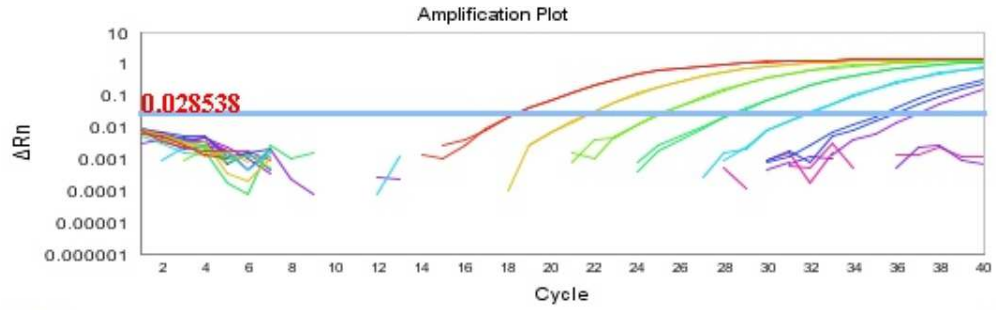


Figure 6-1 Real-time PCR cycle threshold values from DNA standards following serial dilution of 2.55 pg of septoria DNA. Horizontal line 0.028538 represents the baseline fluorescence calculated automatically by the SDS software (Applied biosystems, UK) and marks when the target DNA is sufficiently above the background signal

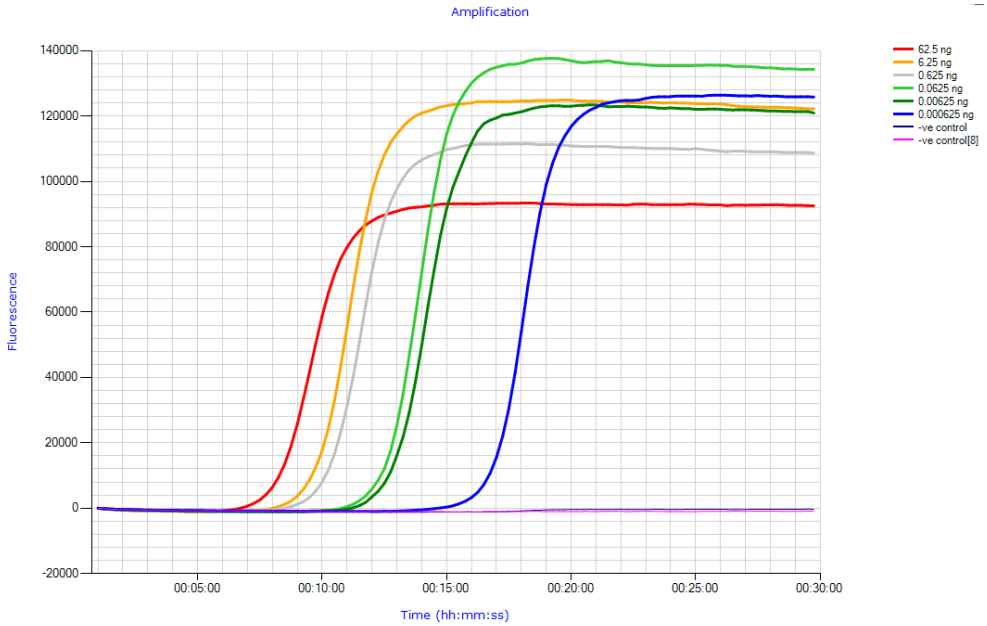


Figure 6-2 Time to positive following LAMP using the calibration standards. Concentration of DNA added to each LAMP reaction is higher compared to qPCR due to the LAMP reaction requiring 2.5 µl sample compared to 1 µl for qPCR

6.4.2 Time course to measure build-up of *Z. tritici* in leaf samples

Individual wheat leaf samples taken daily for a period of 28 days were extracted using conventional techniques and the concentration of septoria DNA determined using qPCR and LAMP TTP. The additional leaf sample taken on the same day was then tested using the 'field extraction' technique and the TTP compared.

6.4.3 Detection of *Z. tritici* DNA using qPCR and LAMP carried out on DNA extracted conventionally

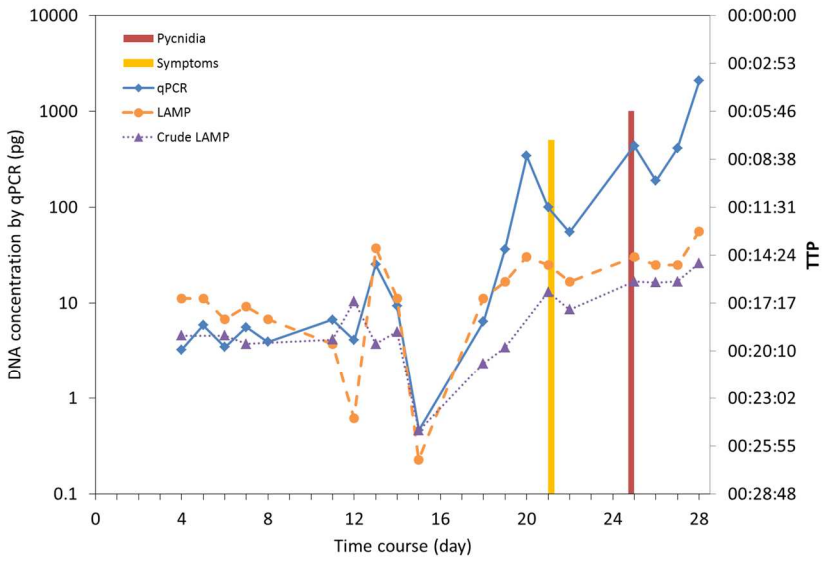
Septoria DNA was successfully detected in wheat leaves by both qPCR and LAMP throughout the time course of infection in both whole plant and detached wheat leaf trials (Figure 6-3). The concentration of septoria DNA determined using qPCR increased quickly in the detached leaf trial with symptoms appearing on day 9 and pycnidia on day 11. Symptoms are likely to have appeared early due to increased temperature, longer infection period (more humid conditions within the agar plate) and potentially increased susceptibility due to the leaf segment being deteached from the seedling. A more gradual increase in septoria DNA was observed in the whole plant inoculated trial with symptoms appearing on day 21 (420 degree days) and pycnidia on day 25 (500 degree days). These latent periods determined by the time of inoculation to necrotic symptoms and pycnidia being observed fits within the broad range of latent periods previously reported in other studies (Lovell, 2004). In general, the concentration of septoria DNA increased over the infection time course throughout both trials. A number of days were observed where the concentration of septoria DNA decreased below the level recorded on the previous day. LAMP tests carried out on conventionally extracted DNA using the TTP was able to closely replicate the changes in septoria DNA observed using the gold standard qPCR.

6.4.4 Detection of *Z. tritici* DNA using LAMP carried out on a crude leaf sample extract

The simplified 'field extraction' detection method was able to successfully detect DNA of septoria within the wheat leaf when tested using LAMP. When the TTP was compared to the concentration of septoria DNA determined using qPCR and LAMP on a separate wheat leaf a similar trend in ability to detect the changes in levels of septoria DNA was evident. Some negative results were obtained using LAMP assays on the detached leaves, which was later identified to be as a result of inhibition of the reaction. To overcome this, further dilution of the lysate was required resulting

in a better level of detection as is observed when carrying out crude LAMP on wheat leaves from the whole plant inoculation trial. To achieve this, an additional 7 ml lysis buffer was added following transferal of the sample into a 15 ml tube.

a



b

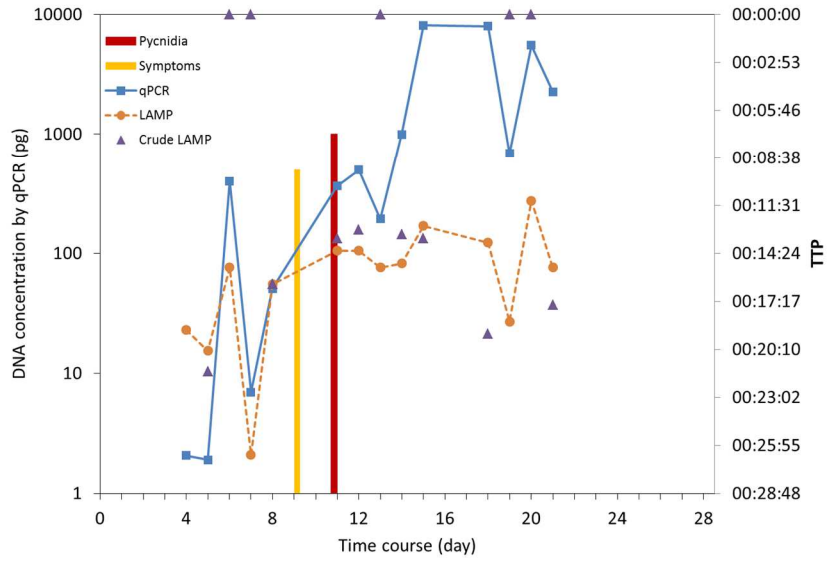


Figure 6-3 Infection time trial in (a) whole plants and (b) detached leaves inoculated with septoria spores and tested using qPCR (using traditional DNA extraction) and LAMP (using crude and traditional DNA extraction)

6.4.5 Detection of *Z. tritici* in field samples

The quantity of septoria in naturally infected wheat leaf samples was determined using qPCR. LAMP was then carried out on the same DNA extract to compare TTP to the quantity of septoria DNA (Figure 6-4). Quantity of latent septoria in field trial samples varied across the different treatments (treated vs. untreated) and varieties. In general, more septoria DNA was detected more frequently in untreated field trial plots. In addition LAMP routinely detected DNA of septoria in field trial samples correlating well with detection using qPCR. TTP was regularly observed to increase when the concentration of septoria DNA increased. Some discrepancies between LAMP and qPCR were observed in terms of detection, usually associated when TTP was in excess of 25 minutes.

A positive correlation existed between LAMP TTP and qPCR (Figure 6-5). However, there were 14 positive LAMP samples with an average TTP of 00:26:00 that were tested as negative using qPCR and 5 samples that tested positive using qPCR that were negative using LAMP. These ranged from 0.1 to 10^3 pg of DNA.

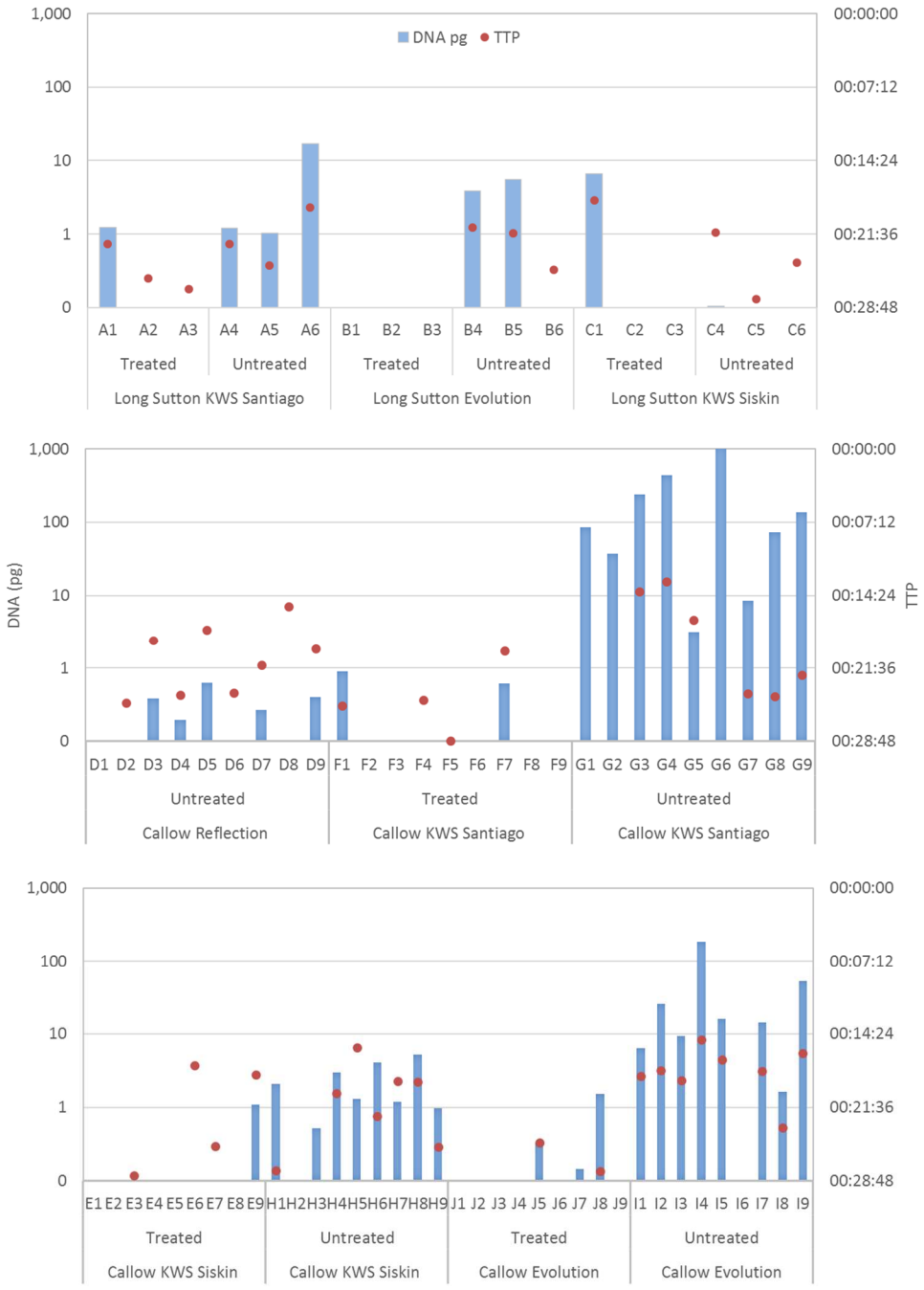


Figure 6-4 Concentration of latent septoria DNA measured by PCR and LAMP TTP in individual wheat leaves sampled from a mixture of varieties of untreated and treated winter wheat field trials at GS38

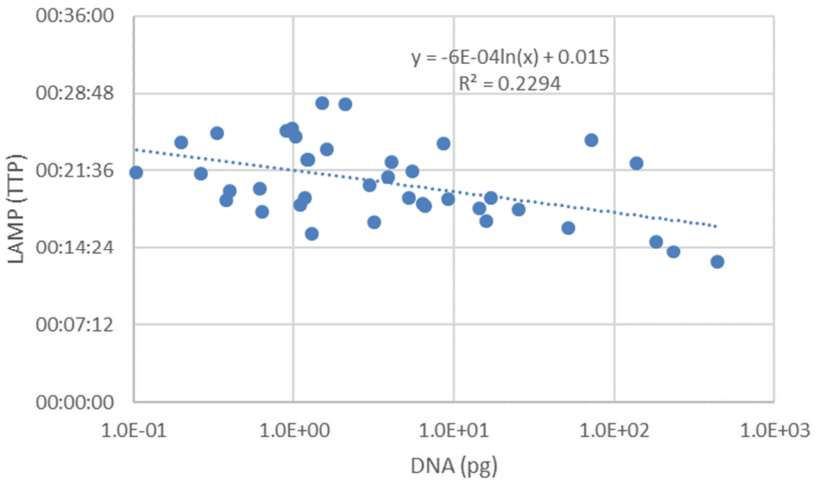


Figure 6-5 Relationship between LAMP and qPCR for the detection of* Z. tritici *DNA in un-symptomatic wheat leaf samples collected at GS38

6.4.6 Comparison between detections made in untreated and treated wheat leaf samples

A small proportion of wheat leaf samples taken from the treated plots tested positive using qPCR (5.2 %) and LAMP (8.2 %) compared to negatives using qPCR (18.3 %) and LAMP (14.4 % (Figure 5-6)). A higher proportion of samples tested positive using qPCR (23.5 %) and LAMP (19.6 %) in the untreated compared to negatives using qPCR (5.9 %) and LAMP (4.6 %).

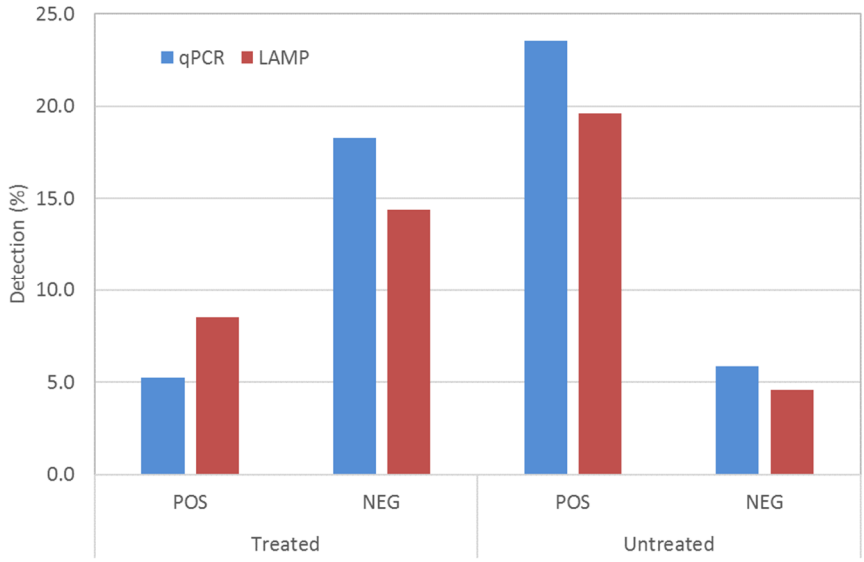


Figure 6-6 Comparison between detections made using LAMP and qPCR in treated and untreated wheat leaf samples

6.5 Discussion

This study has highlighted the ability of LAMP to detect latent septoria within wheat leaves prior to symptom expression using a simple 'field extraction' method. The concentration of septoria DNA recorded using both qPCR and LAMP did not conform to a linear relationship, but did increase significantly prior to symptom expression. Concentration of septoria DNA increased from 10 to >100 pg over a short time period. Successful detection of latent septoria usually occurred within 20 minutes of the LAMP test; however, due to the high level of sensitivity LAMP provides lower concentrations may be detected resulting in TTP >20 minutes. When testing wheat leaf samples from the field trial a reduction in correlation with qPCR was observed when the TTP for LAMP was greater than 26 minutes, suggesting additional leaf samples and LAMP tests are required to confirm latent infection in these cases, or that a limit of detection should be set. Seven of these leaf samples that tested positive using LAMP but negative by qPCR were from untreated wheat plants. It is therefore plausible that septoria DNA was present at a low concentration. Further testing consisting of more replicates and dilution of the DNA extract would be useful to determine if the qPCR test is being inhibited by co-extracted components. This would help explain the late LAMP TTP as this technique is known for maintaining sensitivity when inhibitory compounds are present.

The potential application of the methods developed in this study could be used to aid decision making regarding early in-season disease management decisions to better target fungicide application both in terms of timing and product choice in relation to the pre-symptomatic status of the pathogen at the time a farmer is planning their spray programme. To achieve this, molecular methods have primarily been carried out in the laboratory using qPCR however considerable time is required to process samples under these circumstances. LAMP is a molecular method that has more recently continued to demonstrate its application towards in-field tests offering comparable levels of sensitivity to qPCR. The method developed in this study is simple and quick only requiring shaking a wheat leaf sample in a bottle containing a ball bearing and buffer. The sample can then be transferred into test strips and run on a Genie II with a result achievable within 30 minutes. An increase in the TTP when

target DNA is present indicated septoria DNA being detected. As the concentration of DNA increased the TTP occurred earlier highlighting a quantitative element to the LAMP test. As a result, through further refinement of the test LAMP could be capable of providing an indication of the extent of infection and therefore the stage of latency. This would be particularly useful immediately prior to any spray decision being made. For example, fungicide sprays applied prior to symptom development are considered to be preventative but if applied close to the end of the latent period then they will not be as effective and a product with curative activity should be considered. In addition, better targeting improves overall efficacy of the product.

Techniques developed in this study could be used for a research perspective to study latency more closely in the field. Detailed understanding of latent periods and how they vary according to variety and environmental conditions is lacking and a limiting factor in disease risk model development. Farmers and agronomists could use this instead of spore detection/risk forecasting as an aid to decisions or as a confirmatory aid to provide additional confidence regarding assessment of risk. To implement this effectively a sampling strategy at the field scale would need to be developed and validated. As spray decisions are currently at the field level and a latent infection anywhere in the crop is significant then a protocol which carries out a limited number of tests in areas of the crop where disease is widespread on the lower leaves could give a robust measure of infection status in terms of a decision to spray or not. The LAMP test developed in this study could also be developed for detection of resistant strains of septoria, for example by targeting the CYP51 protein which has undergone rapid evolution in response to the widespread use of azole fungicides (Cools *et al*., 2011). There is also great potential to study other pathogens with a latent period such as rusts (*Puccinia* spp.) and pathogens associated with other crops such as light leaf spot (*Pyrenopeziza brassica)* in oilseed rape.

Author contributions

Conceptualization: IB NB JT

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Formal analysis: IB

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Methodology: IB NB

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Chapter 7 . General discussion

The recent increase in frequency of disease outbreaks in plants can be associated with increasing virulence and extension of a pathogens geographic distribution. Examples of this include wheat black stem rust in Africa and the middle east and ash dieback across Europe (Singh *et al.*, 2011; Gross *et al*., 2014). Overcoming the challenges associated with sustainable control or eradication of plant diseases so that plant health status can be maintained or improved is paramount. The challenge is set to become more formidable due to the potential removal of key active ingredients contained in many of the most effective pesticides used to control diseases. Furthermore, increasing difficulties are faced when predicting epidemics due to variable weather patterns and a build-up of pathogen resistance encouraged by less well-informed use of fungicides. Research in this thesis has focused on addressing some of the recent changes in technological advancements available to better inform disease control strategies at the management scale by being able to provide early detection of airborne fungal plant pathogens. One of the major hurdles in achieving early detection is the ability to sample and test the air containing the inoculum that leads to infection, ideally on the day the pathogen is being dispersed. As a result, particular attention in this thesis has been directed towards spore traps and detection methods that show the most potential to achieve this capability at present.

Fungal plant pathogenic spores seek to colonise fresh plant tissue by using the air as a medium through which to transition. Currently there is a very limited range of aerobiological sampling devices available on the market targeted towards sampling fungal spores and lack of confidence in their capabilities and reliability has significantly impeded their uptake. For those that are used routinely, primarily the slit-type volumetric Hirst-type spore trap, samples acquired tend to be analysed by microscopy. As the surveillance of air spora using spore traps can generate significant numbers of samples the subsequent analysis is both time consuming and prone to errors. To date, molecular detection methods have been implemented to speed up the testing and analysis aspects; however, sample preparation still takes considerable time requiring specialised trained staff.

Advances in hardware involved in air sample collection such as motors, cyclones and batteries are continuing to evolve due to demand for more efficient and powerful products in industry and households such as vacuum cleaners, leaf blowers, fans, hair dryers and heaters. As a result, aerobiological sampling has a lot to benefit from currently available hardware and indeed emerging technologies which span from such industries where large budgets can be reinvested into product development. Current applicable developments include bladeless fans, automated vacuum cleaners, UAVs and self-driving cars.

Routine laboratory-based detection of known plant pathogen using real-time PCR is now extremely reliable, sensitive and can have a relatively fast turnaround time with a diagnostic result achievable within a couple of days. Although field-portable real-time PCR that has the ability to provide a result within two to three hours is possible, the technology has not managed to find routine application, primarily due to the costs involved to set up and maintain the capability, and the complex sample preparation procedures required to achieve a reliable result. Other diagnostic methods showing potential for early plant disease detection exist such as lateral flow microarrays, differential mobility spectrometry and biosensors (Martinelli *et al*., 2015). These however require considerable time and resource to be at a technological readiness level suitable for deployment, and questions governing sensitivity and specificity remain which need further attention.

To address some of the questions and challenges discussed, a novel in-field molecular detection method known as LAMP was developed in conjunction with cyclonic spore traps that are capable of providing samples suitable for rapid testing requiring minimal preparation. In addition to testing air samples, situations will inevitably present themselves where it may not be possible to acquire an air sample or the pathogen may spread in the air at a level out of reach from a spore trap. As a result, the same diagnostic approach was used to evaluate an in-field test to detect latent infection of *Z. tritici* in wheat leaves prior to symptom development, adding additional capability to detect risks from disease before symptoms develop.

Using combined diagnostic and spore trapping approaches, findings in my research have contributed to advancing our current knowledge and capabilities by:

1. Identifying *H. fraxineus* dispersal characteristics at infection sites for the first time in the UK using volumetric spore traps and real-time PCR.
2. Developing, validating and implementing a field deployable LAMP based method capable of detecting low numbers of fungal spores of *P. striiformis, P. triticina* and *Z. tritici* in the field within 40 minutes of acquiring the spore trap sample.
3. Detecting *P. striiformis* and *P. triticina* fungal spores using a fully automated spore trap incorperating the LAMP based methods developed in Chapter 2.
4. Using an infection risk model identified days when infection coincided with the detection of *P. striiformis, P. triticina* and *Z. tritici* spores that is likely to have resulting in infection and subsequent disease incidence observed in winter wheat plots. Identified spore detection can improve risk forecasts and demonstrated the potential for the methods developed in Chapter 2 and 3 to provide in-field real-time spore detection.
5. Developing a rapid in-field test for the detection of latent *Z. tritici* infection.

Research into the monitoring of airborne plant pathogen dispersion has been renewed following the advent of molecular detection techniques. The ability of DNA based detection to distinguish between species and strains also present a significant opportunity to extend in-field diagnostic approaches beyond the species level. This is becoming an important capability to incorporate into diagnostic methods following increased prevalence of strains of fungal plant pathogens with new traits in fungicide or varietal resistance. Traditional molecular diagnostic techniques are now capable of detecting very low levels of pathogen DNA. Research in this project has demonstrated that LAMP can successfully detect as few as 10 fungal spores in air samples and also the initial development of *Z. tritici* infection in wheat leaves, providing comparable results to real-time PCR. Recent developments in mastermix such as Lyse and LAMP has enabled the incorporation of buffers that provide improved lysis of fungal spore cell walls resulting in less power intensive disruption methods required to achieve similar levels of sensitivity to conventional methods. Furthermore, the reduction in power required compared to real-time PCR to meet the conditions for amplification using LAMP at a constant 65 ^{o}C for approximately 30 minutes can be achieved from an internal battery pack. This makes the equipment

light weight, portable and capable of being carried in a brief case sized pelicase. The capabilities of LAMP demonstrated in this research to detect low spore numbers following a simple and quick disruption step in air samples containing inhibitory compounds within 30-40 minutes of sample collection suggests it will play a considerable role in airborne detection of fungal plant pathogens in the future. This research provides proof of concept in the use of an automated spore trap and LAMP-based detection device which has useful application in providing early inoculum detection as a tool for research and as a part of integrated pest management strategies. However, as with real-time PCR, LAMP is restricted to the detection of known pathogens and multiple tests are required if considering more than one target.

Another sector which is driving advancements in technology in this area is human health. Electrochemical PCR has been a focal point of research for many years (Goda *et al*., 2015) and is showing potential for point of care diagnostics as demonstrated by the IO system (Atlas genetics, UK) which is being used routinely to screen for infectious diseases such as Chlamydia and MRSA. The extraction and amplification cartridge is currently able to screen for 24 pathogens and plans to increase this number are in the developmental pipeline. This technology currently delivers advantages over LAMP in the number of targets which can be detected simultaneously, but new developments with the Genie and spore detection device are designed to resolve this.

Recently, attention has been drawn to technologies that can provide the detection of multiple known and unknown plant pathogens through single DNA sequencing. This sector of work has primarily been developed in the laboratory using sequencers such as the 454 pyrosequencer (Roche) and MiSeq (Illumuna) but is now being considered more for in-field testing. A portable sequencing device known as the MinION (Nanopore, Oxford) became available in 2012, which is smaller than a smartphone, connects to a laptop via a USB cable, and is showing considerable potential for this purpose. It can read on average 5 kb by passing sequence through 512 nanopores through which changes in a current applied are used to determine which base has passed through. Difficulties exist however that will limit uptake in the short term, such as high error rates, the requirement for complex laboratory-based DNA preparation and the need for simple solutions to the handling and analysis of

the large volumes of data generated. Improvements are being made since the initial testing was carried out suggesting potential future incorporation into routine diagnostic procedures (Laver *et al.*, 2015; Nezhad, 2014). A particularly useful application with regards to sequencing of environmental samples is the ability to discriminate not just between species but to also detect known and unknown mutations which may confer new resistance and or virulence attributes. For example, a *P. striiformis* race group called 'Warrior' was discovered in the UK in 2011, which was highly virulent on a wide range of winter wheat varieties previously considered resistant to *P. striiformis.* Sequencing of UK isolates highlighted a dramatic shift in the UK *P. striiformis* population in 2013, suggesting diverse assemblage of exotic *P. striiformis* lineages being introduced on an international scale (Hubbard *et al*., 2015). Understanding which strains are present within local populations in a season, detected through means of sampling the spores in the air, would enable timely selection of the most resistant cultivar and most appropriate disease management regime.

Ultimately, predictive weather-based models using highly sophisticated computational algorithms similar to a daily weather forecast are key for guiding control of plant diseases. Due to the advances in in-field detection of inoculum which can now measure inoculum build up more accurately there is the potential in the future to accurately predict spore release and deposition as well as infection through harnessing a range of sensors logging spore loads and weather data in real-time. This may be a number of years away however, and for the technology to mature to be reliable and accurate enough for mass uptake in the agricultural industry, there are a number of key gaps in our knowledge that remain, for example:

- How much area can a single monitoring station accurately service? If spores of a fungal pathogen are detected in one field or farm and a spray application is initiated, should the neighbouring farmer also apply a treatment? Although weather monitoring stations are installed frequently on farms and are relatively cheap and simple to maintain and collect the data once the equipment has been purchased, continual testing for inoculum is expensive and the data requires interpretation. The spatial relevance of a positive spore detection is still largely undetermined and there is emerging evidence that

for some pathogens a threshold spore load may be needed for infection to succeed in a specific host or cultivar; an additional factor which needs to be incorporated into risk models. Pinpointing the best time for sampling could benefit from sophisticated spore dispersion models which attempt to monitor the conditions from maturation leading up to spore release.

- Is it possible to model the entire reproductive life cycle of fungal plant pathogens using meteorological data alone or is the variation too great spatially that inoculum detection is needed to provide accurate risk for guiding management decisions?
- Does air sampling need to be carried out continuously or are there optimal times during the day and, or year when specific spore species disperse that could be targeted guided by weather conditions previously experienced?
- How much of the target species DNA detected in spore trap samples corresponds with viable spores?
- Can a measure of inhibitory compounds be quantified accurately to identify accurately the approximate number of viable spores present within an air sample. Detection which accurately quantifies DNA such as digital PCR could potentially improve quantification if the chemistry became field-portable.

Many of the answers will come from further research which is progressing rapidly, and there will need to be a mixture of *in vitro* and more specifically field-based experiments studying natural dispersal and infection of plant diseases over a number of years to collect the data required for validation. The seasonality aspect of pathogen and host interaction in the environment adds to the difficulty in obtaining the necessary data at pace. In the first instance, detection alone will provide useful information for enhancing risk predictions. Correlations with disease observed in the field can then be made contributing to further validation of the technology. Furthermore, there is potential for integration of the local risk with historic risk information sourced from national monitoring and live monitoring of nearby crops, yielding additional valuable information relating to pathogen virulence and resistance which would allow for selection of most effective variety and product at that point in time.

This work has highlighted for the first time the potential of combined monitoring technologies (Figure 7-1) to provide accurate real-time spore detection and weather data that when analysed alongside infection risk could be used to guide timing and application of treatment. As a result, the actual risk of disease development at the management scale has potential to be incorporated into spatially explicit disease management programs. This technology is still in its infancy; however, the potential for networks of self-reporting spore detection systems located side by side with meteorological monitoring equipment transmitting daily detection data for interpretation by specialists, is becoming a reality.



Figure 7-1 Examples of technology capable of providing real-time spore detection and weather monitoring. Automated spore trap (top left) meteorological monitoring stations (top right & bottom left) and field based LAMP detection using a Genie III (bottom right)

With an extensive array of sensors, uncertainties in predictions can then be modelled on a fine scale and potentially adapt risk predictions to individual farms or even fields. Initially, such a network and associated modelling environment would benefit from being scalable and adaptable (Mahaffee & Stoll, 2016). The cost of such an activity will be considerable and regular maintenance would be required, but will benefit from decreasing costs associated with production and computational power following Moore's law (Mack, 2011). Industries involved in the production of high value crops

such as vines and top fruit could find particular benefit from having their own spore detection and meteorological monitoring system and associated disease risk models based on site. When considering the arable sector, for example, the area of winter wheat cultivated in England and Wales in 2017 was 1.61 Mha approximately (AHDB, media 2017). This massive increase in scale compared to vines and top fruit poses greater variability in weather conditions influenced by topography and microclimates, all of which will influence inoculum behavioural characteristics making predictions more challenging. To accurately model disease risk at the farm or even field scale so that precision disease management can be implemented, weather stations would be best installed in a location representative of the area. For larger farms, more stations could be incorporated, or mobile stations deployed capable of being relocated to areas where high risk crops are being grown on a farm in any given season. Diseases will often appear in the same area of a field over consecutive years and therefore these sentinel locations would be an ideal place for monitoring spores and weather.

Disease risk models are only as accurate as the data provided to them. Further advances in technology will ultimately contribute to their reliability. For example, automated mobile spore traps would offer opportunities to study spore loads at different locations and scales. UAVs are being used extensively in research for mapping disease in crops and can be programmed to survey a whole farm area regularly and autonomously. Attempts have been made to monitor the movement of airborne fungal spores and the UAV can also be programmed to sample at different heights and maintain the same flight path. Depending on which way technology and uptake by industry goes, a more connected automated environment is likely to evolve with treatments automatously and rapidly prescribed to individual fields, and even plants themselves, becoming a possibility.

The outputs of this research have already had significant impact in advancing the use of smart monitoring technologies and is now supporting the establishment of a network of spore monitoring and weather stations across England which are gathering data for validation against weekly surveillance observations in wheat, barley and oilseed rape. The commercial version of the automated spore detection system validated in this study is in development and will be deployed across this monitoring network in the near future (Figure 7-2).



Figure 7-2 Graphical representation of 'SporeSentry'. A fully automated air sampling and spore detection system.

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