Novel insights into the asexual life-cycle of the wheat-leaf pathogen *Zymoseptoria tritici*

William Tudor Kay

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I certify that all material in this thesis which is not my own work has been identified and that no material has previously been submitted and approved for the award of a degree by this or any other University.

Signature: 

(William Kay)

Thesis Abstract

Zymoseptoria tritici, the causal agent of Septoria tritici blotch (STB), is the most economically important pathogen of wheat in temperate climates. EU spending on the control of STB is estimated at more than € 1.3 billion per annum, even when tolerant cultivars are used. This thesis comprises 5 data chapters each looking to ascertain reasons for the success of *Z. tritici*. Using mainly *in vitro* methods combined with confocal microscopy, this thesis explores asexual spore submergence in rain, epiphytic development, the potential triggers for pycnidiation, low-nutrient *ex planta* survival, and soil treatments as a novel method of control. Novel findings include that (i) submergence leads to a form of spore dormancy, (ii) epiphytic development is more extensive than previously thought with hyphal growth and sporulation both occurring readily over a 21 day period, (iii) pycnidiation is a rapid, deterministic developmental process, triggered by contents of lysed plant cells, (iv) asexual spores have the capacity to survive for many weeks in low-nutrient environments, and finally (v) that treating soil-borne environmental spores may be a novel way to reduce field inoculum levels before a new crop is sown. Collectively these results provide many new insights in to the behaviour of *Z. tritici* during its disease cycle. This thesis proposes an updated version of the life cycle for this devastating fungal pathogen and argues that current methods of control are not adequate long-term.

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Abbreviations

% v/v	Percentage volume by volume
% w/v	Percentage weight by volume
%	Percentage
+ve	Positive
µmol m^2 s^{-1}	Photosynthetic photon flux density (micro moles per metre squared per second)
AC	Accessory chromosome
ANOVA	Analysis of variance
ATG8	Autophagy-related protein 8
BODIPY	Boron-dipyrromethene, fluorescent dye for lipid visualisation
bp	Base pairs
CC	Core chromosome
cDNA	Complementary DNA
CWDEs	Cell wall degrading enzymes
D	Dextrose 20 g/l
d	Day
df	Degrees of freedom
dH_2O	Deionised water
DMI	Demethylation Inhibitors
DNA	Deoxyribonucleic acid
dpi	Days post infection
EDTA	Ethylenediaminetetraacetic acid
eGFP	Enhanced green fluorescent protein
ELISA	Enzyme-linked immunosorbent assay
ETI	Effector triggered immunity
ETS	Effector triggered susceptibility
EU	European union
g	Grams
g/l	Grams per litre
GABA	γ-Aminobutyric acid
GASP	Growth advantage in stationary phase
GFP	Green fluorescent protein
GlcNAc	N-Acetylglucosamine
h	Hour
HGCA	Home Grown Cereals Authority
HR	Hypersensitive response
IC50	Half inhibitory concentration
Z. tritici IPO323	Reference strain of *Z. tritici*
IVP	*In vitro* pycnidia
kb	Kilobase pairs
LysM	Lysin motif domain
M	Molar
MAPK	Mitogen-activated protein kinases
Mb	Megabase
MC	Microcycle conidiation (also yeast-like growth)
mg	Milligram
MilliQ	Ultrapure water by Millipore Corporation
min(s)	Minute(s)
ml	Millilitre
mm	Millimetre
mM	Millimolar
mRNA	Messenger RNA
MS	Murashige and Skoog basal salt agar (0.5 x)
N	Number of trials
NBT	Nitroblue tetrazolium
ng	Nanogram
nm	Nanometre
nM	Nanomolar
NMR	Nuclear magnetic resonance
NPLR	N-Parameter Logistic Regression
ºC	Degree Celsius
P	Peptone 20 g/l
PAL	Phenylalanine ammonia lyase
PCD	Programmed cell death
PDA	Potato dextrose agar

PEG	Polyethylene glycol
Phi	Phosphorous acid/phosphite
PI	Propidium iodide
PR	Pathogenesis-related
PRR	Pattern recognition receptor
PTI	Pattern triggered immunity
PtxD	Phosphonate dehydrogenase
QoI	Quinone outside inhibitors
QTL	Quantitative trait loci
R	A free software environment for statistical computing and graphics
RFLP	Restriction fragment length polymorphism
rH	Relative humidity
RNA	Ribonucleic acid
RNase	Ribonuclease
RNAseq	RNA sequencing
ROS	Reactive oxygen species
rpm	Revolutions per minute
rRNA	Ribosomal RNA
SDHI	Succinate dehydrogenase inhibitors
SDI	sterol demethylase inhibitors
SE	Standard error
SEM	Scanning electron microscope
Sso1	Mutant strain of *Z. tritici IPO323* with GFP tagged to the plasma membrane
STB	Septoria leaf blotch
Stb6	Small secreted protein in *Z. tritici* responsible for avirulence on wheat cultivars carrying the Stb6 resistance gene.
TAE	Tris base, acetic acid and EDTA
TBE	Tris base, borate and EDTA
UV	Ultra-violet radiation
V8	V8 vegetable juice
VBNC	Viable but not culturable
-ve	Negative
x G	x Gravity
Y	Yeast extract 10 g/l
YD	Yeast extract 10 g/l and dextrose 20 g/l
YP	Yeast extract 10 g/l and peptone 20 g/l
YPD (a)	Yeast Peptone Dextrose (agar). Yeast extract 10 g/l, Peptone 20 g/l, Dextrose 20 g/l. Agar 15 g/l
µg	Microgram
µL	Microlitre
µm	Micrometre
µM	Micromolar

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1. General introduction

1.1. Wheat and the rise of *Zymoseptoria tritici*

Wheat (*Triticum aestivum*) is a globally important crop. It is estimated to account for around 20 % of global human calorific intake and is a major source of carbohydrate, protein, fibre and vitamins (Shewry and Hey, 2015). The intrinsic yields of wheat have more than doubled since the 1950s (FAOSTAT, 2013). However, the increased reliance on monoculture farming has augmented the potential for losses to microbial pathogens. Wheat has many economically damaging pathogens, these include rusts, smuts, blights, bunts, blasts, rots and mildews (Duveiller *et al.*, 2012). However, a contemporary reliance on winter wheat (rather than spring wheat), coupled with an increasingly warm and wet climate, has brought the blotch fungus, *Zymoseptoria tritici* to the fore (Hýsek *et al.*, 2016, HGCA, 2010, HGCA, 2012, Dean *et al.*, 2012, Fones and Gurr, 2015).

The filamentous ascomycete *Z. tritici*, the causal agent of Septoria leaf blotch (STB) is thought to have evolved in tandem with the domestication of wheat in the Fertile Crescent around 10–11,000 years ago. Wheat is now grown in a number of different climates and thus *Z. tritici* can now be found globally (Stukenbrock *et al.*, 2010). However, it is in temperate parts of the world such as Europe that *Z. tritici* is most prevalent. For this reason this thesis will concentrate on winter wheats in these temperate conditions (HGCA, 2012). Here, infection of wheat leaves can lead to yield losses of up to 50 % if not controlled. However, when modern fungicides are combined with tolerant cultivars, mean yield losses are restricted to between 5 to 10 % (Dooley, 2015, Brown *et al.*, 2015, Fones and Gurr, 2015, Torriani *et al.*, 2015).

Accurate information on a pathogen's life cycle is particularly important for optimising control methods. Historically, *Z. tritici* has been considered a hemibiotroph – a fungus classified by having an initial period of biotrophic growth and a subsequent period of necrotrophic growth (Rudd *et al.*, 2015, Keon *et al.*, 2007). However, as this General Introduction will document, there is little evidence for biotrophy in *Z. tritici*. It is now argued that growth before the necrotrophic switch may simply be asymptomatic/latent (Sánchez-

Vallet *et al.*, 2015, Orton *et al.*, 2011). The following text will collate the current published knowledge of the *Z. tritici* life cycle. Firstly, sexual and asexual spore types and their methods of spread will be explored. Subsequently, the complete infection process, from epiphytic growth, leaf penetration, (latent) apoplastic growth, the switch to necrotrophy, and asexual spore production, will be explored.

1.2. The *Z. tritici* life cycle

Plant pathogenic microbes are reliant on a plant host for all, or a large part, of their life cycle. The host is ultimately a source of nutrition, as well as providing environmental protection. For most plant pathogens, the resources of the host organism are not infinite. After each disease cycle, spread to new host tissue occurs. For this purpose, fungi produce spores, small structures specialised for dissemination, which carry enough resources to germinate and initiate infection on new hosts.

Spores can be the products of either sexual or asexual reproductive events and can be characterised by their physiology, mode of spread, and virulence. For *Z. tritici*, both sexual and asexual forms of reproduction have been widely documented in the field. Sexual spores are produced in perithecia after the meeting of two mating types (Steinberg, 2015). The resulting spores, ascospores, are bicellular and are mostly found within the stubble of harvested wheat crops, 30 to 90 days after initial infection (recently reviewed by Suffert *et al.* (2011)). As a winter wheat season can be up to 11 months long, ascospores can be produced throughout the growing season (AHDB, 2016b, Suffert *et al.*, 2011).

Z. tritici is typical for a *Zymoseptoria* species in the fact it has more than one asexual spore form: pycnidiospores and conidiospores (Quaedvlieg *et al.*, 2011). Pycnidiospores are hyaline, cylindrical, multi-septate conidia, consisting of three or four cells arranged in a long, thin, slightly curved shape – shown in Figure 1A (Verkley *et al.*, 2013). They are produced *in planta*, within the sub-stomatal cavities of leaves in structures termed pycnidia (pycnidial conidiomata). Pycnidia are heavily melanised spheres in which pycnidiospores are suspended in a gel-like cirrus. The cirrus is nutrient rich, composed of proteins and saccharide compounds (Van Ginkel, 1999). Each single pycnidium is estimated, over time, to disperse

between 5 and 10 × 10^3 conidiospores (Eyal, 1971, Gough, 1978). The 'polycyclic' nature of *Z. tritici* allows a single spore to infect, sporulate, and spread, up to six times in the winter wheat growing season (Fones and Gurr, 2015).

Conidiospores, as they will be called in this thesis, are also asexual spores. The nomenclature in the literature is not consistent and thus conidiospores can also be found as microspores, sporidia, microconidia, and micropycnidiospores (Suffert *et al.*, 2011, Steinberg, 2015, Kema and Annone, 1991, Yang, 2015). Conidiospores exist in a much larger range of shapes and sizes than pycnidiospores, growing via a form of growth termed microcycle conidiation. Conidiospores 'bud' from parent cells in a yeast-like manner. Here, cells can separate from the parental cell and survive latterly as single celled conidiospores (Figure 1B, white arrows). However, newly budded cells can also maintain contact with the parental cell, sometimes creating spore structures with cells joined in complex branching patterns (Figure 1B, black arrows).

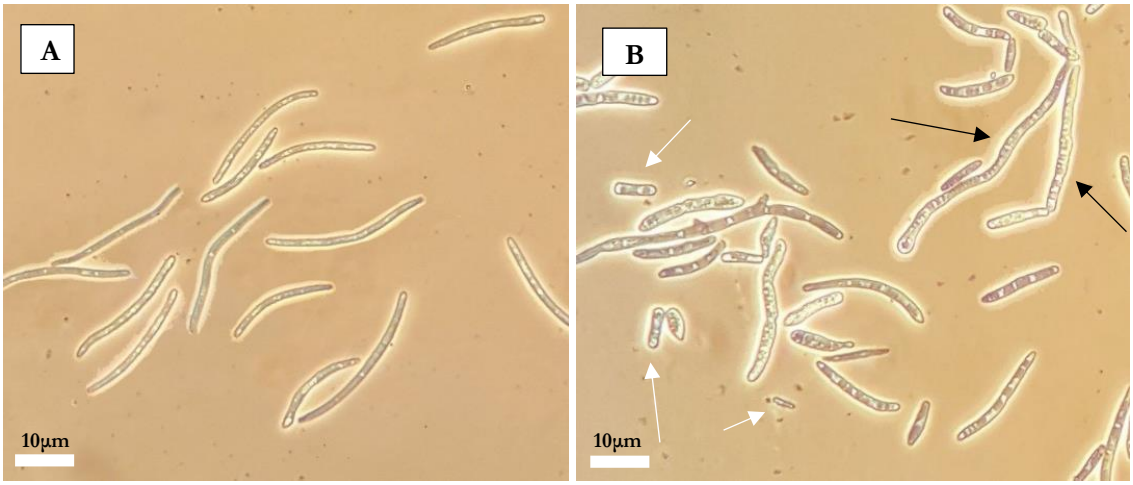

Figure 1: The asexual spore types produced by *Zymoseptoria tritici* IPO323
A: pycnidiospores isolated from wheat leaves. **B:** Conidiospores/microspores/yeast-like growth isolated from rich agar (YPD). Arrows show the diversity of conidiospores produced by microcycle conidiation (yeast-like growth). Spores range from single-celled (white arrows), to multi-cellular (black arrows).

Microcycle conidiation (MC) is the simplification of a typical fungal life cycle. It allows a fungus to produce spores (conidia) directly from spores, rather than from mycelia – as is the standard route (Hanlin, 1994, Jung *et al.*, 2014). The existence of this form of sporulation is thought to surround the avoidance of unfavourable conditions. MC provides a way of

building the population when spore germination and hyphal growth is not favourable. It could easily be hypothesised, however, that even if conditions were favourable for infection, initially building a population via MC would still be a positive strategy if virulence was maintained (Lapaire and Dunkle, 2003).

In the laboratory, many studies have shown that MC is the dominant growth form of *Z. tritici* on rich agar media (Mehrabi *et al.*, 2006, Guo and Verreet, 2008, Kema and Annone, 1991). As such, conidiospores, rather than pycnidiospores, are often used as experimental inoculum (Yang *et al.*, 2015, Rudd *et al.*, 2015, Lee *et al.*, 2014, Kettles *et al.*, 2016, Palma-Guerrero *et al.*, 2015, Shetty *et al.*, 2007, Kema *et al.*, 2008). In the field, however, conidiospores have not been widely reported. A recent review from Suffert *et al.* (2011) highlighted reports of 'microspore' production in stubble after autumn rain in 1950 and 1977. Further evidence of MC on leaves has not been published in *Z. tritici.* However, it has been visualised on leaves, as well as on many other low nutrient situations such as glass, Teflon, water agar (Figure 2).

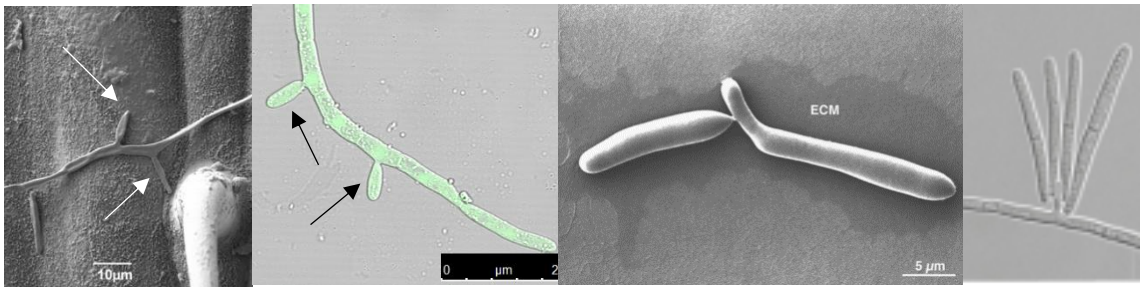

Figure 2: Evidence of microcycle conidiation (MC) in multiple environments.
A: SEM image of MC on the leaf surface (Fones, pers. comm.) **B:** Confocal microscopy of image of MC on a hydrophobic glass slides (Fones, pers. comm.) **C:** SEM image of MC on Teflon while submerged in water (Duncan and Howard, 2000). **D:** Light microscopy of MC on water agar (Mirzadi Gohari *et al.*, 2014).

In fungal pathogens such as *Cercospora*, *Beauveria* and *Metarhizium* spp., conidiospores produced by MC have also been shown to penetrate hosts and cause disease as efficiently as the original primary conidia (Jung *et al.*, 2014). Indeed, no difference in virulence has been shown to exist between the pycnidiospores and conidiospores of *Z. tritici* (Fones *et al.*, 2017b). This indicates that, if occurring in the field, microcycle conidiation in *Z. tritici* may serve as an important source of inoculum.

1.2.1. Spread

Ascospores are spread by wind and thus have the potential to travel large distances (Shaw and Royle, 1993). They are most successfully spread post-harvest as crop canopy removal allows for wind to reach lower parts of the crop. Ascospores are thought to be mainly responsible for disease entering a newly sown crop – the so-called 'primary inoculum' (Shaw and Royle, 1989, Suffert *et al.*, 2011, McDonald and Martinez, 1990). Indeed, this hypothesis was tested experimentally in 1989 when it was shown that disease was reduced when covering a crop with a protective tent (Shaw and Royle, 1989).

Pycnidiospores, depending on the seasonal conditions and cultivar sown, are produced within leaves. They appear after an infection period ranging from 14 to 40 days (Van Ginkel, 1999). A single pycnidium, dependent on its size, is estimated to produce between 5 and 10 $\times$ 10^3 pycnidiospores over its life span (Eyal, 1971, Stewart *et al.*, 2016a). Moisture is very important for pycnidiation for two reasons. Firstly, spore production drops significantly in low moisture environments, ceasing production at below 86 % relative humidity (Gough and Lee, 1985). Secondly, the uptake of moisture by spores relative to the surrounding necrotic leaf tissue, leads to spores swelling and oozing onto the leaf surface. On the leaf surface, pycnidiospores are spread by rain-splash, subsequently dispersing horizontally (> 4 m in windy conditions) and vertically (> 50 cm) through the wheat canopy (Brennan *et al.*, 1985, Bannon and Cooke, 1998, Van Ginkel, 1999, Shaw, 1991, Suffert *et al.*, 2011, Ponomarenko *et al.*, 2011).

It is the pycnidiospores which are thought to be the main source of 'secondary inoculum' – the inoculum that maintains disease within a growing season. A recent estimate from Fones and Gurr (2015) suggested that, per hectare, over a growing season, the pycnidiospore load of a single strain of *Z. tritici* may reach $10^{10\text{-}11}$ conidiospores. However, if MC also occurs in the field, as has been shown in the laboratory (Figure 2), the number of asexual infectious propagules may be significantly higher.

1.2.2. Leaf adhesion

For *Z. tritici*, the relationship with a host plant begins with leaf contact. On the leaf surface, pycnidiospores of *Z. tritici* have been shown to produce cutinases. These are enzymes which break down the leaf waxes which may play a role in the initial leaf adhesion (Rudd *et al.*, 2015, Palma-Guerrero *et al.*, 2015, Brunner *et al.*, 2013, Goodwin *et al.*, 2011).

Duncan and Howard (2000) have visualised an extracellular matrix (ECM) secreted by *Z. tritici* conidiospores and suggested its role in adhesion. The secretion of this ECM has, however, only been documented *in vitro* (agar, glass, Teflon and plastic), so its role in leaf adhesion is only speculative.

Transcriptome analysis has revealed the upregulation of genes responsible for the secretion of four hydrophobins, proteins involved in the interactions between hyphae and hydrophobic surfaces – these may be involved in leaf attachment (Yang *et al.*, 2013a).

Leaf architecture may also play a part in adhesion. An experiment which assessed adhesion through leaf washing, showed that relatively high numbers of trichome-associated *Z. tritici* individuals remained after washing (Fones *et al.*, 2017b). This implies that trichomes may also aid in adhesion. Supportive evidence for this has been presented elsewhere where spores have been visualised by on leaves in stomatal depressions and around trichomes (Rohel *et al.*, 2001).

1.2.3. Germination

Z. tritici is a dimorphic fungus. It can grow in a sporulating form, replicating via microcycle conidiation, or in a hyphal form. The switch from sporulating growth to hyphal growth is termed the dimorphic switch – a process essential to achieving infection and disease (Yemelin *et al.*, 2017).

Spores spread by rain on to the leaf surface are liberated from the high-nutrient cirrus in which they develop. Here, germination events occur quickly and readily, occurring from each end of the spore (Harrower, 1976, Mehrabi *et al.*, 2009). Quantification of asexual spore

germination has consistently reported a rate of between 75 % – 90 % (Cohen and Eyal, 1993, Kema *et al.*, 1996b, Fones *et al.*, 2017b). One study has, however, reported a somewhat lower germination rate of 55 – 60 % (Shetty *et al.*, 2003). The time taken for germination to occur is also disputed. Some studies show no significant increase in the number of germination events after 24 hours (Shetty *et al.*, 2003, Cohen and Eyal, 1993). However, another shows just 20 % of events occurring by the same time-point, rising ultimately to 75 % after around 5 days (Fones *et al.*, 2017b). No *in vitro* quantification of germination has been carried out to date.

1.2.4. Epiphytic growth

On low nutrient media such as water agar, glass, Teflon and plastic, hyphal extension dominates (Duncan and Howard, 2000, Cousin *et al.*, 2006). The leaf surface (phyllosphere) is also a relatively low-nutrient environment and, as expected, hyphal growth has also been confirmed here (Kema *et al.*, 1996b, Fones *et al.*, 2017b, Kettles *et al.*, 2016). The multiple environments in which hyphal growth occurs, strongly suggest the lack of a host specific trigger for the dimorphic switch. However, the slow rate of hyphal extension on tobacco leaves, when compared to wheat leaves, indicates some host-specificity (Kettles *et al.*, 2016).

On nutrient rich surfaces, MC is the dominant growth form in *Z. tritici* (Mehrabi *et al.*, 2006, Guo and Verreet, 2008, Kema and Annone, 1991). The phyllosphere is not nutritionally barren. Leaves are known to exude nutrients such as sugars (Ruinen, 1970, Tukey Jr, 1970). Additionally, trichomes, points of injury, and areas of microbial habitation can all be relatively rich with nutrients such as soluble carbohydrates (Lindow and Brandl, 2003, Derridj, 1996).

It can therefore be hypothesised that conidiation still occurs widely during epiphytic growth. Indeed, evidence for this can be seen in Figure 2 (Fones pers. comm.) and is supported by transcriptomic evidence showing markers of asexual reproduction upregulated from day 3 of the infection process (Palma-Guerrero *et al.*, 2015).

Furthermore, nutrients such as nitrogen which are applied to fields during agricultural fertilisation has been shown to correlate with increased disease. This result may suggest

population growth via epiphytic sporulation (Simon *et al.*, 2003, Leitch and Jenkins, 1995, Lovell *et al.*, 1997).

1.2.5. Epiphytic survival

Leaf contact brings new challenges for the germinating spore such as the plant defence system. A plant defends itself in two distinguishable forms: The first is via the 'passive' defence system. Adaptations here are preformed and often morphological including the primary and secondary cell walls, a thick waxy cuticle, variable leaf openings, and preformed defensive chemicals such as phytoanticipins (VanEtten *et al.*, 1994). The second form is the 'active' defence system. This relies on the recognition of pathogens, specifically, through the foreign molecules associated with them. These molecules, termed elicitors, or Microbe/Pathogen Associated Molecular Patterns (MAMPs/PAMPs), are highly conserved and found either attached to, or secreted by, microbial cells. For fungi, MAMPs include chitin, glucan, xylanase and ergosterol (Henry *et al.*, 2012).

MAMP recognition leads to a form of defence termed pattern triggered immunity (PTI). PTI is characterised by a collection of defensive reactions designed protect the plant from foreign invaders (Nicaise *et al.*, 2009, Schwessinger *et al.*, 2011, Chisholm *et al.*, 2006). Biochemical and morphological changes resulting from PTI are well documented and include reactive oxygen species (ROS) production, localised cell wall reinforcements, the release of systemic antimicrobial chemicals, and the secretion of microbial cell wall degrading enzymes (CWDEs) (Apel and Hirt, 2004, Chowdhury *et al.*, 2014, Ahuja *et al.*, 2012, Collinge *et al.*, 1993). In the wheat-*Z. tritici* interaction, activated defences include the secretion of chitinase, β-1,3-glucanase, phenylalanine ammonia lyase (PAL), peroxidase, and the production of ROS, all strongly induced from around 3 hours post-inoculation (Adhikari *et al.*, 2007, Shetty *et al.*, 2009).

Overcoming these inducible responses is essential if infection is to progress. One documented example of how this is achieved is through the production of ROS scavenging enzymes. Six antioxidant enzymes from the family chloroperoxidases have been confirmed to be secreted by *Z. tritici* at multiple time-points during infection, from as early as day one but peaking around day 3 (Rudd *et al.*, 2015, Yang *et al.*, 2013a, Palma-Guerrero *et al.*, 2015,

do Amaral *et al.*, 2012). The secretion of chloroperoxidases and other antioxidant enzymes at such early time-points strongly suggests involvement in ROS tolerance during epiphytic growth. Heat shock proteins, which are known to be produced in response to a range of environmental stresses including oxidative stress and antifungal compound protection, have also been reportedly upregulated at various time-points during early infection (Keon *et al.*, 2005, Yang *et al.*, 2015).

Z. tritici strains which trigger PTI can be non-pathogenic (Marshall *et al.*, 2011). Consequently, successful strains have evolved to overcome, or avoid, triggering these plant defence responses. This is achieved through the production of effectors: small secreted cysteine-rich peptides with roles in suppressing PTI. The most well-known examples in *Z. tritici* are the chitin binding proteins Zt1LysM and Zt3LysM (Rudd *et al.*, 2015, Sánchez-Vallet *et al.*, 2013, Marshall *et al.*, 2011). Ordinarily, chitin is recognised by two receptors at the plant cell membrane ZtCEBiP and ZtCERK1 (Marshall *et al.*, 2011, Lee *et al.*, 2014). In strains producing these LysM proteins, particularly Zt3LysM, the plant defence response remains untriggered and infection can progress (Marshall *et al.*, 2011). The mechanism for avoidance is achieved through the competitive binding of chitin oligosaccharides released from the fungus during hyphal growth. ZtLysM molecules are released from the fungus which bind rapidly to any chitooligosaccharides released, thus minimising recognition events from the host plant receptors (Sánchez-Vallet *et al.*, 2013, Lee *et al.*, 2014).

There are an estimated 970 genes coding for secreted proteins within the *Z. tritici* genome. 246 of these have been suggested as effectors (Goodwin *et al.*, 2011, do Amaral *et al.*, 2012, Yang *et al.*, 2013a, Rudd *et al.*, 2015). In a 2015 study, 68 genes encoding putative effectors were shown to be differentially expressed (Rudd *et al.*, 2015). However, most of these were expressed at later time-points in the infection process than the epiphytic growth stage.

There are additional biotic factors which may affect *Z. tritici* on the leaf surface, namely epiphytic competitors. Little is known about the relationships between *Z. tritici* and other phyllosphere inhabitants. The aforementioned chloroperoxidases may be involved in the production of antibiotics used to control competitive microbes in the phyllosphere (Rudd *et al.*, 2015, Apel and Hirt, 2004). There is also evidence of defence against competitive microbes via antibiotic detoxification. Specifically, a study by Levy *et al.* (1992), found that

three catalase isozymes and one superoxide dismutase are synthesised by the fungus in response to 1-hyroxyphenazine, an antibiotic secreted by *Pseudomonas aeruginosa*.

Finally, there are abiotic factors which may affect epiphytic growth and survival. An in-field assessment of dominant strains over a growing season, showed that strains which enter the plant more quickly are favoured in dryer (spring) conditions (Suffert *et al.*, 2015). The likely reason for this surrounds the avoidance of abiotic stresses such as UV and low humidity – conditions more prevalent in spring. Again, it can be hypothesised that the chloroperoxidases may also be giving protection from associated ROS generation. Genomic work has highlighted 19 genes coding for putative chloroperoxidases in the *Z. tritici* genome (do Amaral *et al.*, 2012). As highlighted above, only six of these have been confirmed as secreted, leaving 13 which may play a role in detoxification of ROS produced internally. It has also been reported that a large number of genes involved in the perception of light, nutrients and other stresses showed peak transcription at day 3 (Palma-Guerrero *et al.*, 2015).

1.2.6. Epiphytic nutrition

As discussed, the phyllosphere is relatively nutrient poor. However, as discussed earlier, areas of increased nutritional availability do exist such as leaf waxes, secreted sugars, wounds and areas of microbial habitation (Ruinen, 1970, Tukey Jr, 1970, Lindow and Brandl, 2003, Derridj, 1996).

Enzymes potentially involved in acquiring nutrients during epiphytic growth include cutins, peptidases, pectinases, lipases, cellulases, hemicellulases and xylanases (Rudd *et al.*, 2015, Yang *et al.*, 2015, Goodwin *et al.*, 2011, Palma-Guerrero *et al.*, 2015, Brunner *et al.*, 2013). Some of these enzymes may be linked to scavenging freely available nutrients, however, they may also be involved in the acquisition of nutrients via physical breakdown of the plant itself. It should be noted, however, that the molecules released by the breakdown of plant structural proteins (damage associated molecular patterns or DAMPs), can also trigger plant defences, so this strategy may not be beneficial for successful invasion.

The uptake of carbon during the infection process has been studied more closely using a mutant strain of *Z. tritici* with the green fluorescent protein (*GFP* from *Aequorea victoria*) gene

inserted downstream of a carbon source-repressed promoter (the *acu-3* gene from *Neurospora crassa*). Using confocal microscopy and western blot analysis, this study showed that carbon sources (such as glucose, fructose, and sucrose) are not obviously utilised during the epiphytic growth period, i.e. GFP was highly expressed (Rohel *et al.*, 2001). Supportive evidence for lack of hexose or nitrogen assimilation during the first eight days of leaf contact, also can be found in transcriptomic and metabolomic studies (Rudd *et al.*, 2015).

The strongest evidence for an early source of nutrition surrounds lipids. Using a fluorescent stain (Nile Red), lipid granules have been visualised as highly prevalent in pycnidiospores (Cairns, Unpublished). The importance of initial synthesis of lipids in spores has been shown using targeted gene disruption mutants for three cytochrome b5 reductase genes (CBR) genes. In this work, Derbyshire *et al.* (2015) showed the presence and upregulation of CBRs are known to be involved in the biosynthesis of fatty acids and complex lipids., suggesting lipid synthesis is important for asexual spore production.

CBR mutants also displayed attenuated disease, suggesting lipid biosynthesis is important for infection (Derbyshire *et al.*, 2015). However, this result is more likely explained by the fact that mutant spores have a lower initial lipid content than wild type spores, and thus have fewer energy resources to use for hyphal growth. Indeed, Cairns *et al.* (Unpublished) also showed the depletion of lipid granules in hyphal tissue, inferring their importance in early growth. Many papers support this hypothesis by showing the upregulation of genes involved in lipid degradation in *Z. tritici* (M'Barek *et al.*, 2015, Keon *et al.*, 2007, Palma-Guerrero *et al.*, 2015, Rudd *et al.*, 2015). This mirrors other fungi such as *Aspergillus nidulans* and *Magnaporthe oryzae* (Thines *et al.*, 2000, Seong *et al.*, 2008). Genes involved in the breakdown of lipids, and subsequent β-oxidation of constituent fatty acids (such as acyl-CoA dehydrogenase, AMPdep synthetase/ligase, and enoyl CoA hydratase) have been shown to be upregulated as rapidly as day one on leaves, persisting until at least day 4 (Rudd *et al.*, 2015).

1.2.7. Entry into the leaf

Environmental conditions associated with epiphytic growth include drought and UV radiation. To avoid these conditions, some fungi have evolved strategies which aid in rapid plant colonisation. For example, the wheat pathogens *Blumeria graminis* (powdery mildew) and

Puccinia graminis (wheat stem rust) use targeted/directional growth of hyphae to find stomatal openings more rapidly (Brand and Gow, 2009). In the rice fungus *Magnaporthe oryzae*, leaf entry is achieved via high-pressure penetration through the leaf cuticle via specialised hyphal swellings termed appressoria (AHDB, 2016b).

Early research on the colonisation of wheat by *Z. tritici* using light and fluorescence microscopy, reported appressoria-like hyphal swellings (Cohen and Eyal, 1993, Shetty *et al.*, 2003, Kema *et al.*, 1996b). However, these swellings were only found associated with stomatal openings with no signs of cuticular entry. Melanin production, reportedly necessary to create the turgor pressure required to enter a plant through the cuticle, has also been shown to be dispensable in the penetration process, potentially confirming a purely stomatal mode of entry (Mehrabi *et al.*, 2006). Rohel *et al.* (2001) visualised evidence of penetration between epidermal cells in some cultivar–isolate combinations; however, this has not been reported elsewhere.

Evidence of successful stomatal penetration was not found when *Z. tritici* was grown on tobacco (Kettles *et al.*, 2016). Infection by *Z. tritici* may therefore be wheat specific. Indeed, it has been reported twice that hyphal growth is a targeted process on wheat. One paper showed growth to be oriented toward stomatal openings (Duncan and Howard, 2000). Another from Cousin *et al.* (2006), reported a complete loss in stomatal entry after knocking out the MAP kinase gene, ZtFus3. This MAPK gene has previously been linked to extracellular signal perception, therefore this paper indicates that reduced entry is linked to a loss in recognition capabilities. However, other studies provide contradictory evidence to targeted growth and report stomatal invasion as a random process. For example, a study by Kema *et al.* (1996b) showed that although individuals did sometimes grow towards stomata, many just crossed over them with no change in direction – this has also been confirmed more recently (Figure 3, Fones, pers. comm.).

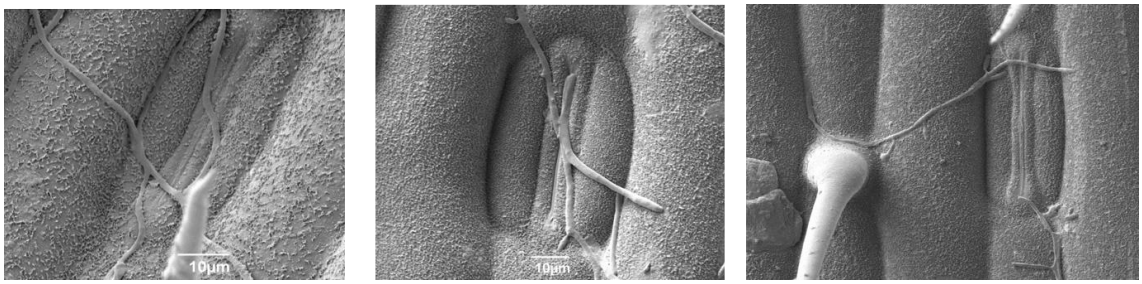

Figure 3: SEM images of *Z. tritici IPO323* hyphae growing over the stomata of a compatible wheat cultivar (Fones, pers. comm.)

Entry has been reported to occur within 24 hours of leaf inoculation (Kema *et al.*, 1996b, Cousin *et al.*, 2006, Duncan and Howard, 2000). However, a 2017 paper by Fones *et al.* (2017b) found that 99 % of individuals remained purely epiphytic up to day 10 post-inoculation. A computer simulation of *Z. tritici* growth using multiple parameters estimated from images of fungal growth on wheat leaves, confirmed that the visualised results closely matched a random model of epiphytic growth.

In planta fungal growth is not detectable using molecular methods until at least 7 days post-infection (Rudd *et al.*, 2015, Kema *et al.*, 1996b, Keon *et al.*, 2007). Additionally, only low levels of H_2O_2, which is known to be released by plants post perception, accumulate until around day 8 (Yang *et al.*, 2013a). Whether this is due to a lack of entry, or due to a slow apoplastic growth rate is disputable.

Further evidence for delayed entry comes from two transcriptomic papers. These studies extracted RNA from infected plant tissue over multiple time-points and found that the percentage of reads which map to the fungus jumps significantly from around 5 % to more than 50 % at around day 9/10 (Palma-Guerrero *et al.*, 2015, Rudd *et al.*, 2015). This growth rate is supported by data showing significant increases in the expression of β-1,3-glucanase and chitinase genes, as well as peak transcription for many ribosomal proteins, tubulin transcripts and an elongation factor, also involved in hyphal extension (Palma-Guerrero *et al.*, 2015, Shetty *et al.*, 2009, Yang *et al.*, 2013a). Whether this result reflects a sudden burst in the number of individuals which have infected the leaf, or whether this reflects a sudden increase in growth is unknown. It should be noted that the speed of entry is likely highly dependent on the strain and cultivar used.

1.2.8. Apoplastic growth

Hyphae have been observed within sub-stomatal cavities at 24 h post-inoculation, initially contracting upon entry but widening after entry to the cavity (Duncan and Howard, 2000, Kema *et al.*, 1996b).

Further growth into the mesophyll, however, has been suggested as a major distinguishing factor between compatible and non-compatible interactions. For example, Mehrabi *et al.* (2006) showed that deletion of the MAP kinase coding gene ZtSlt2 (previously published as MgSlt2) allowed for the maintenance of stomatal penetration but a restriction in mesophyll invasion. Additionally, unpublished data from Haueisen and Stukenbrock, show that when infecting other grass hosts such as *Brachypodium distachyon* or *Agropyron repens*, *Z. tritici* growth stops inside the sub-stomatal cavity (Kellner *et al.*, 2014). The reasons for the cessation in growth in incompatible strains are so far unknown, but it may be linked to a lack of protection from host defences. For example, the deletion of the ABC transporter gene MgAtr4, a gene involved in the protection of the fungus against fungitoxic compounds produced within sub-stomatal cavities, leads to a termination of growth (Stergiopoulos and de Wit, 2009).

Once internal, hyphae have also been shown to reach multiple sub-stomatal cavities within just 4 days of leaf inoculation indicating that growth is not necessarily slow once in the apoplast (Duncan and Howard, 2000). The total hyphal distance in the apoplast has been recorded by Metcalfe *et al.* (1998) at average of 11 mm per pycnidia. These measurements were recorded in a susceptible wheat cultivar shortly before pycnidia were produced. This distance is surprisingly large as leaf inoculations carried out in laboratory show a small distance between inoculated areas of leaves and the final area of symptomatic tissue (Figure 4). It is possible that endophytic hyphal growth is strain and cultivar specific. It is also possible that due to the high concentrations used in these experiments, early necrosis may limit the extent of hyphal growth.

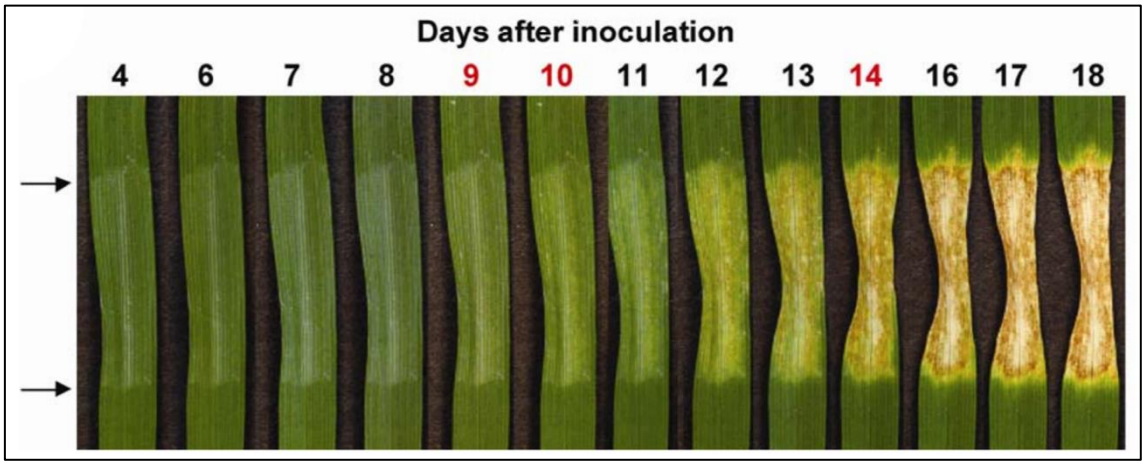

Figure 4: Time course for *Z. tritici* symptom development in winter wheat.
Image highlights that symptoms do not spread significantly outside the inoculated area of the leaf (marked by arrows), suggesting limited apoplastic hyphal growth (Keon *et al.*, 2007).

1.2.9. Apoplastic survival

Z. tritici must be protected from many stresses within the apoplast. These would include abiotic stress such as a relatively low apoplastic pH and myriad active plant defences such as antimicrobial chemicals and ROS. An assessment of total gene regulation shows that 9 dpi is the time-point where most fungal genes are differentially regulated compared to growth on rich agar medium (Rudd *et al.*, 2015).

Some genes encoding some of the chloroperoxidases show peak induction around day 4 of the infection cycle (Rudd *et al.*, 2015). Others peak around day 10, suggesting protection of ROS stress during apoplastic growth (Yang *et al.*, 2013a, Keon *et al.*, 2007). Other proteins involved in ROS protection such as thioredoxin peroxiredoxin are also upregulated around this time but peak around day 13, suggesting greater importance at later time-points (Yang *et al.*, 2013a).

Perhaps most significant, is that the number of secreted proteins peak between days 7 and 11 (Palma-Guerrero *et al.*, 2015). In fact, it is estimated that 50 % of all transcripts at day 9 are thought to be secreted effectors (Rudd *et al.*, 2015). These effectors are likely important for protecting fungal tissue from the active plant defences. For example, the LysM effectors. The general timing and host specificity of the LysM proteins seems to be relatively generic. Transcriptomic evidence of their upregulation is found on tobacco as well as wheat (Kettles

et al., 2016). They are also produced throughout the infection cycle, still being produced at least up to 15 days post-infection (Rudd *et al.*, 2015, Shetty *et al.*, 2009).

Due to the plant cell membrane integrated nature of the receptors which bind to PAMPs such as chitin, it is likely that these LysM proteins play a larger role with regards to hiding during growth within the apoplast rather than on the leaf surface, as previously discussed. Rudd *et al.* (2015) showed that the LysM proteins are the 12th and 27th most abundantly upregulated genes at day 9, suggesting their importance at this time-point, but also confirming a high rate of hyphal growth. A second study confirmed this showing peak transcription around 11 days post inoculation (dpi), and confirming that LysM proteins are amongst the top 100 genes transcribed at this time point (Palma-Guerrero *et al.*, 2015).

1.2.10. Apoplastic nutrition

Many apoplastic fungal plant pathogens are biotrophs. These can feed from cells using highly invaginated arbuscular structures called haustoria (Stotz *et al.*, 2014). For *Z. tritici*, however, there has been no documented evidence of haustoria. This suggests that any nutrition gained from this point comes from within the plant apoplast.

Evidence for the uptake or assimilation of apoplastic nutrients is conflicting. Genes coding for secreted enzymes such as proteases, peptidases and alpha amylases, all thought to be involved in metabolising apoplastic nutrients have been found to be upregulated in high numbers in the *Z. tritici* genome (Goodwin *et al.*, 2011). However, apoplastic proteomics show no conclusive evidence for uptake during the latent stage of apoplastic growth (Yang *et al.*, 2015, Yang *et al.*, 2013b). An additional study using ^{1}H-nuclear magnetic resonance (NMR) analysis, in fact found small increases in apoplastic carbohydrate levels between days 6 and 9 (Keon *et al.*, 2007). This increase may be due to host manipulation by the fungus to release more carbohydrates into the apoplast. This has been shown in *Cladosporium fulvum* which triggers an almost three fold rise in the amino acid γ-Aminobutyric acid (GABA) during infection, while also producing the enzymes necessary to utilise it (Solomon and Oliver, 2001, Solomon and Oliver, 2002). The overall increase in carbohydrates seen in *Z. tritici*, however, indicates that increased uptake is not occurring.

The aforementioned work from Rohel *et al.* (2001) which used a mutant *Z. tritici* strain with the *GFP* gene inserted downstream of carbon source-repressed promoter, showed that fluorescence decreased during the apoplastic phase when compared to the epiphytic growth stage. This result signified the presence, uptake and metabolism of apoplastic carbohydrates. Additionally, enzymes required for nitrate and hexose uptake are downregulated during *in planta* growth compared to growth on rich agar. This implies that other sources of nutrition are used this period.

Fungal lipases, fatty acid desaturases, alcohol dehydrogenases (ADH), and sterol desaturases have all been found to be highly upregulated during apoplastic growth, indicating the continued use of internal or external lipids to at least day 9 post-inoculation (Keon *et al.*, 2007, Palma-Guerrero *et al.*, 2015). It can be suggested therefore, that the nutritional needs of fungal individuals during this period are met by internal sources of energy within the spore itself. However, many of these genes are no longer upregulated by day 10, suggesting a switch in nutritional use (Rudd *et al.*, 2015).

1.2.11. Necrotrophic switch

Successful apoplastic growth before the necrotrophic switch is generally asymptomatic. It is thereby generally known as the 'latent' stage of *Z. tritici* development. This period is reported to last from anywhere between 9 days to 4 weeks post-infection (Shetty *et al.*, 2003, Kema *et al.*, 2008, Rudd *et al.*, 2015). Around this time a clear switch occurs and symptoms such as chlorosis and necrosis begin to appear – a change termed 'the necrotrophic switch'. From 12 dpi, there is massive H_2O_2 accumulation in the mesophyll (Yang *et al.*, 2013a). For the plant, 14 dpi is when the maximum defence response is recorded (Rudd *et al.*, 2015). Wide scale changes occur, including the beginning of plant programmed cell-death (PCD). This has been confirmed by showing the laddering of plant DNA and leakage of cytochrome-c from the mitochondria into the cytoplasm (Keon *et al.*, 2007, Cousin *et al.*, 2006).

The trigger for PCD is disputed. One explanation surrounds the secretion of CWDEs by the fungus. The number of putative CWDEs found in the *Z. tritici* reference genome is, however, relatively low for a necrotrophic plant pathogen (Goodwin *et al.*, 2011, do Amaral *et al.*, 2012). Some CWDEs have been found to be highly upregulated at around 11 dpi (Palma-Guerrero

et al., 2015, Yang *et al.*, 2015). The timing of this upregulation allows for the hypothesis that, rather than triggering an immune response, CWDEs are produced to aid in plant cell lysis, this yielding the nutrition therein.

A more established theory on triggering plant cell-death surrounds the secretion of effectors. As discussed, effectors are generally produced to interfere with, or avoid, the active defence system in plants. However, in what has been dubbed the 'Zig-Zag model' of plant defence (Jones and Dangl, 2006), effectors themselves can, in turn, be recognised directly by plant resistance (R) proteins. Recognition leads to effector triggered immunity (ETI) (Flor, 1942, Jones and Dangl, 2006). Classically, the outcome of this recognition event is the hypersensitive response (HR) – a form of defence which leads to programmed cell-death in the plant (PCD). For biotrophic plant pathogens, PCD would restrict growth through the leaf, thus minimising continued infection and resulting in immunity for the plant. For necrotrophic pathogens such as *Z. tritici*, however, PCD in plant cells brings with it the release of nutrients, thus allowing for continuation of the fungal life-cycle. For this reason, not all effectors have evolved to restrict the effects of plant recognition. Some are thought to be produced with the opposite aim of triggering PCD – a theory termed effector triggered susceptibility (ETS) (Stotz *et al.*, 2014).

A published example of ETS surrounds the tan spot fungus of wheat – *Pyrenophora tritici-repentis*. This pathogen secretes the effectors ToxA, ToxB and ToxC into plants during apoplastic growth (Lamari *et al.*, 2003). These proteins are recognised by wheat plants in a gene for gene manner, leading to chlorosis and/or necrosis in cultivars which contain the corresponding resistance genes (Tan *et al.*, 2010, Moffat *et al.*, 2014).

246 proteins in the *Z. tritici* genome have been highlighted as potential effectors (Goodwin *et al.*, 2011, do Amaral *et al.*, 2012, Yang *et al.*, 2013a, Rudd *et al.*, 2015, McDonald *et al.*, 2016). The secretion of many of these putative effectors has been confirmed at numerous time-points during infection (Rudd *et al.*, 2015, Palma-Guerrero *et al.*, 2015). The search for an effector which may be responsible for the triggering of PCD has, however, so far been elusive. For example, the capacity of 63 of these putative effectors to induce symptoms such as chlorosis and necrosis in plants has been assessed by Kettles *et al.* (2016). This study created used *Agrobacterium*-mediated transient expression mutants to screen these effectors

for recognition in *N. benthamiana*. Fourteen of the effectors tested resulted in chlorosis or cell-death. None, however, caused cell-death when subsequently injected into the wheat leaf apoplast. Additionally, multiple effectors have been knocked out of the *Z. tritici* genome, with no loss in pathogenicity recorded (M'Barek *et al.*, 2015).

1.2.12. Plant cell lysis

Irrespective of the trigger, plant PCD leads to a loss of plant cell membrane integrity, subsequently leading to cell wall degradation and the collapse of the mesophyll tissue. The associated cell lysis creates a hostile environment for the pathogen which includes the release of ROS, and the induction of defence signalling and metabolism-related genes (Keon *et al.*, 2007, Rudd *et al.*, 2008, Shetty *et al.*, 2003, Yang *et al.*, 2013a). Genes coding for ROS-scavenging enzymes from the plant are downregulated, allowing for ROS accumulation to occur (Yang *et al.*, 2013a). Here, the fungus must protect itself to complete its life-cycle. Genes encoding ROS-scavenging enzymes and stress-response proteins such as thioredoxin, peroxiredoxin, and a number of chloroperoxidases show peak induction during these symptomatic infection stages (Yang *et al.*, 2013a, Keon *et al.*, 2007).

Additionally, plant cell lysis leads to the release of intracellular sources of nutrition. Between day 9 and 13 post-infection, both carbohydrate and aliphatic compounds are released in large quantities into the apoplastic fluid, producing an environment rich in proteins, metabolites, sugars and ions (Keon *et al.*, 2007). Fungal effector proteins are now found to be downregulated, suggesting that any active relationship with the plant has come to an end (Rudd *et al.*, 2015). Instead, *Z. tritici* produces proteases, peptidases, lipases and amylases with high abundance. This indicates that *Z. tritici* now takes advantage of external sources of nutrition (Goodwin *et al.*, 2011, do Amaral *et al.*, 2012, Yang *et al.*, 2013a, Rudd *et al.*, 2015). The data also suggest that, during this late infection stage, plant substrates such as cellulose, hemicellulose and pectin are metabolised (Rudd *et al.*, 2015).

1.2.13. Pycnidiation

After a period ranging from 14 to 40 days post-infection, (depending on the seasonal conditions and the cultivar sown), asexual spore formation begins to occur. Rapid hyphal extension combined with right angle branching, leads to the formation of a basket like lining around the base of sub-stomatal cavities (Duncan and Howard, 2000).

Enzymes associated with asexual spore production such as mannitol dehydrogenase and Zn^{2+}-containing alcohol dehydrogenases are produced. These are hypothesised to be involved in the generation of spore storage compounds for subsequent periods of survival during entry into a future host (Rudd *et al.*, 2015). These compounds are accompanied by a high degree of melanisation (Cousin *et al.*, 2006, Mehrabi *et al.*, 2006), before the subsequent production of pycnidiospores (Eyal, 1971).

Upon leaf wetting, pycnidiospores swell with the uptake of water. The relative swelling compared to the necrotic leaf tissue in which the spores are contained, leads to them oozing out onto the leaf surface. Here, rain-splash distributes the pycnidiospores outwards and upwards through the canopy, thus completing the life cycle.

1.2.14. Environmental survival

Plant pathogenic fungi are reliant on hosts for survival and replication. However, hosts are not always available. They may be geographically distant, seasonally unavailable or already occupied by competitors. The capacity to survive away from the preferred niche is, therefore, often necessary. Hence, fungal spores, the propagules of spread between hosts, can be highly specialised in their survival potential. For example, a 2016 study on 135 saprotrophic and mycorrhizal fungi highlighted that even after 30 years of sterile cold water storage, 42 % of isolates maintained their culturability (Richter *et al.*, 2016). Conidia of *Aspergillus* spp. have been shown to be resistant to UV-light, extreme temperature changes (6–55 °C), and dehydration, with spores surviving without water for at least a year (Krijgsheld *et al.*, 2013). There are many examples of extended off-host survival for plant pathogenic organisms. Examples include the oomycete potato pathogen *Phytophthora infestans* (Pittis and Shattock, 1994) which produces thick-walled overwintering oospores, and the fungal pathogens *Mucor*

racemosus, Fusarium culmorum and *Peacylomyces varioti* which produce chlamydospores with features evolved for long term survival. Fungi may also survive on secondary hosts during the off-season, as does the rust fungus *Puccinia graminis* on barberry (Jin, 2011).

For *Z. tritici*, survival in both the sexual and asexual spore form has been documented, particularly when spores remain on, or in, host tissues. For example, pycnidiospores, when held within the leaf in the cirrus-containing pycnidium, have been found to remain viable for up to 132 days (Gough and Lee, 1985). However, the longevity of spores within leaves is dependent on many factors including humidity, moisture, UV, heat, microbial competition, and soil submergence. For example, harvested pycnidiospores have been shown to remain viable for around 2 months at 15–30°C, but 9 months at 5 – 15°C (Hilu and Bever, 1957). Therefore, actual survival rates in the field are likely less than those quoted (Djerbi, 1977, Hilu and Bever, 1957, Brokenshire, 1975, Suffert *et al.*, 2011, Gough and Lee, 1985).

Z. tritici is thought to be highly host specific, causing infection only on wheat and only on specific cultivars. However, a literature review from Suffert *et al.* (2015) presented that the fungus has in fact been isolated from another 26 different grasses, with 6 being probable secondary hosts. Additional work has also highlighted survival on the non-graminaceous plant, tobacco, although, this study only assessed spores up to 8 days, so longer survival cannot be inferred (Kettles *et al.*, 2016).

Away from plant tissue, conidiospores have been shown to survive in sterilised soil in the dark at 4 °C for at least 20 weeks (Shearer *et al.*, 1974). On water agar, liberated conidia can survive for around 15 days in the laboratory (Arsenijević, 1965, Gough, 1978). In the field however, when freely exposed to sunlight, ascospores and pycnidiospores been shown to survive for just 2 days (Brown *et al.*, 1978, Arsenijević, 1965). Spore survival in field-realistic conditions needs further assessment.

1.3. A final life-cycle

Below (Figure 5) is a current representation of the *Z. tritici* life-cycle by Ponomarenko et al (2011). This will be re-assessed in the thesis Discussion.

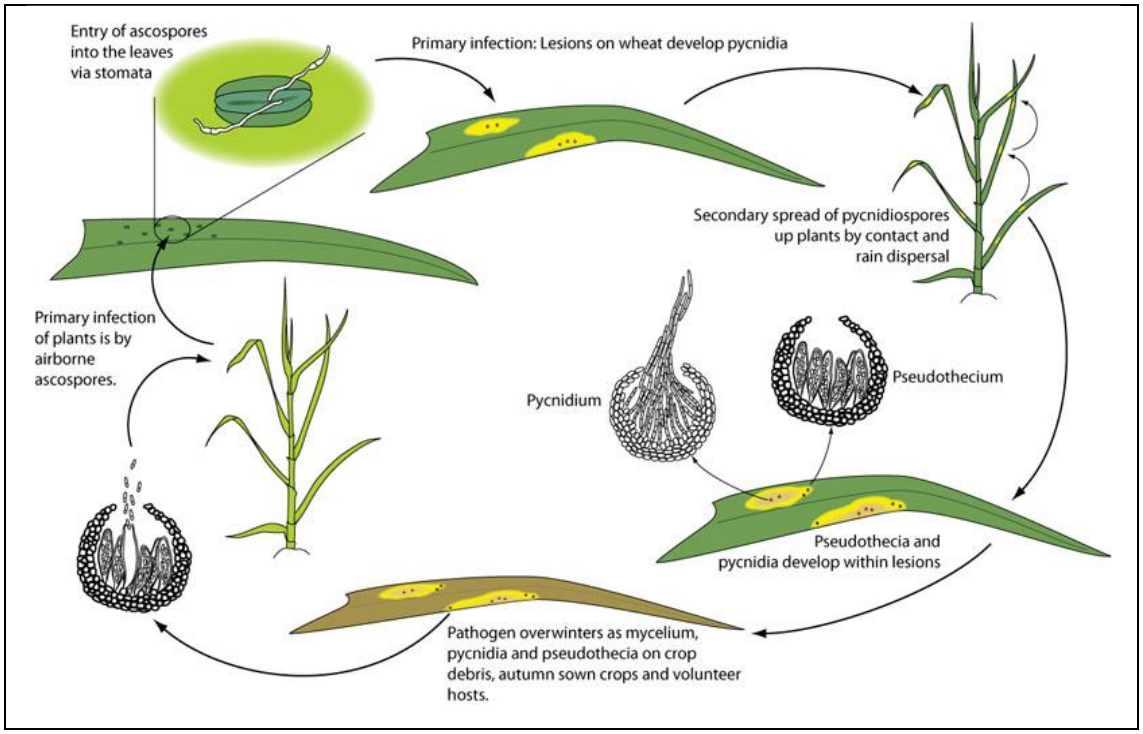

Figure 5: *Z. tritici* disease cycle by Alisa Ponomarenko, Stephen B. Goodwin, and Gert H. J. Kema (Ponomarenko *et al.*, 2011)).

2. General materials and methods

2.1. *Zymoseptoria tritici* inoculum

Unless otherwise stated, *Zymoseptoria tritici* strain Sso1, a mutant strain of *Z. tritici* IPO323 was used for all experiments. Sso1 was chosen due to ease of visualisation under microscopy due to the fusion of eGFP to a syntaxin 2 homologue, a gene which codes for a protein that localises in the fungal plasma membrane (Kilaru *et al.*, 2017). The strain was maintained long term as conidiospores stored in 50 % glycerol at -80 °C. Strains also used in this thesis were chosen due to their compatibility with the wheat cultivar used (Consort), these strains were: (i) IPO323: The reference strain, isolated in 1981 from Western Brabant cv. Arminda, (ii) IPO94269: A Dutch field isolate from wheat stubble, Kraggenburg, Netherlands, (iii) IPO97001: Isolated from leaves of bread wheat, Czech Republic, 1997 (Kema and van Silfhout, 1997, Kema *et al.*, 1996a) and (iv) Ku70: A strain developed by Sidhu et al (2015) deficient in the non-homologous end joining pathway of DNA double stranded break repair, by inactivating the KU70 gene.

To produce conidiospores for inoculum, *Z. tritici* was cultured on YPD agar (recipe below) for 7 days. For work entailing quantification such as leaf infections, conidiospores were suspended in MilliQ water. Concentrations were estimated by haemocytometer and adjusted to concentrations listed in each experiment. Conidiospore suspensions were used within 60 minutes of suspending, unless otherwise stated.

2.2. Agar-based media

YPD Agar: prepared as per Sigma recipe (Y1500 – YPD Agar): Yeast Extract 10 g/l (Sigma-Y1625), Bacteriological Peptone 20 g/l (Sigma-Aldrich-91249), Dextrose 20 g/l (Sigma-D9434), Bacteriological Agar 15 g/l (Sigma-Aldrich-A5306).

Where appropriate, YPD was supplemented with 50 mg/l of Penstrep (P4333 SIGMA) and 100 mg/l of Ampicillin (A9393 SIGMA) were added to agar media by filter sterilisation before pouring.

Minimal media agar: MilliQ water was supplemented with 6 g $NaNO_3$, 0.52 g KCl, 0.52 g $MgSO_4.7H_2O$, 1.52 g KH_2PO_4, 10 g glucose, 1.0 ml HUTNER'S trace elements, The pH was adjusted to 6 with NaOH or HCl before sterilisation by autoclaving at 121 °C for 15 minutes (Pontecorvo *et al.*, 1953).

V8 agar: V8 vegetable juice 50 % v/v. Bacteriological Agar 15 g/l (Kema and Annone, 1991).

PDA: Potato extract 4 g/l, Glucose 20 g/l, Bacteriological agar 15 g/l.

Wheat leaf agar (WLA) was adapted from (Guo and Verreet, 2008). Leaves were taken from plants grown for at least 4 weeks. Harvested leaves were frozen in liquid nitrogen, before being broken into small pieces and stored at -80 °C for a period of less than 6 months. To prepare media for autoclaving, 1 litre of sterile distilled water was heated to boiling in a 5-litre conical flask under continuous magnetic stirring. 100 g frozen leaf pieces were added directly from the -80 °C freezer and boiled for 20 minutes. Water was then added to replace any loss by evaporation. Leaf suspensions were supplemented with 20 g/l dextrose and 15 g/l bacteriological agar (Sigma-Aldrich-A5306) before immediately autoclaving at 121 °C for 15 minutes. To ensure homogeneous wheat leaf agar plates, agars were cooled to 45 °C in a water bath before gently swirling and pouring. Any alterations to this wheat leaf agar (WLA) recipe are outlined in individual experimental results.

Arabidopsis thaliana Col0, Durum wheat (*Triticum durum* cv. Volcani), Barley (*Hordeum vulgare* cv. Golden Promise) and Rice (*Oryza sativa* cv. Co39) were also used for leaf agars.

2.3. Seeds, soil and growth cabinet conditions

J. Arthur Bower's John Innes No. 2 Compost and *Triticum aestivum* cv. Consort winter wheat seeds (kindly donated by Nick Palmer of RAGT seeds) were use in all experiments unless

otherwise stated. Compost was stored frozen at -20 °C for 3 weeks before use. Two seeds were sown in each cell of a 24 modular seed tray (Vacapots from H.Smith Plastics, plantcell.co.uk) containing compost, and loosely covered. Inserts were then placed into a Whitefurze 38 cm Gravel Tray and filled with 750 ml distilled water.

Plants were placed onto one of the three shelves of a Panasonic MLR-352-PE growth cabinet. A long-day light cycle (16 hours of light at 20 °C and 8 hours of darkness at 15 °C) was used with 90 % relative humidity, using the maximum light setting (~5 x 10 μmol m^2 s^{-1} at leaf level). Plants were left uncovered for 5 days until growth was visible above the soil and subsequently grown for at least 14, and a maximum of 21 days before use.

2.4. Foliar inoculations and plant incubation periods

For all foliar applications, conidiospores were suspended in autoclaved MilliQ water. Concentrations were estimated by haemocytometer and adjusted to concentrations described in each experiment. Before use, suspensions were supplemented with 0.01 % (v/v) Silwet L-77 (Momentive Specialty Chemicals, UK). Suspensions were then applied using a paintbrush to the abaxial side of fully expanded leaves, until complete coverage was obtained.

Post-inoculation, all wheat plants were stored under standard growth cabinet conditions for 28 days. For the first 5 days, plants were sealed in autoclave bags to maintain maximum humidity. Plants were assessed for disease by counting pycnidia per cm^2 of inoculated leaf tissue.

2.5. Experimental repeats and statistical analysis

Unless otherwise stated, all individual experiments contained at least 3 replicates. Experiments were also repeated 3 times. For data analysis and graphs, the means from each experimental replicate was used. Where appropriate, residuals from datasets were analysed for normality using a Shapiro-Wilk test. If necessary, non-normal datasets were transformed using either a square root, $(N + 1)^{\wedge 2}$ or natural log transformation before parametric or

nonparametric tests were carried out accordingly. Where used, transformations are outlined in experimental results.

2.6. Photography and scanning

All photography was carried out using a Nikon D7000 with an 18 – 105 vr lens kit. Overhead images were taken using a Kaiser RS1 copy stand. Aperture and shutter settings differed depending on light conditions. Scanning of leaves for staining or disease assessments were carried out on a Canon CanoScan LiDE 210 at a setting of 1200 dots per inch.

2.7. Confocal microscopy

Confocal images were obtained using a 63x oil immersion lens on a Leica SP8 microscope. GFP fluorescence from *Z. tritici* Sso1 was detected at 510-530 nm using an argon laser with emission at 500 nm. Images were obtained using LAS-X software and processed as batches using macros written in Adobe Photoshop®.

2.8. Assessing growth on agar media

To assess growth and colony formation on a semi-solid surface, 1.5 % agar plates were made with nutrients outlined in each experiment. Conidiospore germination and colony growth were assessed by plating a known quantity of a 1 x 10^4 conidiospore solution onto agar plates in triplicate. Plates were stored under a long day cycle (18 hours light, 6 hours dark) at 20 °C. Colony number and size were measured after 7, 8, 10 or 14 days incubation (Methods 2.6). Finally, at 14 days, plates were scraped with 2 ml of water and resulting conidiospore solutions were counted on a haemocytometer to quantify levels of conidiation over time.

3. Leaving the pycnidium: Conidiospore culturability

3.1. Introduction

The pycnidiospores (asexual spores) of *Zymoseptoria tritici* are produced within the substomatal cavities of wheat leaves. They are protected from desiccation and inhibited from germination by a nutrient rich suspension termed the cirrus (Van Ginkel, 1999, Gough and Lee, 1985). In humid or wet conditions, spores imbibe water. This causes them to swell and subsequently ooze out of the stomatal space onto the leaf surface. During rain fall these spores are spread by rain-splash through the canopy, finally landing on nutrient-poor areas such as the leaf surface. Here, germination ensues and hyphal growth is induced (Duncan and Howard, 2000, Cousin *et al.*, 2006, Kettles *et al.*, 2016). However, there has been little study of how the environmental changes associated with spore dispersal, which include submergence in water (rain drops) and a rapid reduction in nutritional availability, affect these asexual spores.

We can mimic the natural life cycle of an asexual spore by submerging/suspending conidiospores grown on YPD agar into MilliQ water. Water-suspended spores can then be monitored over time by assessing changes in viability, culturability, and virulence.

3.2. Methods

3.2.1. Assessing the culturability of water-suspended conidiospores

Three *Z. tritici* strains were tested: *Z. tritici* IPO323, Sso1 and Ku70 (further detailed in Methods 2.1), for assessments of conidiospore culturability in water over time. All strains were incubated from -80 °C glycerol stocks onto YPD agar 7 days before use. For spore suspensions, conidiospores were suspended in autoclaved MilliQ water. Concentrations were subsequently estimated by haemocytometer and adjusted to 1 x 10^7 conidiospores/ml. Aliquots were then diluted by 10^{-3} before spreading 100 µl (~ 1000 spores) onto YPD agar.

Changes in culturability were assessed by colony counting after 7 days. Culturability was calculated as: number of colonies/number of spores plated.

YPD agar medium and constituent agar media (YP, YD, PD, P, D, Y) were all made in same quantities as Sigma recipe: Yeast extract 10 g/l (Sigma-Y1625), Bacteriological peptone 20 g/l (Sigma-Aldrich-91249), Dextrose 20 g/l (Sigma-D9434). All agars were supplemented with 15 g/l bacteriological agar (Sigma-Aldrich-A5306): Wheat leaf agar was made as per Methods 2.2.

3.2.2. Assessing the effects of osmotic & nutrient shock during submergence in water

Six suspension media were tested to assess if culturability is linked to the reduction of available nutrients and/or osmotic shock: (i) Autoclaved MilliQ water, (ii) Autoclaved MilliQ water supplemented with sorbitol to a final concentration of 0.6 M (110 g/litre) and (iii) Autoclaved MilliQ water supplemented with PEG 4000 to a final concentration of 20 % w/v, (iv) Autoclaved MilliQ water supplemented with YPD (Yeast Peptone Dextrose, prepared as per Sigma recipe Y1500 – YPD), (v) Autoclaved MilliQ water supplemented with both YPD and sorbitol (0.6 M), (vi) Autoclaved MilliQ water supplemented with both YPD and PEG 4000 (20 % w/v). The water used in all solutions was supplemented with 10% Tris-HCl to act as a buffer against pH changes. Spore suspensions were made by suspending at 1 x 10^7 conidiospores/ml (concentrations were estimated by haemocytometer). At the time-points listed in each experiment, aliquots were taken and diluted by 10^{-3} before spreading 100 µl (~ 1000 spores) onto YPD agar. Changes in culturability were assessed by colony counting after 7 days. Culturability was calculated as: number of colonies/number of spores plated.

3.2.3. Assessing spore viability over time in water cultures

Cell-death/viability was quantified using confocal microscopy, using propidium iodide (PI) to stain spores. One ml aliquots of 1 x 10^7 conidiospores/ml suspensions were supplemented with PI at a final concentration of 1 µl/ml. Stained suspensions were used within 60 minutes.

2-dimensional confocal images (Methods 2.7) were taken every 24 hours using excitation/emission maxima of wavelength 493/636 nm. Cells were scored as dead/non-viable if PI was visualised to have completely stained the cytoplasm (pink fluorescence). The total number of cells per conidiospore over time was also assessed from these images.

3.2.4. Assessing the virulence of conidiospore populations over time

Three *Z. tritici*: strains, IPO323, IPO94269 and IPO97001 were grown on YPD agar for 7 days before suspending in autoclaved MilliQ water at 1 x 10^5 conidiospores/ml. Suspensions were then supplemented with 0.01 % (v/v) Silwet L-77 (Momentive Specialty Chemicals, UK).

At either 10, 60, 120 or 240 minutes post-suspension, conidiospore suspensions were applied by paintbrush (as per Methods 2.4) to wheat plants (cv. Consort) which had been grown at standard growth cabinet conditions (Methods 2.2) for 14 days. Disease was assessed as pycnidia/cm^2 of leaf after 28 days.

3.2.5. RNA: Extractions

For RNA analysis of conidiospores during submergence, conidiospores were suspended at 1 x 10^7 conidiospores/ml in 50 ml tubes of MilliQ water. These tubes were shaken vigorously for 10 seconds every 24 hours to maintain oxygenation. At four time-points (1 hour, 4 hours, 24 hours and 7 days), four randomly selected 50 ml tubes were centrifuged for 1 minute at 2000 x g before freezing pellets in liquid nitrogen. Pellets were subsequently ground to a powder in a pestle and mortar using liquid nitrogen and were not allowed to thaw.

Total RNA extractions were carried out using the Qiagen RNeasy Kit (Cat No./ID: 74903) protocol 'Purification of Total RNA from Plant Cells and Tissues and Filamentous Fungi' with an on-column DNase step using Qiagen RNase-Free DNase Set (Cat No./ID: 79254). Samples were finally suspended in 50 µl RNase-free water before storing at -80 °C for analysis.

3.3. Results

3.3.1. Culturability of water-suspended conidiospores is < 30 %

On leaf tissue, *Z. tritici* conidiospores are reported to germinate with about 75 % efficiency (Cohen and Eyal, 1993, Kema *et al.*, 1996b, Shetty *et al.*, 2003, Fones *et al.*, 2017b). To confirm this on agar, conidiospores from three strains of *Z. tritici* were suspended in water for approximately 90 minutes. Around 1000 conidiospores were subsequently plated onto YPD agar. Changes in culturability were assessed by colony counting after 7 days. Culturability was calculated as: number of colonies formed/number of spores plated. Additionally, to assess the suitability of YPD agar as a medium with which to assess culturability, *Z. tritici IPO323* was also plated onto 9 agars which differ in their nutrient content.

Results (Figure 6A) show that, in all three *Z. tritici* strains tested, < 30 % of conidiospores formed colonies when plated onto rich agar (YPD) plates. No significant differences were recorded between the culturability of the 3 strains tested (One-way ANOVA, p-value = 0.21, n = 2. Data were normally distributed). These results indicate that the conidiospores of *Z. tritici* have a significantly lower culturability when plated on YPD agar than previously reported on leaves. Figure 6B shows that the nutritional content of the agar onto which conidiospores are plated, does not significantly affect culturability (One-way ANOVA, p-value = 0.05, n = 4. Data were normally distributed). The agars tested included a complete and nutrient rich agars (YPD), agars rich in particular nutrients (e.g. yeast extract, peptone or dextrose) but also contained a low nutrient agar (water agar) and a host specific agar (wheat leaf agar).

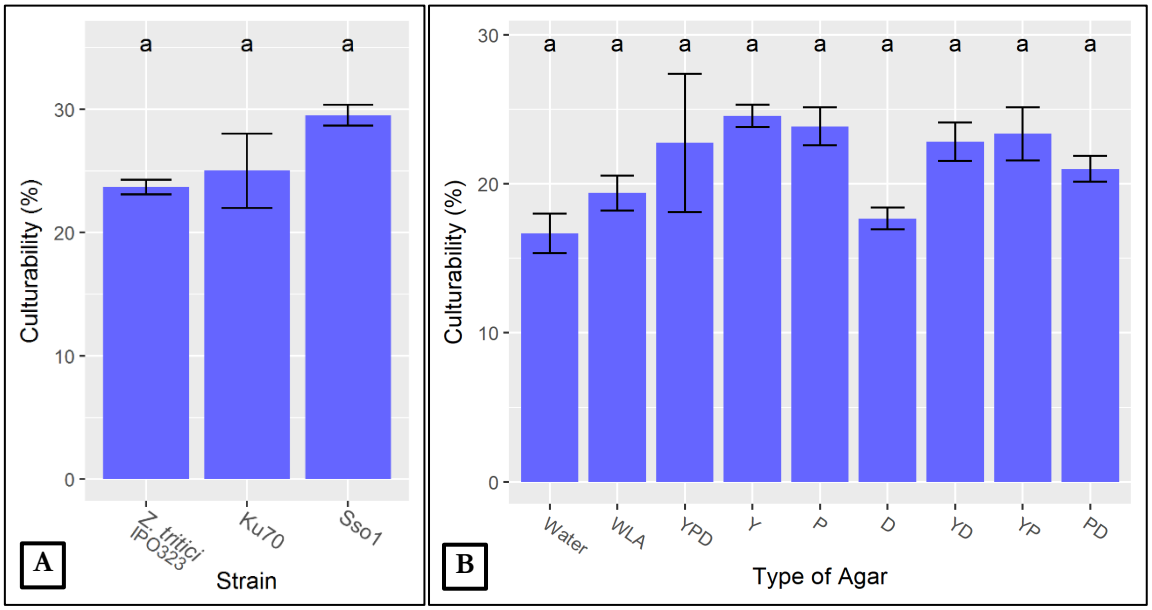

Figure 6: An assessment of conidiospore culturability in *Z. tritici*
A: An assessment of the conidiospore culturability of three *Z. tritici* strains (plated onto YPD). ~ 1000 conidiospores of three *Z. tritici* strains were plated from 1 x 10^7 /ml water cultures onto YPD agar. Colonies were counted after 8 days. Culturability was calculated as: (number of colonies formed / number of spores plated) x 100. Data are means from two independent experiments. Error bars show SE. Results show that in all three strains, culturability was < 30 %. No significant differences were recorded between the three strains tested (ONE WAY ANOVA), P-VALUE = 0.21. Letters indicate no significant differences between treatment groups as determined by post-hoc Tukey test; $p < 0.05$). **B:** An assessment of culturability of *Z. tritici* IPO323 conidiospores plated onto multiple agars which differ in their nutrient content. ~ 1000 *Z. tritici IPO323* conidiospores were plated from a 1 x 10^7 /ml water culture onto various agars. Colonies were counted after 8 days. Culturability was calculated as: number of colonies formed/number of spores plated. Data are means from four independent experiments. Error bars show SE. Agars tested are constituents of YPD, WLA = Wheat Leaf Agar (100 g/l wheat leaves), and water agar. All agars contained 15 g/l bacteriological agar (Sigma-Aldrich-A5306). No siginificant differences were recorded in the number of CFUs (ONE WAY ANOVA). Letters indicate no significant differences between treatment groups as determined by post-hoc Tukey test; P-VALUE = 0.05. Data were normally distributed.

3.3.2. Conidiospore culturability is not affected by suspension concentration

The culturability of *Z. tritici* conidiospores stored at 1 x 10^7 spores/ml has been shown to be unexpectedly low as compared to previous, leaf-based assessments (Results 3.3.1). The spore densities contained in raindrops in the field would likely be lower than that tested here. To assess if the high spore concentration used here may have affected culturability, an experiment was conducted up to assess suspensions ranging from 10^4 – 10^7

conidiospores/ml. At ~ 2 hours and 24 hours, conidiospores of each suspension were plated onto YPD agar. Changes in culturability were assessed by colony counting after 7 days. Culturability was calculated as: (number of colonies formed/number of spores plated) x 100.

Results in Figure 7 support previous data (Results 3.3.1) showing low culturability in previously water-suspended conidiospores. In all trials and treatments, the number of conidiospores which formed colonies after 2 hours of suspension in water, in comparison to spores kept in water for 24 h, was significantly lower. It can be suggested, therefore, that the culturability of conidiospores might have already dropped significantly by the 2-hour time-point. Culturability may therefore be affected by the initial shock of submergence in water.

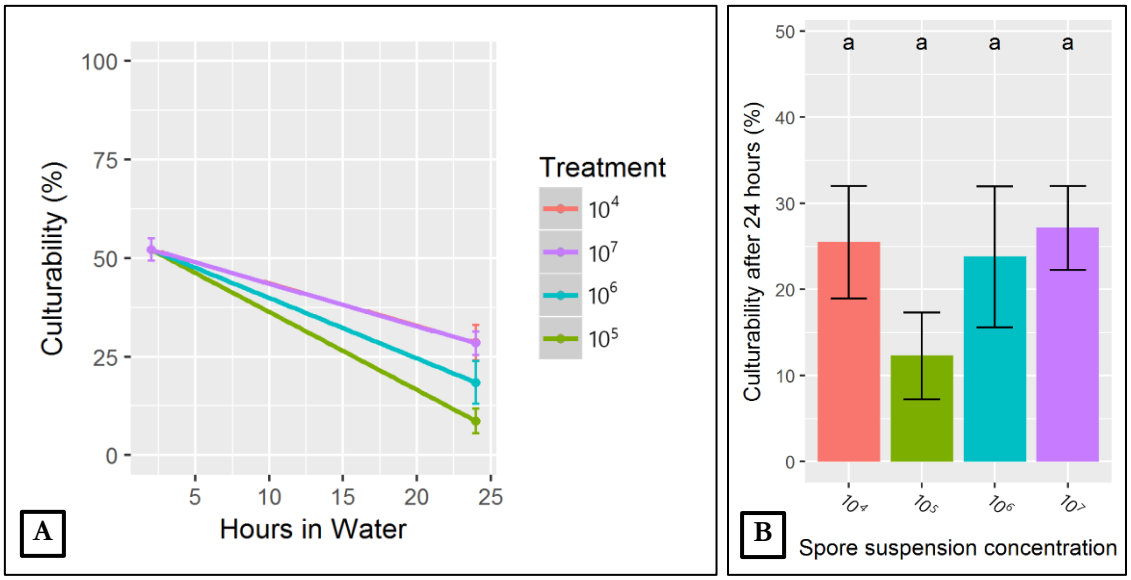

Figure 7: Culturability of *Z. tritici* conidiospores suspended at different concentrations over a 24-hour period.
A: Conidiospore stored at all suspension concentrations significantly lose culturability over a 24 hour period. ~1000 conidiospores were plated from water cultures onto YPD agar after 2 hours or 24 hours. Colonies were counted after 8 days. Culturability was calculated as: (number of colonies formed / number of spores plated) x 100. Data are means from 9 independent experiments. Error bars show SE. **B:** Culturability of conidiospores after 24 hours in water is not significantly effected by the concentration of the conidiospore suspension from which they were plated (One-way ANOVA, p-value = 0.35, n = 9. To achieve normality, data were transformed using a Log(10) transformation – Shapiro-Wilk, p-value = 0.33).

3.3.3. Reduction in conidiospore culturability is rapid upon suspension

Previous results have shown that culturability of conidiospores is below 40 % within 2 hours of suspension in water (Results 3.3.1). It has also been shown that 24 hours later, culturability had fallen further still to below 25 % (Results 3.3.2). From this it can be argued that the decline in culturability likely begins before the time-points tested in this assay. To assess earlier time-points, an experiment was conducted which plated water-suspended conidiospores onto YPD agar at multiple points during a 4-hour period. Changes in culturability were assessed by colony counting 7 days after plating. Culturability was calculated as: (number of colonies formed/number of spores plated) x 100.

Results (Figure 8) show that conidiospores lose culturability rapidly when suspended in water. After 10 minutes, the reduction was not statistically significant from that measured in conidiospores aliquoted after 1 minute (Students *t*-test, t = 1.63, df = 10, p-value = 0.134). Within 60 minutes, however, significant differences were recorded (Students *t*-test, t = 2.45, df = 10, p-value = 0.034). The culturability continues to drop over all time-points tested. In conclusion, results show that upon submergence in water, the culturability of *Z. tritici* conidiospores drops rapidly and continues to reduce over a 4-hour period. These results confirm that the culturability of conidiospores is rapidly and significantly affected by submergence in water.

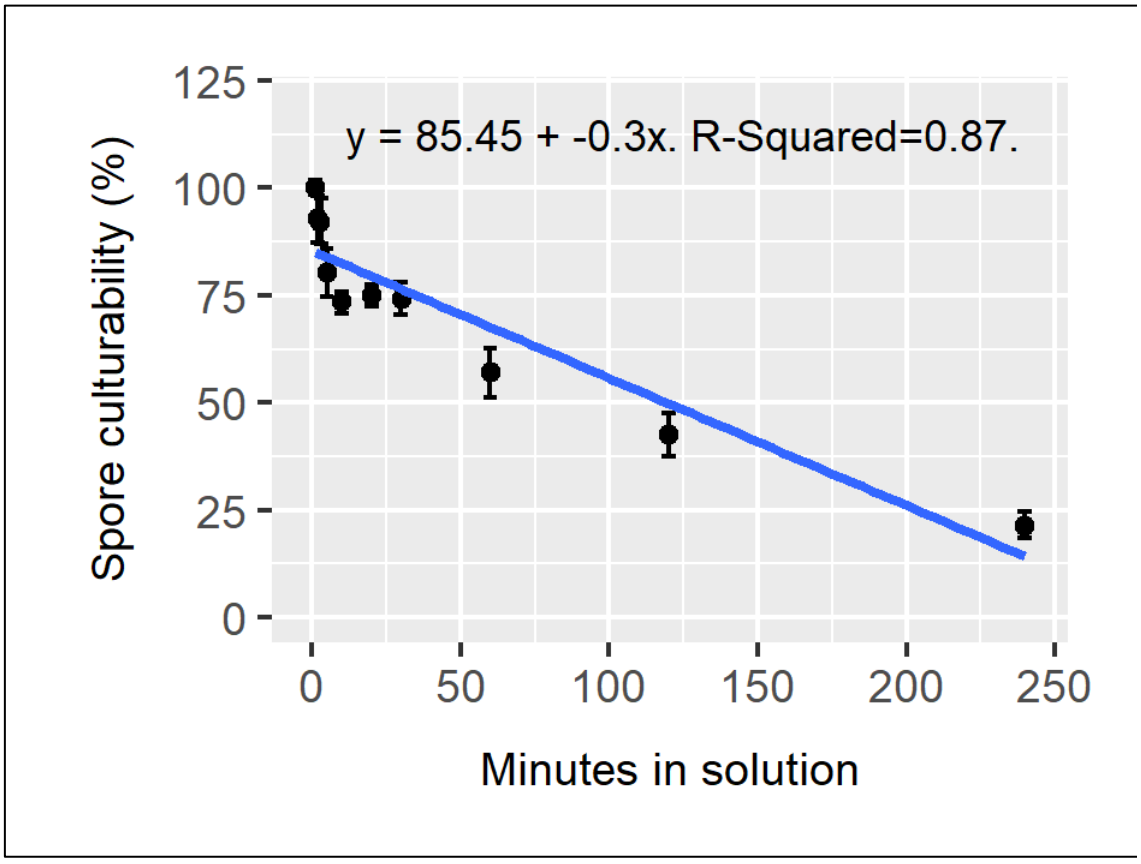

Figure 8: Culturability of *Z. tritici* conidiospores from water cultures over a 4-hour period. At each time-point ~ 1000 conidiospores were plated from 10^6 spore suspensions onto YPD agar. Colonies were counted between 4 and 8 days post plating. Data shown uses counts relative to counts at first time point for each independent experiment. Culturability was calculated as: (number of colonies formed/number of spores plated) x 100. Data are means from 16 independent experiments. Line of best fit is derived from a linear regression ($R^2 = 0.87$). Error bars are CI.

3.3.4. Osmotic and nutrient shock contribute to reduction in culturability

The previous experiment showed that conidiospore culturability is lost rapidly upon submergence in water. In this process, conidiospores are moved from a high-nutrient agar-based environment (YPD agar), to a low-nutrient submerged environment (water-suspension). It can be suggested, therefore, that any loss in culturability may be linked to either the osmotic or nutrient shock occurring during suspension in water. To test this hypothesis, an experiment was conducted to compare conidiospores suspended in either pure water, YPD, or the osmolytes polyethylene glycol 4000 (PEG 4000) (20 % w/v) or

sorbitol (0.6 M). During a 4-hour period, conidiospores were plated from each suspension onto rich (YPD) agar. Changes in conidiospore culturability were assessed by colony counting after 7 days. Culturability was calculated as: (number of colonies formed/number of spores plated) x 100.

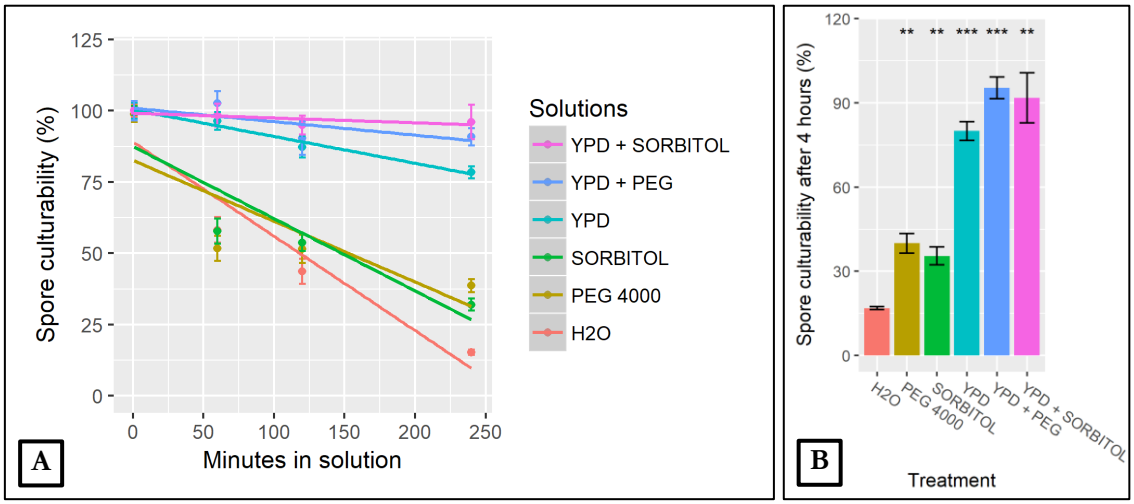

Figure 9: Assessment of the effects of osmotic and nutrient shock on the culturability of *Z. tritici* conidiospores suspended in water over a 4-hour period.
At each time-point ~ 1000 conidiospores were plated from suspensions onto YPD agar. Colonies were counted after 8 days. Culturability was calculated as: (number of colonies formed/number of spores plated) x 100. **A:** A comparison of conidiospore culturability in six different culture media over a 4-hour period. H_2O suspensions show the most significant reductions in culturability over time compared with sorbitol, PEG 4000 or YPD supplemented suspensions. Lines of best fit are derived from linear regression fits (R^2: H_20=0.75, Sorbitol=0.64, PEG=0.50, YPD=0.33, YPD+Sorbitol=0.002, YPD+PEG =0.09). **B:** A comparison of conidiospore culturability in four different cultures after 4 hours of suspension. The addition of the osmolytes PEG 4000 (20 % w/v) and sorbitol (0.6 M) significantly maintain the culturability of conidiospores compared to H_2O (Students two Sample *t*-test, compared to water only, t = -7.8023, df = 7, p-value = 0.0001069 and t = -3.7284, df = 8, p-value = 0.005801 respectively). Suspending in YPD further maintains culturability compared to H_2O (Students two Sample *t*-test, compared to water only, t = -18.45, df = 8, p-value = 7.671e-08). Suspending conidiospores in YPD supplemented with PEG 4000 or sorbitol almost completely maintains the culturability of conidiospores over a 4-hour period (Students two Sample *t*-tests, compared to water only, t = -12.021, df = 8, p-value = 2.116e-06 and t = -11.739, df = 8, p-value = 2.535e-06 respectively).

Figure 9 shows that the culturability of conidiospores suspended in water decreases from around 100 % at 1 minute, to around 16 % by 4 hours. Suspending conidiospores into a nutrient rich YPD solution, however, maintains culturability of conidiospores at more than 75 %. The addition of either of the osmolytes sorbitol or PEG 4000 (20% w/v), chemicals used to alleviate osmotic stress, also helps to maintain conidiospore culturability compared

to those suspended in water alone. Conidiospores added to a solution of both YPD and sorbitol or PEG 4000 showed only around a 10 % loss in culturability over a 4-hour period.

3.3.5. Reductions in culturability are not due to conidiospore death

As shown in Results 3.3.3, conidiospores rapidly lose culturability upon suspension in water. A possible explanation for this reduction is that spores rapidly lose viability. To assess this, an aliquot of each conidiospore suspension was stained with propidium iodide (PI). PI is a membrane impermeant dye that stains only the plasma membrane of viable cells, but floods the cytoplasm of non-viable cells (Thermo Fisher, R37108). Using confocal microscopy, at least 4 images were taken from each of the 4 experimental repeats. For each image, the mean viability was calculated as: the mean number of spores with at least one live cell/total number of spores. Cells were scored as viable if the cell cytoplasm is not flooded with dye.

Results (Figure 10C) show that the number of viable conidiospores (measured as conidiospores with > 1 viable cell) is maintained for at least 4 days post-water-suspension. Microcycle conidiation (MC) can be visualised at all time-points (Figure 10B, white arrows), confirming active, and therefore viable cells.

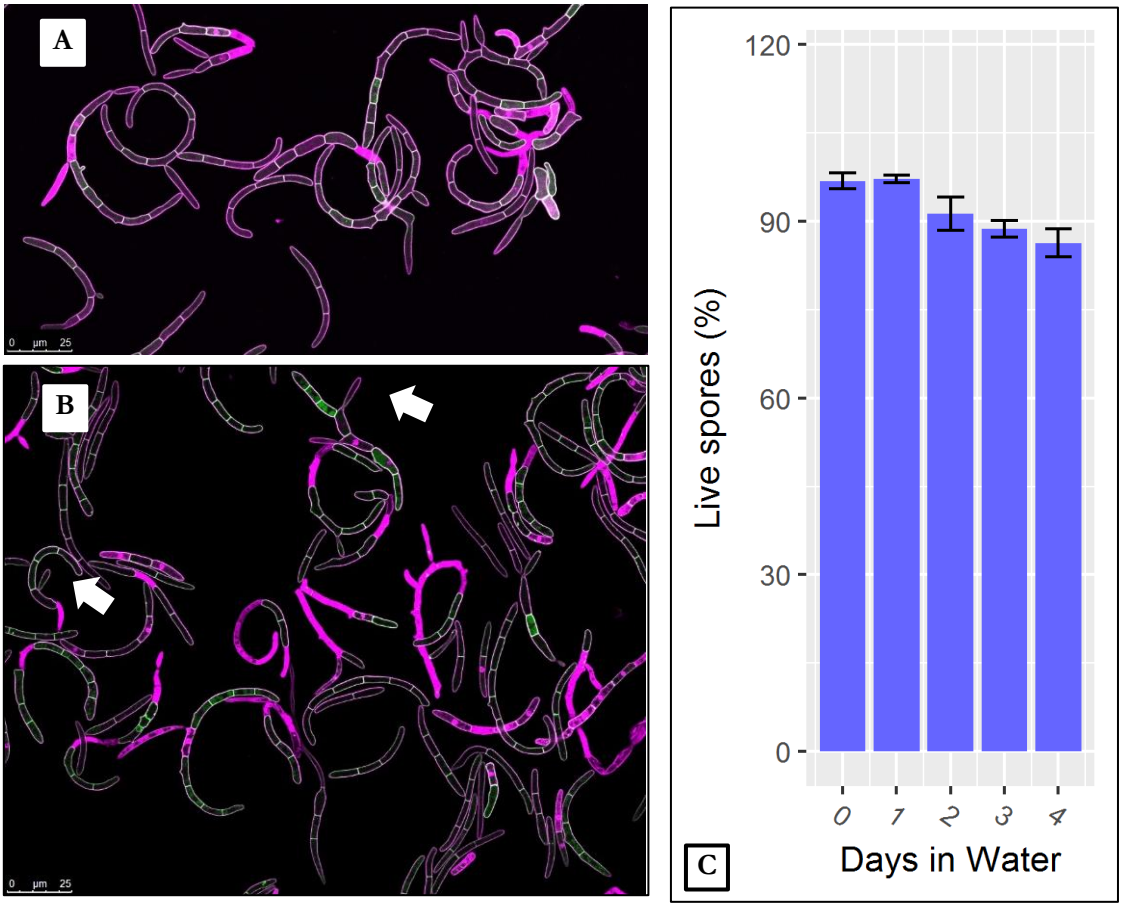

Figure 10: An assessment of the viability *Z. tritici* conidiospores post- suspension in water. Spores stained with propidium iodide (PI). Live spores contain at least one live cell (unstained cytoplasm).
A: Representative image of a conidiospore population at 1-hour post-suspension in water. Image shows > 90 % of conidiospores have at least 1 live cell. B: Representative image of a conidiospore population at 1-day post-suspension in water. Image also shows > 90 % of conidiospores have at least one live cell (unstained cytoplasm). White arrows show microcycle conidiation confirming active, and therefore viable cells/spores. C: Graph shows the percentage of conidiospores with at least one live cell over time. There is no significant decrease in the number of viable conidiospores during the first 4 days post-suspension in water (Students t-tests. df = 5, Bonferroni corrected p-values = 1.0, 0.85, 0.05, 0.09 respectively). Data are means of viability assessments from 4 independent experiments, each containing at least 4 images of conidiospores populations. For each image, the mean viability was calculated as the mean number of spores with at least one live cell/total number of spores. Cells were scored as viable if the cell cytoplasm was not flooded with dye. Error bars show SE.

3.3.6. Virulence of conidiospores reduces over time in water cultures

Results have shown that conidiospore culturability drops upon suspension in water (Results 3.3.3). However, spore viability is retained for at least 4 days (Results 3.3.5). It could be argued that the culturability of a spore on agar, is not akin to culturability on a plant. Indeed,

high spore germination has been recorded in plant studies elsewhere (Cohen and Eyal, 1993, Kema *et al.*, 1996b, Fones *et al.*, 2017b). To assess if suspension in water also affects conidiospore germination/growth on a leaf, conidiospores were inoculated onto plants at different time-points post-suspension. Three *Z. tritici* strains were used to assess this: (i) *Z. tritici* IPO323: (ii) IPO94269 and (iii) IPO97001 (Kema and van Silfhout, 1997, Kema *et al.*, 1996a).

Results (Figure 11) show that in all three strains tested, disease (measured as number of pycnidia per cm^2 of leaf on day 28) declined with time suspended in water. After 60 minutes post-suspension, the conidiospores of all three strains caused less disease when compared to those which had been water-suspended by just 10 minutes (letters indicate significant differences between treatment groups as determined by post-hoc Tukey test; $p < 0.05$).

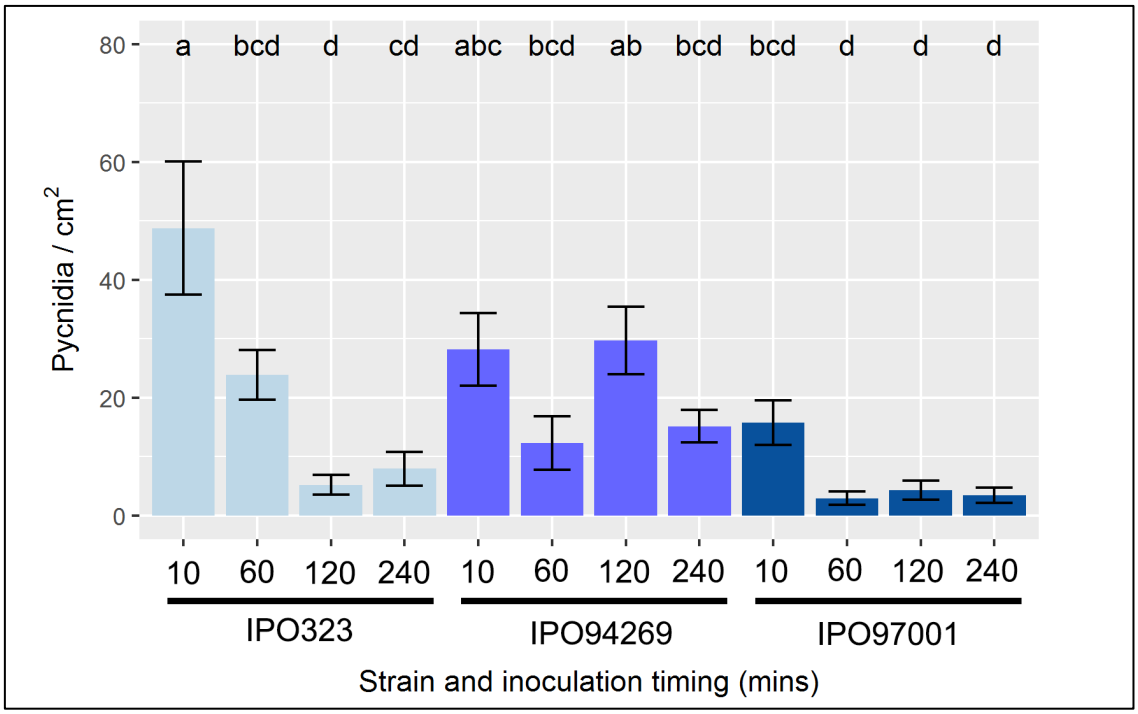

Figure 11: Assessment of water-suspended *Z. tritici* conidiospore virulence over time. Conidiospore suspensions were made for three strains of *Z. tritici*. Suspensions were then inoculated on to plant leaves by paintbrush method (Methods 2.4) at either 10, 60, 120 or 240 minutes. The effect of time suspended in water on subsequent disease (pycnidia/cm^2 at 28 days) was significant: (One-way ANOVA, p-value < 0.0001, n = 3). Data are means of pycnidia counts from 3 independent experiments, each containing at least 7 leaves. Error bars show SE. Letters indicate significant differences between treatment groups as determined by post-hoc Tukey test; $p < 0.05$).

3.3.7. RNA extractions

To assess if the observed reduction in culturability leads to a reduction in gene transcription, total RNA extractions were implemented using the Qiagen RNeasy Plant Kit. Extractions were carried out using ~ 5 * 10^8 conidiospores either isolated from YPD plates, or from water-suspended populations. An initial assessment of extracted RNA was made using the Thermo Scientific NanoDrop™ 1000 Spectrophotometer. Figure 12 shows the concentration in ng/µl of all samples. RNA yields fall significantly after suspending conidiospores in water. Previous results have shown that conidiospores retain their viability over all the time-points assessed here (at least up to day 4, Results 3.3.5).

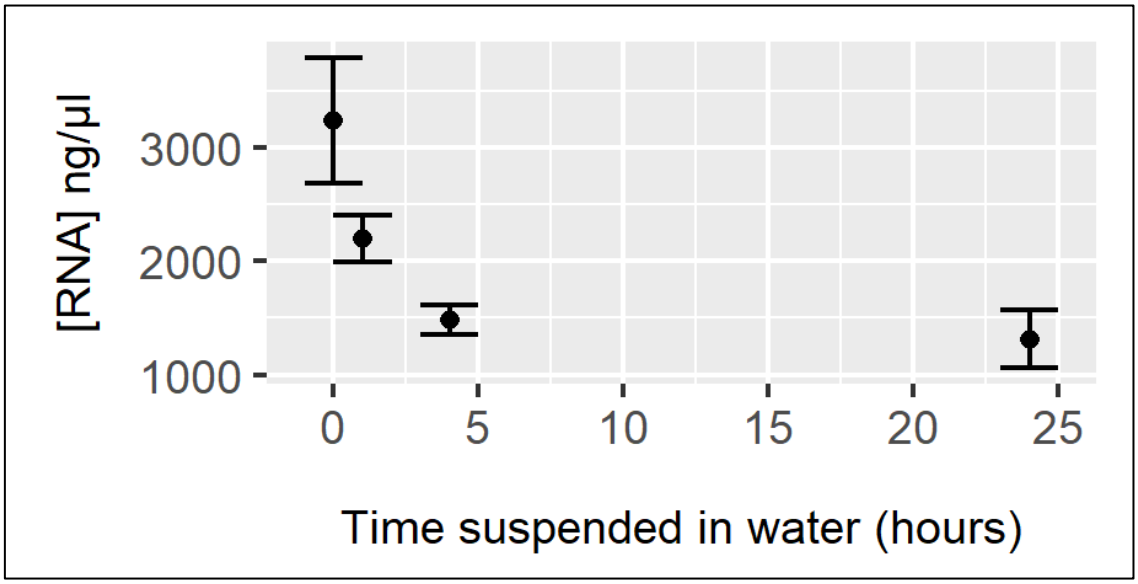

Figure 12: RNA yields (ng/µl) from conidiospore extractions (NanoDrop™ data).
Total RNA extractions were implemented using the Qiagen RNeasy Plant Kit. Extractions were carried out using ~ 5 x 10^8 conidiospores, either isolated from YPD plates, or from water-suspended populations.

3.3.8. RNA: NanoDrop™ quantification

An initial assessment of extracted RNA was made using the Thermo Scientific NanoDrop™ 1000 Spectrophotometer. Table 1 shows the concentration in ng/µl of all samples, as well as an assessment of both protein and carbohydrate contamination. RNA absorbs at around 260 nm. Proteins and phenol (often used in extractions) often absorb at 280. We can therefore

assess protein contamination using the 260/280 ratio. For a pure RNA sample a 260/230 of 2.0 is expected. This was achieved in all samples except for two samples after 7 days submergence. Carbohydrates absorb around 230. We can therefore also assess carbohydrate contamination using the 260/230 ratio. For a pure RNA sample a 260/230 of above 2.0 is expected. This was achieved in the majority of samples.

Table 1: RNA yields and quality (NanoDrop™ data) from suspended conidiospores.

Time	YPD	YPD	YPD	YPD	1h	1h	1h	1h	4h	4h	4h	4h	24h	24h	24h	7d	7d	7d	7d
Rep	1	2	3	4	1	2	3	4	1	2	3	4	1	3	4	2	4	5	6
RNA ng/µl	3508	4048	1620	3784	1040	2770	1790	2160	612	1519	680	1821	808	1504	1622	49.8	28.6	37.7	23.8
$A_{260/280}$	2.18	2.16	2.18	2.17	2.17	2.12	2.18	2.17	2.21	2.19	2.24	2.19	2.21	2.20	2.21	2.13	2.16	1.06	1.98
$A_{260/230}$	2.49	2.43	1.62	2.48	1.41	2.29	2.44	2.42	0.74	1.73	2.44	2.15	0.84	2.41	2.24	0.58	0.11	1.08	1.48

3.3.9. RNA: Quantification by agarose gel electrophoresis

To assess DNA contamination in samples, and to visualise RNA degradation, a known concentration of RNA from each sample was supplemented with loading dye and run on a 0.8 % agarose gel in TAE buffer (40 mM Tris, 20 mM acetic acid, and 1 mM EDTA) at 80 V for 90 minutes. Bright bands shown in Figure 13 represent ribosomal RNA (rRNA). This was expected to show two bands, 18S and 28S, however, samples contain three bands. Brightness of rRNA bands can be used as a proxy for the presence of total concentration of messenger RNA (mRNA) in samples. Due to the size of the 28S rRNA (3306 bp) compared to the 18S rRNA (1743 bp) in *Z. tritici*, it is expected that the relative brightness would be 1.9:1. This indicates that the middle band in Figure 13 is the 28S rRNA, with the 18S band found just below. In most samples, mRNA (expected to be ~5 % of total cell RNA) can be visualised as a faint band at the bottom of the gel. Genomic DNA contamination is not obviously present. This would be seen as a band at the top of the gel, due to the large (and therefore heavy) size of genomic DNA in comparison to RNA.

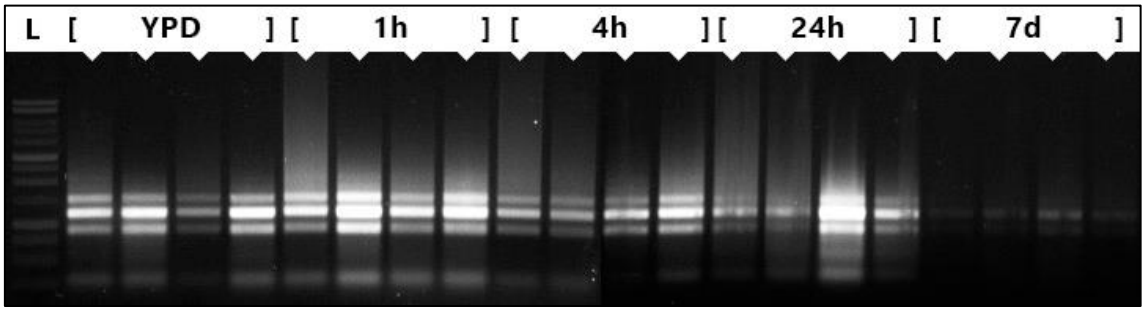

Figure 13: Agarose gel electrophoresis of RNA extractions from water-suspended *Z. tritici* populations.
Ladder (lane 1) is Promega™ 1kb DNA Ladder Molecular Weight Marker. Extractions are from YPD plates (lane 1-4) and from water cultures at 1 hour (lane 5 – 8), 4 hours (lane 9 – 12), 24 hours (lane 13 – 16) and 7 days (lane 17 – 20).

3.3.10. RNA: Quantification by Agilent 2100 Bioanalyser

Samples were further analysed on the Agilent 2100 Bioanalyser: a microfluidic system for the electrophoresis-based analysis of biomolecules such as RNA. 1 μl of each sample was used to assess purity and total concentration. Figure 14 shows that all samples contain both the 28S and 18S rRNA peaks. In all samples, as expected due to its size, the 28S peak is larger than 18S peak. As estimated by height of peaks, RNA concentrations of extractions significantly fall over time. RNA Integrity Number (RIN) scores shown in Table 2 (scored from 1 – 9) are used to assess degradation of samples. These scores show a generally low level of degradation (over 7 is requested for sequencing). However, 24-hour samples have lower scores.

Table 2: RIN (RNA Integrity Number, scored from 1 – 9) for extracted samples.
A quantification of the quality of RNA extracted from conidiospore suspensions over time. RIN numbers are results from Agilent 2100 Bioanalyser assessments using the total RNA protocol.

Time	YPD	YPD	YPD	YPD	1h	1h	1h	1h	4h	4h	4h	4h	24h	24h	24h	7d	7d	7d	7d
Rep	1	2	3	4	1	2	3	4	1	2	3	4	1	3	4	2	4	5	6
RIN	8.4	7.7	8.5	8.2	8.5	8	7.9	8.2	8.8	8.2	8.8	8.2	6.6	6.8	6.8	8.9	8.1	8.4	8.8

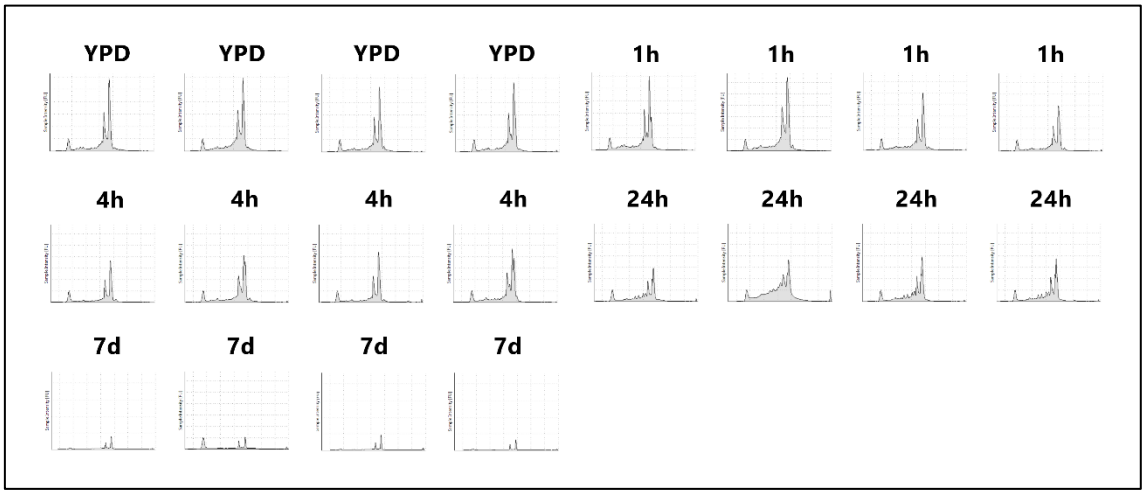

Figure 14: Agilent 2200 Tapestation assessment of RNA extractions.
Sample sizes taken for extractions were equal number of cells but RNA yield extracted drops sharply over time. By 14 days, no RNA was isolated so the experiment was terminated.

3.3.11. RNA: Quantification by Qubit™

Finally, using concentration data from the Qubit™ Fluorometric Quantitation System (Table 3), samples were normalised to 200 ng in 48 µl RNase free water. A further 2 µl of spike-ins was added before submission for sequencing – hybridisation between the spike-ins and the control probes are used to normalise the hybridisation measurements of the sample RNA.

Table 3: RNA concentrations using the Qubit™ Fluorometric Quantitation system.

Time	YPD	YPD	YPD	YPD	1h	1h	1h	1h	4h	4h	4h	4h	24h	24h	24h	7d	7d	7d	7d
Rep	1	2	3	4	1	2	3	4	1	2	3	4	1	3	4	2	4	5	6
RNA ng/µl	364	191	286	201	312	176	130	263	211	97	223	96	228	119	108	38	5.2	5.1	4.6

3.3.12. RNA: cDNA Library preparation and sample pooling

cDNA Library preparation and sample pooling was carried out by Miss Audrey Farbos from the University of Exeter sequencing service. Illumina® TruSeq® RNA Sample Preparation Kit v2 was used to convert the mRNA into a library of template molecules suitable for subsequent cluster generation and DNA sequencing. This process involved 6 steps: (i) purifying poly-A containing mRNA molecules using oligo-dT attached magnetic beads, (ii)

fragmentation of mRNA using elevated temperature, (iii) transcription of first strand of cDNA using reverse transcriptase and random primers, (iv) synthesis of second strand cDNA using DNA Polymerase I and RNase H, (v) the addition of a single 'A' base, and then ligation of adapters to cDNA (end repair process), and (vi) PCR with 15 cycles to purify and enrich the final cDNA library. Individual sample libraries were 'barcoded' using specific adapter sequences allowing samples to be pooled, or multiplexed, before sequencing (Table 4). This multiplexing process allowed all samples to be run on the same lane on the same flow cell, while later allowing RNAseq data to be separated based on sample.

Table 4: Libraries for extracted RNA samples listed with barcode adapters.

Sample	Library	Protocol	PCR	Barcode	Conc ng/µl
01_YPD	1_YPD-l2	TruSeq HT	15	TCTCGCGC-TATAGCCT	47.6
02_YPD	2_YPD-l2	TruSeq HT	15	TCTCGCGC-ATAGAGGC	38.1
03_YPD	3_YPD-l2	TruSeq HT	15	TCTCGCGC-CCTATCCT	39.5
04_YPD	4_YPD-l2	TruSeq HT	15	TCTCGCGC-GGCTCTGA	46.8
05_1Hour	5_1Hour-l2	TruSeq HT	15	TCTCGCGC-AGGCGAAG	40.1
06_1Hour	6_1Hour-l2	TruSeq HT	15	TCTCGCGC-TAATCTTA	62.9
07_1Hour	7_1Hour-l2	TruSeq HT	15	TCTCGCGC-CAGGACGT	23.9
08_1Hour	8_1Hour-l2	TruSeq HT	15	TCTCGCGC-GTACTGAC	60.2
09_4Hours	9_4Hours-l2	TruSeq HT	15	AGCGATAG-TATAGCCT	7.51
10_4Hours	10_4Hours-l2	TruSeq HT	15	AGCGATAG-ATAGAGGC	14.1
11_4Hours	11_4Hours-l2	TruSeq HT	15	AGCGATAG-CCTATCCT	5.06
12_4Hours	12_4Hours-l2	TruSeq HT	15	AGCGATAG-GGCTCTGA	27.3
13_24Hours	13_24Hours-l2	TruSeq HT	15	AGCGATAG-AGGCGAAG	2.7
15_24Hours	15_24Hours-l2	TruSeq HT	15	AGCGATAG-TAATCTTA	5.27
16_24Hours	16_24Hours-l2	TruSeq HT	15	AGCGATAG-CAGGACGT	4.09
18_7Days	18_7Days-l1	TruSeq HT	15	ATTCAGAA-GTACTGAC	12.3
20_7Days	20_7Days-l1	TruSeq HT	15	GAGATTCC-TATAGCCT	10.5
21_7Days	21_7Days-l1	TruSeq HT	15	GAGATTCC-ATAGAGGC	20.3
22_7Days	22_7Days-l1	TruSeq HT	15	GAGATTCC-CCTATCCT	29.3
Negative	Negative-l2	TruSeq HT	15	AGCGATAG-GTACTGAC	0.25

3.3.13. RNA: data analysis by MiSeq Nano

A MiSeq Nano Run was first run to assess the quality of the sample pool before HiSeq sequencing. The Illumina MiSeq sequencer was used to generate 150-bp paired-end reads. 20 libraries were run on a single lane totaling 1,078,682 reads. An average yield of 56,769 reads per sample (range: 45,748 – 68,887 reads per sample, Table 5). Data for demultiplexed samples can be viewed by following this link: https://1drv.ms/f/s!Ak4wo8SKoFJaupdlbSPRqRXyC85mew. This included 'Adapter content', 'Contamination', 'Kmer Profiles', 'Krona plots', 'Per Base N Content', 'Per Base Quality', 'Per Base Sequence Content', 'Per Sequence Content', 'Per Base GC Content', 'Per Sequence Quality', 'Per Tile Quality' and 'Sequence length distribution'.

Table 5: Demultiplexed MiSeq Nano results for RNA samples.

Sample	Count	Perfect	OneMismatch	Yield (Mb)	% > Q30	Mean Q
01_YPD	61255	60286	969	18	92	36
02_YPD	64791	63582	1209	19	90.8	35.7
03_YPD	57510	56580	930	17	90.7	35.7
04_YPD	57765	56606	1159	17	91.9	36
05_1 Hour	59345	56523	2822	17	92.9	36.2
06_1 Hour	67794	66693	1101	20	91.8	36
07_1 Hour	57481	56263	1218	17	90.5	35.7
08_1 Hour	58984	57887	1097	17	92.7	36.2
09_4 Hours	45748	45003	745	13	94.1	36.5
10_4 Hours	47950	47140	810	14	92.5	36.1
11_4 Hours	52752	51901	851	15	93.9	36.5
12_4 Hours	51270	50293	977	15	92.3	36.1
13_24 Hours	46033	43945	2088	13	93.4	36.4
15_24 Hours	47528	46709	819	14	93.1	36.3
16_24 Hours	51483	50377	1106	15	94	36.5
18_7 Days	55979	54629	1350	16	88.5	35.2
20_7 Days	68887	67547	1340	20	89.5	35.5
21_7 Days	62888	61639	1249	18	88.9	35.4
22_7 Days	63178	61875	1303	18	88.9	35.4
Negative	61	43	18	0	52.8	27

3.4. Discussion

The majority of laboratory work involving *Z. tritici* uses conidiospores as the source of inoculum (Kettles *et al.*, 2016, Fones *et al.*, 2017b, Rudd *et al.*, 2015, Yang *et al.*, 2015, Lee *et al.*, 2013). Stored as glycerol freezer stocks, conidiospores are subsequently suspended in water before quantification and application to either leaves or agar for assessment. Conidiospores are reported to have a germination rate above 75 % (Cohen and Eyal, 1993, Kema *et al.*, 1996b, Fones *et al.*, 2017b). These studies, however, have likely scored the presence of hyphal growth, not necessarily the continuation of growth past this point. Quantitative details of conidiospore germination and colony formation *in vitro* have not been published.

Initially, data in this chapter was expected to confirm the high conidiospore germination rate previously recorded on leaves and in culture. However, results from assessments of three strains of *Z. tritici,* showed that less than 30 % of conidiospores plated formed colonies on agar (Results 3.3.1A). This unexpectedly low colony formation led to a series of experiments exploring the viability and culturability (measured as the number of colonies formed on agar/number of spores plated) of *Z. tritici* conidiospores. Further results showed that the low rate of culturability was not affected by the nutritional content of the media on which the spores were plated (Results 3.3.1B). The agar media tested included wheat leaf agar, therefore the likelihood of culturability being low due to needing a host specific trigger seems unlikely. Additionally, culturability was not affected by the concentration of the suspension from which the spores were plated (Results 3.3.2). In some fungi, such as the wheat rust *Puccinia graminis* and the bean rust *Uromyces phaseoli*, chemical inhibitors of germination are present on spores (Macko and Staples, 1973, Hazen and Cutler, 1983). Washing is known to remove these inhibitors. In the experiment carried out here, conidiospores suspended at 10^4 spores/ml had been diluted (and therefore washed) 4 times, so it might be expected that auto-inhibitors would have been removed. The fact no higher colony formation rates were recorded likely rules out chemical inhibition.

An important observation from these experiments, however, was that between the initial plating at around 2-hours after suspending conidiospores in water, and a secondary plating

24-hours later, culturability decreased. This suggested that spores were losing culturability over time. An examination of earlier time-points confirmed both an initially higher level of culturability (Results 3.3.3) and a rapid and steady reduction in the number of colonies was recorded, with culturability dropping to less than 25 % within 4 hours.

3.4.1. The stress of suspending conidiospores in water

Suspending in water significantly reduces the ability of a conidiospore to form a colony – even when plated on nutrient rich agar. The removal of conidiospores from a nutrient rich, non-submerged environment (an YPD agar plate, or the pycnidium), to a submerged low nutrient one (water). This change would impose large alterations in osmotic potential and nutrient availability for conidiospores. To assess if these changes affect conidiospore culturability, suspensions were supplemented with either (i) an osmolyte, (ii) nutrients (in the form of YPD), or (iii) a combination of both. Results (Results 3.3.4) showed that in all cases, the supplementation of water led to a maintenance of culturability.

(i) Adding an osmolyte (sorbitol) to *Z. tritici* spore suspensions partially maintained culturability over a 4-hour period, suggesting an osmotic shock response (Results 3.3.4, Students *t*-test, t = -2.88, df = 7.62, p-value = 0.021). Upon suspending conidiospores in water, cells experience a hypo-osmotic shock. The water potential between the cell and the water in which the cells are suspended, may lead to an initial influx of water into the cell (Hohmann, 2002). This can lead to swelling and potentially, although protected by the cell wall, bursting and death. Adding an osmolyte, such as sorbitol, to water reduces the intensity of this interaction by interacting with water molecules and reducing the water potential of the solution compared to the cell cytoplasm, thus reducing the hypo-osmotic shock. This result indicates that the hypo-osmotic shock experienced by conidiospores entering water is a small but significant factor in the maintenance of culturability. To asses this result more accurately, it would be important to work out the specific osmotic potentials for the solutions used here.

(ii) Sorbitol is also a source of carbon. It is obtained by the reduction of glucose to glucitol. The addition of sorbitol to water cultures therefore increases the nutrient availability of the suspensions. Potentially then, the maintenance of culturability may be linked to nutrient

availability. To test this further, conidiospores were suspended in a rich nutrient solution (YPD) before plating. Here, conidiospore culturability was maintained for much longer periods, reducing by only 20 % after 4 hours of suspension in culture – a highly significant difference when compared to conidiospores from pure water cultures (Results 3.3.4, Students *t*-test, $t = -9.01$, $df = 7.39$, p-value < 0.001). This result indicates a rapid recognition of low nutrient conditions.

(iii) Finally, suspending in water cultures supplemented with both YPD and sorbitol resulted in complete maintenance of conidiospore culturability (Results 3.3.4). This result suggests that nutrient and osmotic stress are both (independently or interactively) significant factors that affect the maintenance of culturability during suspension in water.

Water-based survival and spread is common in plant pathogenic fungi and oomycetes. Examples of *Verticillium* spp., *Rhizoctonia* spp., *Fusarium* spp. *Pythium* spp., *Phytophthora* spp., and *Botrytis* spp., can all tolerate extended periods of suspension in water while remaining pathogenic (Kang *et al.*, 2015, Palacios *et al.*, 2014, Pullman and DeVay, 1981). In *Z. tritici*, water is essential for the spread of asexual spores (rain-splash). Any drop in culturability related to submergence would therefore be a major hindrance for successful leaf-infection and subsequent reproduction.

It is possible, therefore, that the recognition of this change in environmental conditions, and subsequent entry into a non-culturable state may be an evolved response. One explanation could be that some conidiospores, upon spread by water, enter a dormancy phase. This might allow for a sub-population of spores to infect plants later in the growing season, particularly when conditions favourable for germination occur. Dormancy is common in fungi and ensures populations can survive for periods when conditions are not favourable. For successful spore germination, fungi need specific conditions. Moisture is essential for the process, due to the fact it is needed for hyphal extension. However, germination cues can be quite specific. *Aspergillus niger* is known to need carbon and nitrogen sources to form germ tubes (Hayer *et al.*, 2014). *Neurospora crassa* requires an extended period of heat (Plesofsky-Vig and Brambl, 1985). It has been suggested that *Z. tritici* can survive as dormant mycelium to overwinter on wheat stubble (Bayer, 2017). This dormancy may also occur in asexual spores. Further study, such as an assessment of transcriptomic markers of dormancy

(Hagiwara *et al.*, 2016), would allow for a better assessment of whether this was occurring here. However, the fact that *Z. tritici* conidiospores do not germinate and form colonies on YPD agar, wheat leaf agar, or any other agars tested, suggest that spores are not dormant, but instead are in a viable-but-not-culturable state (VBNC).

3.4.2. Conidiospores are viable but not culturable: The VBNC state

To assess the viability of conidiospore populations over time, cells were stained with PI, a membrane impermeant dye that is excluded from viable cells. Images were taken using confocal microscopy and images were assessed for viability by counting the number of spores which contained at least one viable (unstained) cell. Results (3.3.5) show that the number of viable conidiospores within the population does not drop for at least 4 days of suspension in water. Culturability has been shown to drop significantly within minutes. It can therefore be suggested that upon suspension in water, *Z. tritici* conidiospores enter a VBNC state.

VBNC cells are defined as "living cells that have lost the ability to grow on routine media, on which they normally grow" (Oliver, 2000). This state is thought to occur when organisms enter a state of low metabolic activity in the face of unfavourable conditions (Heim *et al.*, 2002, Weaver *et al.*, 2010, Grey and Steck, 2001). VBNC cells can be compared to dormant cells but the two differ in their metabolic activity. VBNC cells have measurable metabolic activity, yet dormant cells do not. VBNC cells are reported to have increased tolerance to stresses such as high temperatures and chemical changes, including those which would be present during suspension in water such as a starvation, oxidative stress, and osmotic stress (Wong and Wang, 2004, Nowakowska and Oliver, 2013, Du *et al.*, 2007).

The VBNC state has been studied mostly in bacteria where more than 60 species have been documented (Oliver, 2005). In fungi, VBNC cells have been found in drinks industry research, where yeasts such as *Saccharomyces cerevisiae* and *Candida stellate* are thought to enter this state after conditions such as electrolytic low amperage shock, pasteurisation and alcoholic fermentation (Salma *et al.*, 2013, Guillou *et al.*, 2003, Bleve *et al.*, 2003, Mills *et al.*, 2002). Studies on 'hospital waters' have also highlighted VBNC spores from water-borne fungi such as *Candida albicans, Aspergillus fumigatus, Fusarium solani* and *Saccharomyces cerevisiae* (De Vos and Nelis, 2006, Salma *et al.*, 2013). VBNC has not been shown in plant pathogenic

fungi such as *Z. tritici*, the prevalence of this state in other microorganisms allows it to be hypothesised here.

3.4.3. Resuscitation and the maintenance of virulence in VBNC cells

VBNC cells are known to resuscitate when the source of stress is removed (Lleo *et al.*, 2001, Su *et al.*, 2015, Li *et al.*, 2014, De Vos and Nelis, 2006, Salma *et al.*, 2013). For example, *Ralstonia solanacearum*, a soil-borne plant pathogenic bacterium, has around 99% of cells in a VBNC state in late stages of infection in the tomato vascular system. If these non-culturable bacteria are then distributed into sterile soil in the presence of tomato seeds, resuscitation (and infection) commences (Grey and Steck, 2001). In *Z. tritici*, however, when taking potential VBNC cells from water cultures and plating onto rich agar media (YPD), resuscitation does not occur. If spore containing plates are incubated over longer time periods (assessed up to 21 days), resuscitation still does not occur. Potentially then, the conidiospores of *Z. tritici* need a specific trigger to resuscitate. However, plating onto wheat leaf agar (and 8 other nutrient agars) resulted in no significant differences in culturability when compared to YPD (Figure 6B: One-way ANOVA, p-value = 0.05. Data were normally distributed).

Post-resuscitation, VBNC cells are known to retain their ability to infect and cause disease (Oliver and Bockian, 1995, Baffone *et al.*, 2003, Du *et al.*, 2007, Rahman *et al.*, 1994). Although resuscitation conditions had not been replicated on agar, it can be hypothesised that a host plant should provide the ideal conditions for resuscitation. To assess this in *Z. tritici*, conidiospores were applied to wheat leaves at different periods after suspension in water. Results (0) showed that, with all three strains tested, infection and disease were significantly reduced when spores from water-suspended cultures were used for leaf inoculations. After just 60 minutes of suspension in water, all three strains displayed reduced virulence. This finding confirms that (i) *Z. tritici* conidiospores do not resuscitate, even on a host plant, and (ii) due to this, the virulence of *Z. tritici* conidiospore populations is not maintained.

VBNC cells in *Escherichia coli* have been shown to have a lower metabolic rate (Zhao *et al.*, 2016). To assess this in *Z. tritici*, RNA extractions were carried out using Qiagen RNeasy Plant Kit. It would be expected that a lower yield of mRNA would be extracted from the

same number of cells if the metabolic rate was lower. Indeed, yields show a rapid reduction in available RNA upon conidiospore suspension in water, continuing to drop over time (Results 0). This indicates that transcription reduces with time suspended in water, and may suggest a VBNC state is occurring here. Further transcriptomic analysis may also show any differential gene expression linked to VBNC/dormancy when compared to culturable cells (Su *et al.*, 2015, Asakura *et al.*, 2007, González-Escalona *et al.*, 2006). Contrastingly, if cells are not in a VBNC state, biochemical markers of cell-death such as those linked to necrosis, programmed cell-death, or apoptosis might be found to be upregulated (Shlezinger *et al.*, 2012).

In conclusion, *Z. tritici* conidiospores can be shown to have certain hallmarks of VBNC cells. They have intact membranes and viable cells for at least 4 days after suspension in water (Results 3.3.5). Cells also continue gene transcription, although at a lower rate (Results 0). However, the lack of resuscitation on agar, or on leaves, coupled with the loss of virulence, indicates that water-suspended *Z. tritici* conidiospore populations are not entering a VBNC state. Further time-points would be needed to conclude that resuscitation does not occur. The recovery of culturability may simply be slow, or precise conditions may not have been replicated here.

3.4.4. Implications for experimental methodology

The research detailed in this chapter was designed to mimic *in vivo* conditions. The use of water agar may not, however, be relevant in a field-based scenario. Nevertheless, the use of water-suspended conidiospore suspensions for *Z. tritici* on pathogenicity and virulence is widespread, therefore these results have relevance for the scientific community.

Most important are the implications for studies which assess differences in virulence between *Z. tritici* strains. In this chapter, experiments have shown that within just 60 minutes of suspension in water, populations of conidiospores from three strains of *Z. tritici* all displayed significantly attenuated virulence (Results 0). Therefore, if the conidiospores of all three strains are water-suspended at the beginning of an experiment, the order at which plant leaves are infected has the potential to result in very different experimental conclusions. For example, if the conidiospores of IPO94269 were applied to leaves within 10 minutes, and *Z.*

tritici IPO323 conidiospores after 60, it would be concluded that there is no significant difference between the virulence of these strains. However, if plants were inoculated in reverse, strains would be concluded to have significantly different levels of virulence (Results 0).

It should be noted, that Results 0 highlight that levels of disease do not always correlate well with culturability over time. For example, leaves inoculated with IPO94269 conidiospores after 120 minutes water-suspension, produce more disease on leaves when compared to those suspended for just 60 minutes. The reason for this increase is unknown, but the general low level of disease in these experiments includes a large amount of variation. If this experiment was to be repeated, a greater conidiospore number could be used for inoculations, likely leading to less varied low-level infections.

In conclusion, the results presented here indicate that the time between preparing conidiospore suspensions and inoculating leaves is a major factor in obtaining reproducible results in pathogenicity and virulence assays. To minimise variability, conidiospore suspensions should be made individually, with leaf inoculations carried out after exactly the same time post-suspension for each strain tested. Additionally, obtaining results which reflect the true virulence of each strain, conidiospores should be applied to leaves rapidly. Realistically, after quantification of spore populations, this should be within 10 minutes of suspension preparation.

The use of high nutrient media (such as YPD) for suspensions would potentially alleviate the rapid loss in culturability which occurs upon suspension in water. The subsequent application of high nutrient spore suspensions to leaves would, however, likely affect experiments in unforeseen ways. Further work is needed to find a medium which maintains conidiospore culturability without compromising the integrity of the experiments.

4. The capacity for epiphytic growth and sporulation

4.1. Introduction

Rain-splash facilitates the spread of asexual spores (pycnidiospores) from infected leaves to new host tissue (Brennan *et al.*, 1985, Bannon and Cooke, 1998, Van Ginkel, 1999, Shaw, 1991, Suffert *et al.*, 2011, Ponomarenko *et al.*, 2011). Subsequent germination events are reported to occur mostly within the first day of leaf contact, but can continue up to 5 days post-arrival (Shetty *et al.*, 2003, Fones *et al.*, 2017b). Following germination, foliar growth is reported to be hyphal (Kema *et al.*, 1996b, Fones *et al.*, 2017b, Kettles *et al.*, 2016).

On low nutrient *in vitro* environments such as hydrophobic glass slides and water agar, growth is also reported to be predominantly hyphal, although microcycle conidiation (MC) has been visualised (Mirzadi Gohari *et al.*, 2014, Derbyshire *et al.*, 2015, Guo and Verreet, 2008, Kema *et al.*, 1996b, Fones *et al.*, 2017b). On high nutrient *in vitro* environments such as YPD (yeast peptone dextrose) and PDA (peptone dextrose agar), however, MC is the dominant growth form (Mehrabi *et al.*, 2006, Guo and Verreet, 2008, Kema and Annone, 1991).

The leaf surface is not a nutritionally barren environment. Therefore, it can be hypothesised that *Zymoseptoria tritici* undergoes both hyphal growth and MC on the surface. Due to these dimorphic capabilities, it could even be suggested that epiphytic sporulation may be essential to maintaining an infectious population throughout a growing season.

This chapter uses water agar to mimic the low-nutrient semi-solid leaf surface, subsequently focussing on the quantification of factors such as colony growth, hyphal elongation over time, and continued MC. Subsequently, nutrient supplemented agars are used to make predictions about natural conditions, including the likelihood of increased disease after periods of agricultural fertilisation. Finally, the period at which *Z. tritici* remains primarily epiphytic is also evaluated.

4.2. Methods

4.2.1. Water agar and nutrient supplemented agar plate cultures

Water agar and all nutrient agar media were made with sterile distilled water and supplemented with 15 g/l of bacteriological agar (Sigma-Aldrich-A5306). Concentrations of all nutrients are outlined in experimental results. Conidiospores were cultured for 7 days on YPD agar before use. For all experiments, agar plates were inoculated with conidiospores and cultured under a long-day light cycle at 20 °C. Water agar was used as a control to compare against nutrient agars. Colony morphology and sporulation were assessed after 7, or 10 days depending on the experiment.

For spore visualisation, propidium iodide (PI) was used to stain live/dead cells. 1 ml aliquots of 1 x 10^7 conidiospores/ml suspensions were supplemented with PI at a final concentration of 1 µl/ml. Stained suspensions were used within 60 minutes. 2-dimensional confocal images (Methods 2.7) were taken using excitation/emission maxima of wavelength 493/636 nm.

4.2.2. Fertiliser supplemented disease trials

Plants were grown at standard growth cabinet conditions (Methods 2.2) for at least 14 days, and a maximum of 21 days, before inoculations. Conidiospores were cultured for 7 days on YPD agar before suspension in autoclaved MilliQ water. Concentrations were estimated by haemocytometer, adjusted to 1 x 10^5 conidiospores/ml and supplemented with 0.01 % (v/v) Silwet L-77 (Momentive Specialty Chemicals, UK). Suspensions were applied to leaves at as per Methods 2.4.

24 hours post-inoculation, nitrate or ammonium sprays, also supplemented with 0.01 % (v/v) Silwet L-77 (Momentive Specialty Chemicals, UK), were applied to the abaxial side of inoculated leaves using an Iwata Revolution SAR airbrush. Solutions were applied at a rate of 1 ml per cell of a 24-cell plant tray (1 plant per cell, avoiding run off). Concentrations of $(NH_4)_2NO_3$ and $NaNO_3$ are outlined in the experimental methods. Disease was assessed after 28 days by counting pycnidia/cm^2 of inoculated leaf tissue.

4.2.3. Carbendazim based confirmation of epiphytic growth

Plants were grown at standard growth cabinet conditions (Methods 2.2) for at least 14, and a maximum of 21 before inoculations. Conidiospore suspensions, supplemented with 0.01 % (v/v) Silwet L-77 (Momentive Specialty Chemicals, UK), were applied to leaves at 1 x 10^5 conidiospores /ml as per Methods 2.4.

At 7, 14 or 21 dpi, a 100 µM carbendazim solution, a benzimidazole fungicide, targets the fungal cytoskeleton by inhibiting of ß-tubulin polymerisation (FRAC, 2017), was supplemented with 0.01 % (v/v) Silwet L-77 (Momentive Specialty Chemicals, UK) and applied to both the abaxial and adaxial sides of inoculated leaves using an Iwata Revolution SAR airbrush.

Solutions were applied at a rate of 1 ml per cell of a 24-cell plant tray (2 plants per cell, avoiding run-off). Disease was assessed after 28 days by counting pycnidia/cm^2 of inoculated leaf tissue.

4.3. Results

4.3.1. Growth on water agar is hyphal and yeast-like

Hyphal growth has been documented consistently on low nutrient surfaces such as a leaf and water agar (Kema *et al.*, 1996b, Fones *et al.*, 2017b, Kettles *et al.*, 2016, Derbyshire *et al.*, 2015, Guo and Verreet, 2008). However, yeast-like growth (microcycle conidiation (MC)) has also been visualised on water agar (Mirzadi Gohari *et al.*, 2014). To assess these growth forms over time, conidiospores were plated onto water agar. The rate and distance of hyphal growth, and the number of spores produced by MC, were assessed every 7 days for a 28-day period.

Firstly, results (Figure 15A) confirm previous reports that hyphal growth occurs under low nutrient conditions. For the first 7 days of colony development, however, hyphal extension is slow, with mean colony diameter measured at 1.28 mm (Figure 15B). Between 7 and 14

days colony diameter more than doubles to a mean of 3.17 mm (Figure 15B-C). The most rapid period of hyphal growth is between day 14 and day 21. Here, the mean colony diameter increases to a mean of 6.68 mm. This period of radial growth is coupled with extensive branching (Figure 15C-D). No subsequent radial hyphal growth is recorded between day 21 and day 28.

Somewhat unexpectedly, fungal individuals undergo extensive MC during the first 7 days of colony development. Each conidiospore produces more than 5,000 additional conidiospores (Calculated by scraping plates and counting spores divided by colonies, Figure 15F). Conidiospores produced during this 7-day period are almost exclusively single celled (Figure 15H). After 7 days, the rate of conidiation decreases, with little significant sporulation.

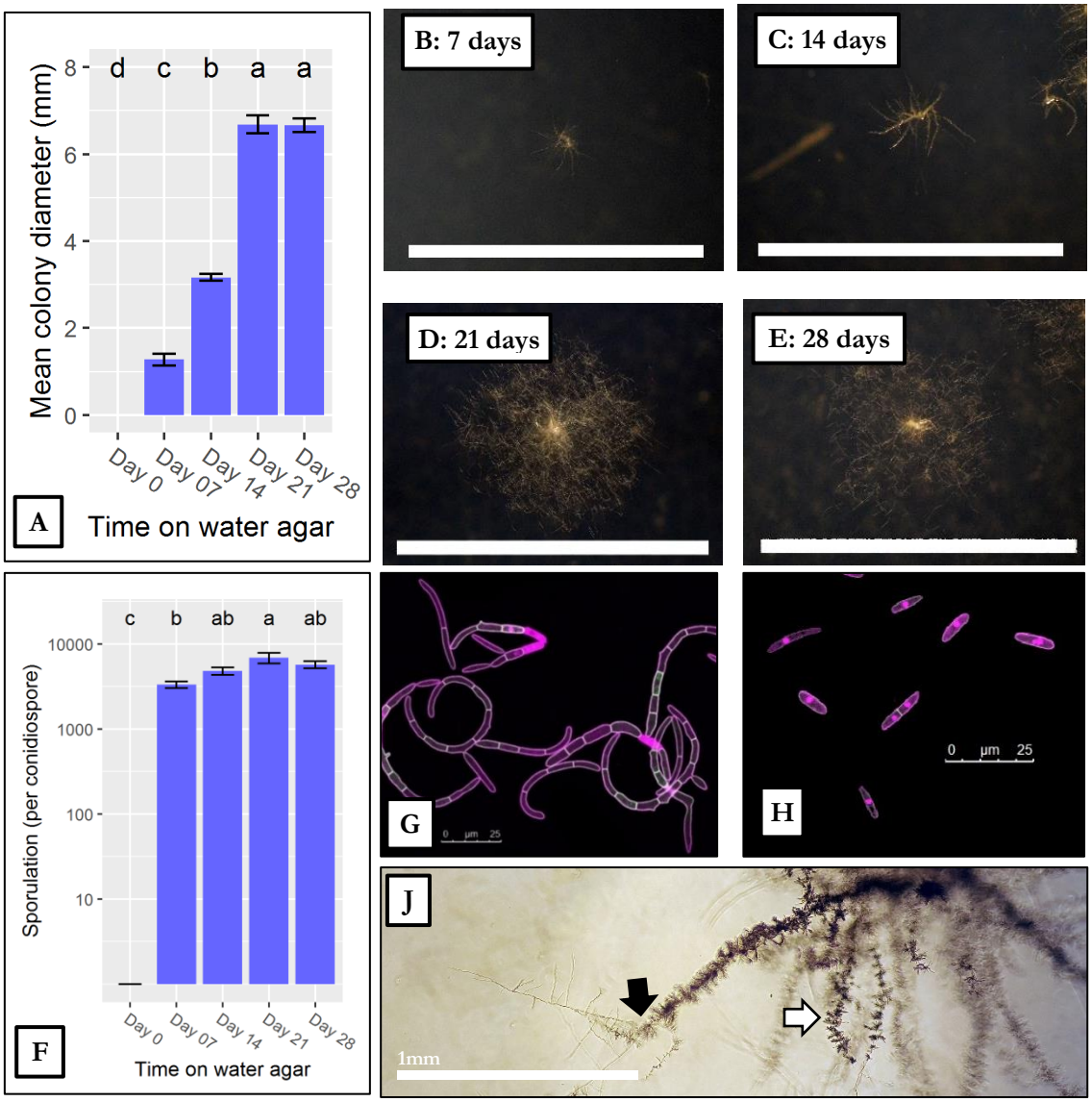

Figure 15: An assessment of conidiospore growth on water agar over a 28-day period.
A: Mean colony diameter on water agar over time. Hyphal growth is slow for the first 7 days but extensive and rapid later, particularly between day 14 and day 21. No increase in colony diameter was recorded after 21 days (Representative images in **B, C, D** and **E**). Data used are mean colony diameters from 5 independent experiments (plates). From each plate, at least 5 colonies were measured. Error bars show SE. **F**: Sporulation over time (conidiospores isolated from plate at specified time/conidiospores plated on day 0). Results shows that each conidiospore plated can produce > 5,000 spores on water agar in just 7 days. Between day 7 and day 28 further sporulation is minimal. Data are means of spore counts, estimated by haemocytometer, from 8 replicate plates from 4 independent experiments. Error bars show SE. **G:** Representative image of day 0 conidiospore population. **H:** Representative image of day 14 conidiospore population. At day 14 conidiospores are almost exclusively single-celled. Conidiospores are stained with propidium iodide for visualisation (1 µl/ml final concentration). Results suggest that microcycle conidiation dominates over the first 7 days, with hyphal growth dominating from day 7 to day 21. No significant hyphal growth or microcycle conidiation was recorded after 21 days. **J:** White arrows show evidence of microcycle conidiation from a colony growing on water agar. Image also shows change in morphology after around 7 days where conidiation ceases and subsequently, hyphal growth dominates (black arrow).

4.3.2. Sporulation increases with glucose, fructose and sucrose availability

As already shown using water agar (Results 4.3.1), MC continues to occur in environments with a low level of exogenous nutrients. However, the leaf surface potentially provides varied nutrients. Waxes are hypothesised to provide nutrition for *Z. tritici* (Yang *et al.*, 2013a, Rudd *et al.*, 2015). Additionally, sugars such as glucose, sucrose and fructose are known to be naturally exuded on to the surface of leaves (Ruinen, 1970, Tukey Jr, 1970). Epiphytic sugars have been shown to be used as a nutritional source by various plant pathogens, aiding in both growth and colonisation (Mercier and Lindow, 2000, Leveau and Lindow, 2001). To determine whether *Z. tritici* can take advantage of carbon based leaf exudates, water agar was supplemented with three sugars: glucose, sucrose and fructose. Conidiospores were plated on these agars and incubated for 7 days. Conidiospore production and hyphal growth were assessed after 7 days. Results were compared to water agar.

Results (Figure 16A) show that sporulation significantly increased with the availability of all three sugars tested. These results suggest that *Z. tritici* would be able to take advantage of any sugars available on the leaf surface. In doing so, the population of infectious propagules will likely increase via MC. Hyphal growth was also significantly promoted by glucose and sucrose (but not fructose) over the same 7-day period when compared to water agar (Figure 16B1-4).

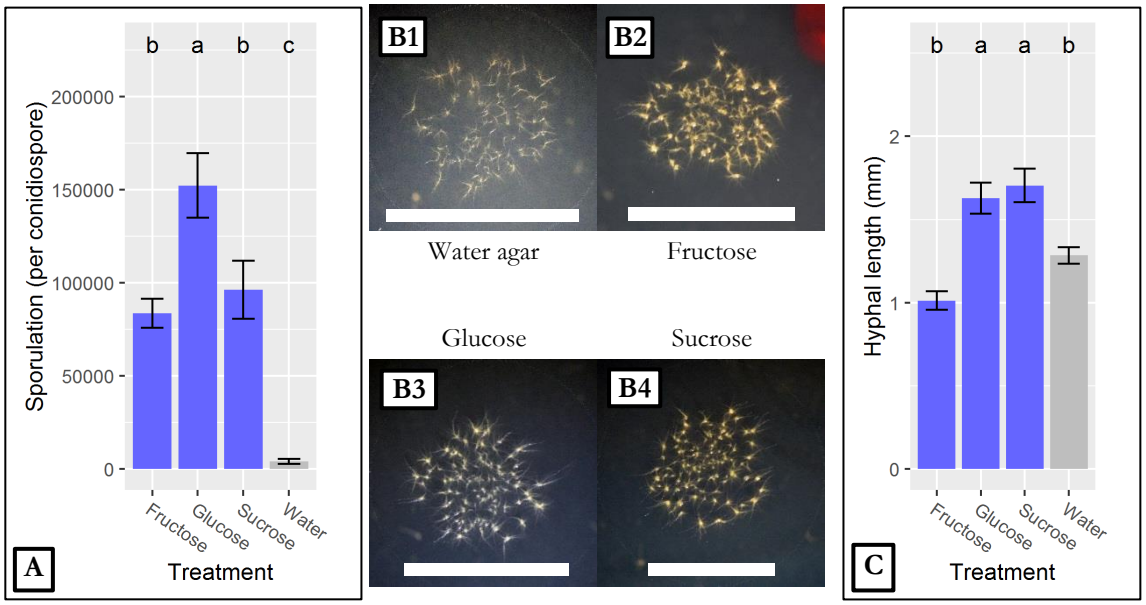

Figure 16: An assessment of conidiospore growth on glucose, fructose and sucrose agar. **A:** Compared to water agar, microcycle conidiation is significantly increased on agar supplemented with either glucose, sucrose or fructose. Within 7 days, conidiospores on water agar produced ~ 4,000 conidiospores per spore plated. Each conidiospore produced significantly more spores on sugar supplemented agar. Fructose produced a mean 83,666 spores per spore plated (One-way ANOVA, p-value = 9e-08, n = 3. Data were normally distributed. Letters indicate significant differences between treatment groups as determined by post-hoc Tukey test; $p < 0.05$). Data are mean spore counts, estimated by haemocytometer, from 6 replicate plates from 3 independent experiments. Error bars show SE. **B1, B2, B3, B4:** Representative images of hyphal growth on agar media. Images are taken after 10 days incubation under a long-day light cycle at 20 °C. Scale bars show 10mm. **C:** Hyphal length is significantly increased when cultured on agar supplemented with glucose or sucrose. (One-way ANOVA, p-value = 1.1e-06, n = 1. To achieve normality, data were transformed using a square root transformation (Shapiro-Wilk, p-value = 0.12). Letters indicate significant differences between treatment groups as determined by post-hoc Tukey test; $p < 0.05$. Data are mean hyphal measurements from > 20 hyphae from > 10 colonies. Error bars show SE.

4.3.3. Sporulation increases with the availability of agricultural fertilisers

Z. tritici grows hyphally but continues to sporulate when cultured on water agar (Results 4.3.1). The addition of a carbon source, in the form of either fructose, glucose or sucrose, increases both sporulation rates and hyphal growth over a 7-day period. In the field, high concentrations of nutrients such as those found in lab-based media are rare. However, in some agricultural situations, such as during crop fertilisation, plant macronutrients such as nitrogen and phosphorous can be found in unusually high concentrations. To assess if nitrogen (in the form of nitrates or ammonium ions) or phosphorous (in the form of

phosphate ions), could trigger increased sporulation or hyphal extension, an experiment was conducted in which conidiospores were plated onto various nutrient supplemented agars. Plates were incubated for 7 days in the light at 20 °C before assessing photographs and total conidiospore counts.

Results (Figure 17A) show that, as with sugar supplemented agar, *Z. tritici* conidiation increases with the availability of nitrate, ammonium and phosphate ions when compared with water agar. Hyphal growth over a 7-day period, however, does not seem significantly affected.

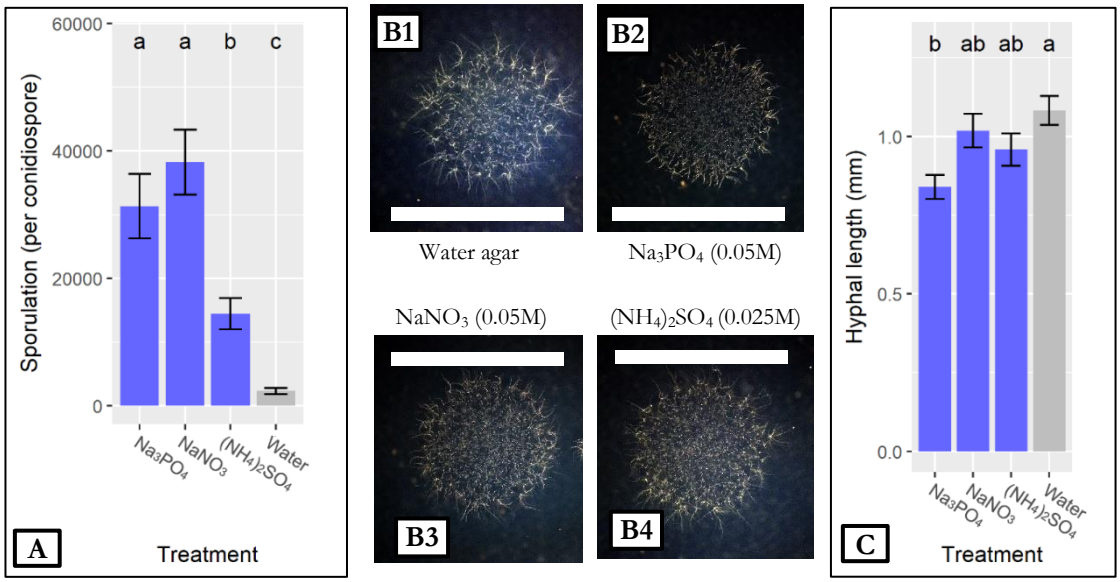

Figure 17: An assessment of sporulation (after 7 days) and hyphal growth (at 7 days) on nutrient fertiliser supplemented agar (0.05 M phosphate, 0.05 M nitrate and 0.025 M ammonium).
A: Microcycle conidiation significantly increases from a mean of 2333 conidiospores produced per spore plated on water agar, to 31,333 on 0.05M phosphate (One-way ANOVA, p-value = 7.3e-08, n = 3. Data were normally distributed. Letters indicate significant differences between treatment groups as determined by post-hoc Tukey test; $p < 0.05$). Data are mean spore counts, estimated by haemocytometer, from 6 replicate plates from 3 independent experiments. Error bars show SE. **B1, B2, B3, B4:** Representative images of hyphal growth on agar media. Images are taken after 10 days incubation under a long-day light cycle at 20 °C. Scale bars show 10mm. **C:** Hyphal length is significantly reduced on agar supplemented with phosphite ions but not significantly affected by supplementation with 0.05 M nitrate or 0.025 M ammonium ions (One-way ANOVA, p-value = 0.0049, n = 1. To achieve normality, data were transformed using a square root transformation Shapiro-Wilk, p-value = 0.34. Letters indicate significant differences between treatment groups as determined by post-hoc Tukey test; $p < 0.05$).

4.3.4. Foliar fertiliser applications do not significantly increase disease

It has been shown previously (Results 4.3.1), that *Z. tritici* sporulation occurs readily in low nutrient conditions (water agar). It has also been shown that the availability of nitrogen sources, such as nitrate ions and ammonium ions, significantly increase this sporulation capacity (Results 4.3.3). This sporulation, if also occurring on leaves, might suggest a link between fertiliser use and *Z. tritici* disease outbreaks. Indeed, this has previously been reported (Simon *et al.*, 2003, Leitch and Jenkins, 1995, Lovell *et al.*, 1997).

This experiment was conducted to assess whether the presence of nitrogen on leaves may also increase disease. Conidiospores were applied to leaves by paintbrush method. After 24 hours, nitrates (in the form of $NaNO_3$ 0.02 M) or ammonium ions (in the form of $(NH_4)_2NO_3$ 0.02 M) were applied as foliar sprays. Disease was assessed by pycnidia counting 28 dpi.

Results (Figure 18) show that foliar fertiliser treatments did not significantly affect the levels of disease after 28 days. Application of nitrate ions, in the form of $NaNO_3$ (0.02 M), 24 hours after *Z. tritici* inoculation, leads to elevated pycnidia counts when compared to leaves treated with surfactant (Silwet L-77) alone. However, a Students *t*-test shows this difference to not be statistically significant ($t = -0.50641$, $df = 2.9831$, p-value = 0.6476). The application of ammonium ions in the form of $(NH_4)_2NO_3$ also leads to elevated disease. However, this increase was also found to not be statistically significant when compared to Silwet only (Students *t*-test, $t = -0.80712$, $df = 2.6171$, p-value = 0.4864).

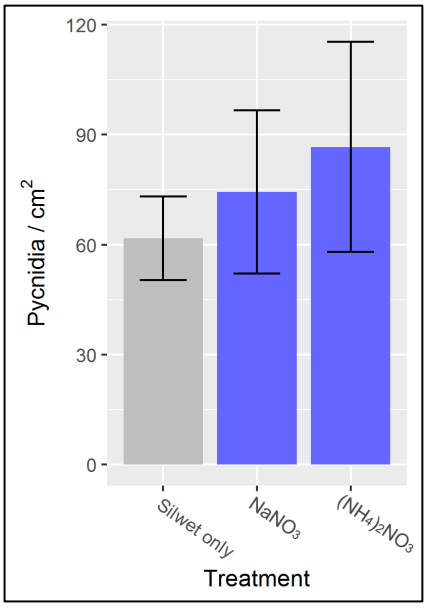

Figure 18: An assessment of the effect of foliar fertiliser applications on disease by *Z. tritici*
No significant differences in disease were recorded when leaves were treated with either 0.2 M $NaNO_3$ (Students *t*-test, t = -0.50641, df = 2.9831, p-value = 0.6476) or 0.2 M $(NH_4)_2NO_3$ (Students *t*-test, t = -0.80712, df = 2.6171, p-value = 0.4864) when compared to leaves treated only with the surfactant Silwet L-77. Disease was assessed after 28 dpi. Data are mean pycnidia/cm² counts from 3 independent experiments, each treatment containing at least 7 leaves. Error bars show SE.

4.3.5. *Z. tritici* growth remains epiphytic for more than 7 days

To complete this chapter on the behaviour of *Z. tritici* during epiphytic growth, an experiment was conducted to assess the point at which *Z. tritici* becomes endophytic. Carbendazim, a benzimidazole fungicide which targets the fungal cytoskeleton by inhibition of ß-tubulin polymerisation, was used to assess this (FRAC, 2017). Infected leaves were sprayed with carbendazim at different time-points post-inoculation. To ensure that conidiospores were not simply washed off leaves, 'water only' sprays were included in the experiment.

Results (Figure 19) show that carbendazim significantly reduces disease if applied to leaves within 7 days of fungal inoculations (Students *t*-tests: t = 7.17, df = 73.98, p-value = 4.707e-10). However, disease still occurs. Therefore, this result indicates that at least some *Z. tritici* individuals enter the plant apoplast within 7 days. However, due to the significantly reduced disease recorded at this time-point, it can be concluded that the majority of *Z. tritici* individuals likely remain epiphytic at 7 dpi (i.e. are accessible to the fungicide).

By 14 dpi, the levels of disease recorded have increased. This suggests that additional fungal individuals have entered the plant and are thus no longer affected by the fungicide application. However, disease levels are still significantly lower than non-fungicide-treated leaves (Students *t*-tests: t = 2.07, df = 32.06, p-value = 0.047). Therefore, it can be concluded that many *Z. tritici* individuals remain purely epiphytic, and are thus controlled by carbendazim fungicide sprays. Any MC during this period is also controlled. If carbendazim is sprayed at 21 dpi, no significant reduction in disease is recorded (Students *t*-test, t = -0.38, df = 26.82, p-value = 0.702).

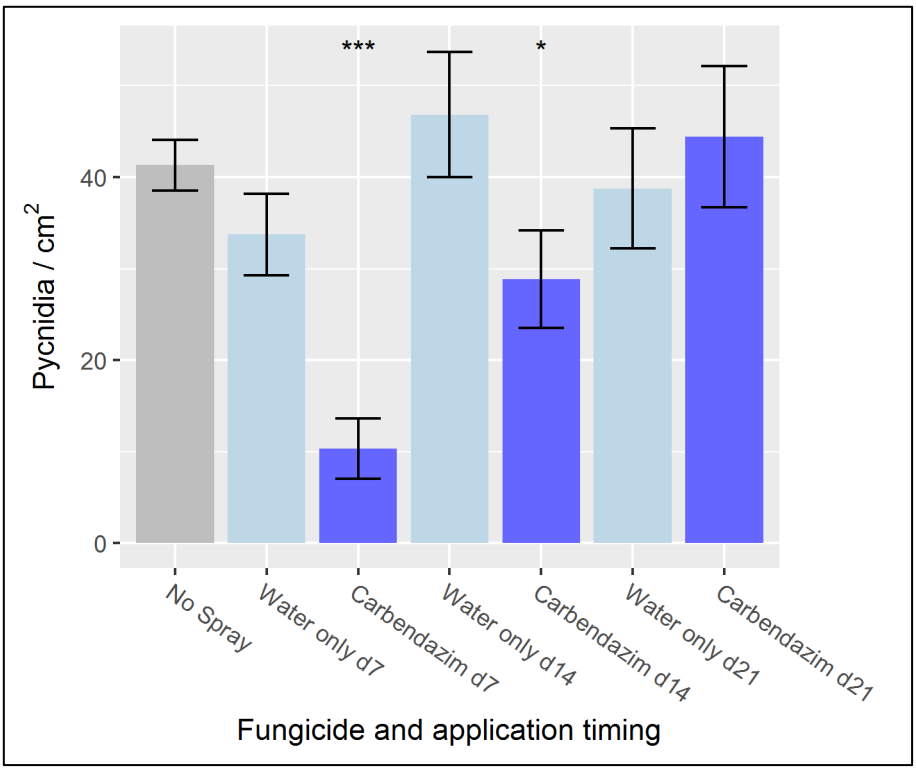

Figure 19: An assessment of *Z. tritici* disease after fungicide (carbendazim) sprays over 21d. Results show that foliar treatments of carbendazim significantly reduce disease if applied within 7 days (Students *t*-test, t = 7.17, df = 73.98, p-value = 4.707e-10) and 14 days of *Z. tritici* inoculation (Students *t*-test, t = 2.07, df = 32.06, p-value = 0.047). However, if treating leaves with carbendazim at 21 days, there is no significant reduction in disease (Students *t*-test, t = -0.38, df = 26.82, p-value = 0.70).

4.4. Discussion

Z. tritici is a dimorphic fungus. It can grow either in a hyphal form, or a yeast-like sporulating form. On a plant leaf, growth is reported to be hyphal. The yeast-like growth form has not been documented (Kema *et al.*, 1996b, Shetty *et al.*, 2003, Fones *et al.*, 2017b). *In vitro,* colony morphology is dependent on the nutrient availability of the medium on which *Z. tritici* is cultured. On low-nutrient media such as water agar and glass slides, growth is reported to be predominantly hyphal (Duncan and Howard, 2000, Cousin *et al.*, 2006). On high-nutrient media such as YPD agar, growth is reported to be predominantly yeast-like (Mehrabi *et al.*, 2006, Guo and Verreet, 2008, Kema and Annone, 1991). Under intermediate levels of nutrition, both morphologies are recorded.

In the case of *Z. tritici*, most nutrition is thought to be acquired within the leaf after the necrotrophic switch. Therefore, the ultimate target for hyphae is leaf entry/infection. For *Z. tritici* this occurs through natural openings such as stomata. The rapidity at which *Z. tritici* enters a host is debated. It was previously reported to be within 3 days (Kema *et al.*, 1996b, Keon *et al.*, 2007, Duncan and Howard, 2000, Cousin *et al.*, 2006). However, it has recently been argued that many previous reports were taken from experiments designed only to assess the *mode* of entry, not the timing. The use of more realistic conditions has led to the finding that, post-germination, 99 % of individuals remain exclusively epiphytic for at least 10 days (Fones *et al.*, 2017b). Indeed, using methods such as RNAseq and enzyme-linked immunosorbent assay (ELISA), no significant evidence of the fungus has been found internally until at least 7 days (Rudd *et al.*, 2015, Kema *et al.*, 1996b, Keon *et al.*, 2007).

To assess the capacity for an extended period of epiphytic growth, water agar was used as a low-nutrient leaf mimic. Conidiospores were cultured on water agar and assessments were made of hyphal growth and sporulation over a 28-day period. Growth forms were also assessed on agar supplemented with nutrients that may be found on a host leaf during a growing season. These included the sugars: glucose, fructose, sucrose; as well as nitrogen and phosphorous, macronutrients applied during agricultural fertilisation.

4.4.1. The capacity for growth on water agar

To assess hyphal extension, colony development on water agar was photographed every 7 days over a 28-day period. Results showed that, although initially slow (~1 mm colony diameter within 7 days), hyphal growth dominates the colony morphology on water agar (Figure 15). Colony growth was radial, continuing up to 21 days post-germination. The continuation of growth over this time period strengthens the previous finding that *Z. tritici* has the capacity for extended periods of epiphytic growth (Fones *et al.*, 2017b).

The current dogma is that the majority of *Z. tritici* hyphal extension during infection is apoplastic (Orton *et al.*, 2011). Indeed, for a plant pathogenic fungus, epiphytic growth means increased periods in contact with stresses such as UV and drought. For this reason, epiphytic hyphal growth in many fungi is targeted for rapid leaf entry. Examples include *Puccinia graminis*, which follows the anticlinal cell walls of leaves until a stomata is found, and *Magnaporthe oryzae* which ignores stomata, instead entering the leaf using a high pressure injection system composed of an appressorium (Brand and Gow, 2009, Wilson and Talbot, 2009). However, in *Z. tritici* no such adaptations for rapid entry have been found. Most findings have reported random/untargeted growth, with hyphae often growing over stomata if they are closed – shown in Figure 3 (Kema *et al.*, 1996b, Shetty *et al.*, 2003, Fones *et al.*, 2017b).

This lack of specialised infection mechanisms can lead to the hypothesis that an extended period of epiphytic growth may be beneficial. For example, due to complex plant-pathogen interactions, epiphytic growth might be less restricted than endophytic growth. If so, the distances recorded here suggest that epiphytic individuals could build up large hyphal networks on the leaf surface. These networks could span hundreds of stomata. Each germinated spore could, therefore, be responsible for multiple separate infection events. Potentially then, epiphytic growth, in addition to endophytic growth, may have a role to play in lesion size and development.

The rate of colony growth on water agar also increases significantly over time during this 21-day period. After 7 days, colony area rises from around 1.6 mm^2 on day 7, to around 10 mm^2 by day 14, terminating at 44 mm^2 (6.68 mm diameter) by day 21. Between 14 and 21 days

significant hyphal-branching also occurs. *In planta*, two rapid periods of hyphal growth have been recorded. Firstly, directionally through the apoplast once the fungus becomes endophytic (Hilu and Bever, 1957, Metcalfe, 1998), and additionally in a rapid branching pattern prior to pycnidiation (Shetty *et al.*, 2003). Both these events have been attributed to increases in nutrient availability (Keon *et al.*, 2007, Sattelmacher, 2001). Firstly, from the apoplast in comparison to the leaf surface, and secondly, post plant cell lysis. Results here, however, show that both rapid extension, and widespread branching, occur with only low amounts of nutrient availability. These results suggest that *Z. tritici* conidiospores have enough internal resources to complete an extensive part of the life cycle without high levels of exogenous nutritional input.

4.4.2. The capacity for epiphytic sporulation

A second potential benefit of an extended epiphytic growth stage surrounds sporulation. As discussed previously, *Z. tritici* is a dimorphic fungus with both hyphal and yeast like (MC) growth. In many fungi, the presence of MC is often attributed to the avoidance of unfavourable conditions. It is defined as being a simplification of a typical fungal life cycle, allowing a fungus to build a population directly from conidia rather than from mycelial structures (Hanlin, 1994, Jung *et al.*, 2014). In the case of *Z. tritici*, however, MC is not necessarily linked to unfavourable conditions. In fact, the more nutrients are available, i.e. the richer the agar on which it is grown, the more MC is recorded (Mehrabi *et al.*, 2006, Guo and Verreet, 2008, Kema and Annone, 1991).

The leaf surface is relatively low in available nutrients. As might be expected, hyphal growth has been confirmed to dominate (Duncan and Howard, 2000, Cousin *et al.*, 2006). However, MC has also been shown to occur on the leaf surface (Figure 20). Other low nutrient environments where MC has been confirmed include water agar, glass slides and Teflon (Figure 20). The experiments used in this chapter mimicked the low nutrient availability of leaf conditions by plating on low nutrient (water) agar. To assess if sporulation continued in these conditions, plates were inoculated with a known quantity of conidiospores and, at 7, 14, 21 and 28 days, individual plates were flooded with water, scraped, and counted by haemocytometer. Interestingly, within just 7 days, the number of spores per plate had

increased by more than 5,000 times (Figure 15F). This result confirms that MC can occur to highly significant levels even in low nutrient conditions.

Agar is in fact a relatively complex substance obtained from marine algae named Rhodophyceae (Sigma, 2017). Agar is therefore plant derived which suggests that some of the constitute compounds, such as cell wall materials may be ideal nutrient sources for an evolved epiphyte. *Z. tritici* has several glycoside hydrolases in its genome and thus is likely to be able to metabolize any polymers such as long chain carbohydrates found in agar. To further assess this, *Z. tritici* could be cultured on agarose, a purified form of agar, instead of agar.

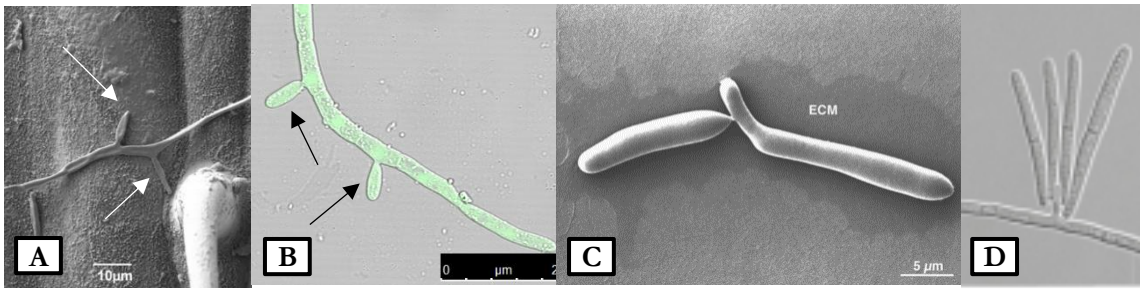

Figure 20: Evidence of microcycle conidiation in multiple environments.
A: SEM image of budding on the leaf surface (Fones, pers. comm.) **B:** Confocal microscopy of image of bussing on a hydrophobic glass slides (Cowper & Fones, pers. comm.) **C:** SEM image of budding suspended in water on Teflon (Duncan and Howard, 2000). **D:** Light microscopy of budding on water agar (Mirzadi Gohari *et al.*, 2014).

4.4.3. Nutrient availability in the field

Water agar was used in these experiments to assess low nutrient growth. On a leaf, *Z. tritici* individuals may encounter more complex pockets of nutrition. For example, sugars such as glucose, sucrose and fructose are naturally exuded on leaves (Ruinen, 1970, Tukey Jr, 1970). In epiphytic bacterial plant pathogens, the availability of foliar nutrients has been shown to promote plant colonisation and bacterial population growth (Mercier and Lindow, 2000, Leveau and Lindow, 2001). The availability of external carbon sources has also been shown to enhance growth in fungi such as *Botrytis cinerea* (Vercesi *et al.*, 1997).

Further evidence that *Z. tritici* makes use of externally available nutrients during epiphytic growth comes from transcriptomic work. Enzymes linked to the acquisition of nutrition from external sources have been shown to be upregulated. These include secreted enzymes such as cutinases, peptidases, pectinases, lipases, cellulases, hemicellulases and xylanases (Rudd *et al.*, 2015, Yang *et al.*, 2015, Goodwin *et al.*, 2011, Palma-Guerrero *et al.*, 2015, Brunner *et al.*, 2013). Conversely, two papers have suggested that carbon and nitrogen uptake are not occurring during early growth. Firstly, Rudd *et al.* (2015) reported a lack of transcriptomic and metabolomic evidence for hexose and nitrogen assimilation during the first 8 days post germination. Additionally, a study which used the upregulation of a GFP signal to assess carbon assimilation reported no increased fluorescence during epiphytic growth. Fluorescence was only recorded when the fungus became apoplastic (Rohel *et al.*, 2001).

To assess if nutrient availability affects hyphal growth, agar was supplemented with the carbon sources glucose, fructose or sucrose (Figure 16C). The availability of 20 g/l glucose or sucrose resulted in significantly increased hyphal growth when compared to water agar (1.63 mm, 1.70 mm and 1.28 mm respectively). The availability of 20 g/l fructose, however, did not significantly affect hyphal extension. Plates were also assessed for sporulation after a 7-day period. Results showed that, in all nutrient agars tested, MC is significantly increased in the presence of all three carbon sources (Results 4.3.2). This result allows for the suggestion that, during an extended period of epiphytic growth, increased sporulation may allow for the increase in pathogenic spores over a growing season.

4.4.4. The effect of agricultural fertilisation on epiphytic growth and subsequent disease

As well as any naturally exuded nutrients such as sugars, agricultural inputs such as nitrates, ammonium ions and phosphates are used to maintain the macronutrient content of soils during the growing season (Sylvester-Bradley *et al.*, 2008). This means that relatively high levels of nutrients will be intermittently available during the growing season. In many plant pathogens a link has been shown between agricultural fertilisation and disease (Veresoglou *et al.*, 2013). In *Z. tritici* specifically, a positive correlation has also been reported (Simon *et al.*, 2003, Leitch and Jenkins, 1995, Lovell *et al.*, 1997). One paper speculated a link between the

increased spread of asexual spores to the larger well-fertilised leaves, however, the reasons could surround the behaviour of *Z. tritici* on the leaf. For example, previous results have shown that hyphal growth and sporulation are increased in the presence of carbon sources (Results 4.3.2). This affect may also be seen with nitrogen.

To assess these factors, hyphal growth and sporulation were investigated by culturing *Z. tritici* conidiospores on agar supplemented with either nitrogen sources (nitrates and ammonium ions), or phosphorous (phosphites). Sporulation was found to be significantly increased in all supplemented agar media tested (Figure 17). Hyphal extension, however, was reduced in all cases when compared to water agar. These results imply that if nutrition is high enough, sporulation is favoured over hyphal growth. Potentially then, building a population by epiphytic MC may be may be important to the fungus in its own right, as well as possibly facilitating plant infection or increasing infection loads. It can be hypothesised that the increases in nutritional availability linked with modern farming, may have shaped the evolution of *Z. tritici* by favouring sporulating strains as opposed to hyphal strains. This may have led to a dominance of strains with extended periods of epiphytic growth.

Collectively, these results confirm that agricultural fertilisation could be influencing disease levels at a field scale. To further assess this, an experiment was carried out using foliar sprays of ions found in nutrient fertilisers. Ammonium ions ($(NH_4)_2NO_3$) or nitrates ($NaNO_3$) were sprayed on leaves 24 hours after the application of *Z. tritici* conidiospores. After 28 days, disease on fertiliser treated plants (pycnidiation) was found to be slightly elevated (Results 4.3.4). Over 3 independent experiments, however, pycnidia counts were not found to statistically differ for leaves treated with either nitrates (Students *t*-test, t = -0.50641, df = 2.9831, p-value = 0.6476) or ammonium ions (Students *t*-test, t = -0.80712, df = 2.6171, p-value = 0.4864). Collectively, these results suggest that either (i) ammonium ions and nitrate ions are not utilised for sporulation when on a leaf as opposed to agar, (ii) spores are indeed produced, but leaves were already saturated with fungal material so no more disease was recorded, or (iii) spores produced on the leaf have a different role compared with their infectious parental colony – such as survival for future infection events.

4.4.5. Spores as 'survival' structures

An interesting observation is that the conidiospores produced on water agar are almost exclusively single celled (Figure 15H). MC is the production of conidia from conidia, rather than from mycelia. In the case of *Z. tritici*, conidiospores can be uni- or multi-cellular. Therefore, conidiation can occur in two ways: either the budding of single-celled spores, or via continued extension and subsequent breakage of multicellular conidiospores. When nutrients are plentiful, such as on YPD agar, conidiospores are mostly multicellular, suggesting extension from single celled spores (Figure 15G). Results here on water agar, however, show that under nutrient depletion, little extension occurs and single-celled conidiospores dominate the population (Figure 15H). Staining with propidium iodide confirms that the conidiospores produced all remain viable (Figure 15H). The lack of hyphal growth in these single-celled spores also suggests germination is repressed.

Spores produced via MC have been previously reported to have increased tolerance to stress conditions such as heat (Lapaire and Dunkle, 2003, Hanlin, 1994, Zhang *et al.*, 2010). It can be hypothesised, therefore, that single-celled, ungerminated conidiospores produced on water agar are propagules of survival. They may remain viable for extended periods, potentially retaining their pathogenicity until rain-splash spreads them to a new host. The validity of this suggestion will be studied in more detail in chapter 5.4, where long term survival, spread, and virulence are all assessed.

4.4.6. Conclusion

In conclusion, the extent of this growth, measured here as colony diameter, suggests that rapid infections/entry events are not essential for disease as hyphal growth can continue for up to 21 days even with no external nutrition (water agar). The epiphytic growth stage can therefore continue for relatively long periods before infection events. During these periods, sporulation is also extensive, suggesting an additional benefit to epiphytic growth – population increase. *Z. tritici* also has the capacity to take advantage of nutrition during epiphytic growth. This could potentially reduce infection times via increasing hyphal growth rates, but also enhance disease over-time due to increasing sporulation rates.

Interestingly, results presented here show that growth on water agar seems to mimic the natural life cycle of the fungus *in vivo*:

1. For the first 7 days post-germination, hyphal growth on water agar is slow and sporulation dominates. *In vivo* work has shown this period to consist of purely epiphytic growth with few recorded entry events for *Z. tritici* IPO323 (Fones *et al.*, 2017b).
2. Between 7 and 14 days post-germination, hyphal extension on water agar is increasingly rapid. This emulates the reported period *in vivo* where most fungal individuals enter the plant via stomata (Fones *et al.*, 2017b).
3. Between day 14 and 21 post-germination, hyphal extension on water agar is at its most rapid. This mimics what is seen in the apoplast, where hyphae rapidly colonise the apoplastic space (Keon *et al.*, 2007, Shetty *et al.*, 2003). Additionally, during this 14 to 21-day period, hyphal growth on water agar branches significantly, also reflecting what has been reported of fungal development directly preceding pycnidiation (Shetty *et al.*, 2003).
4. Between day 21 and 28 there is no more growth on water agar. *In vivo*, this period is dominated by pycnidiation. It is possible that nutrition, which *in planta* would be provided by plant cell lysis, is needed for this final stage to occur. A plant specific trigger may also be necessary to initiate sporulation. This will be examined in the following chapter.

The timings recorded here may simply be coincidental. However, what can clearly be concluded is that *Z. tritici* has enough internal resources to germinate, grow, enter, and proliferate within a host leaf with little external nutrient availability.

One potential internal resource is lipid granules which have been shown to be present in high number in *Z. tritici* pycnidiospores (Cairns *et al*, Unpublished). In *Z. tritici*, many papers have confirmed the upregulation of genes involved in lipid degradation during infection. Examples, upregulated as rapidly as day 1 on leaves, include enzymes such as Acyl-CoA dehydrogenase, AMPdep synthetase/ligase, and enoyl CoA hydratase (Rudd *et al.*, 2015, M'Barek *et al.*, 2015, Keon *et al.*, 2007, Palma-Guerrero *et al.*, 2015). Currently, the hypothesis

that the expression of these genes leads to the breakdown and depletion of lipid stores has not been tested through visualisation of lipids. This will be further assessed in chapter 6.

5. Pycnidiation: Searching for a trigger for asexual spore formation

5.1. Introduction

Pycnidiation is the *in planta* production of asexual spores termed pycnidiospores. These spores can be differentiated from conidiospores by the fact they are exclusively produced within a pycnidium. In *Zymoseptoria tritici* pycnidiation occurs only within the sub-stomatal cavities of wheat leaves, appearing after a period of plant cell-death and necrosis.

Thus far, the work described in this thesis demonstrates that conidiospores plated onto agar-based media produce colonies formed by hyphal growth and/or microcycle conidiation (MC). In this work, there have not been pycnidia-like structures. Agar-based experiments from other groups have, however, reported these structures. It has been reported that the process is dependent on light, nutrient availability, an initial period of starvation. Additionally, contact with the tissues of plants such as wheat, maize or carnation (Guo and Verreet, 2008, Cousin *et al.*, 2006, Kema and Annone, 1991).

As pycnidiation in the field is a purely seen *in planta*, the signal(s), or trigger(s), to initiate the process is/are likely to be linked to the conditions within, or features of, a wheat leaf. Additionally, as plant cell-death occurs prior to pycnidiation, the trigger may be linked to this necrosis. Using *in vitro* agar-based experiments, the work described herein assesses the conditions required to trigger pycnidiation, firstly aiming to confirm that wheat leaf agar is sufficient for pycnidiation *in vitro*, and subsequently exploring the properties of the trigger/s.

5.2. Methods

5.2.1. Plant leaf agars

Leaf agars were made as per Methods 2.2.

Any alterations are outlined in the experimental results.

5.2.2. Plant leaf agar inoculations and colony assessments

Conidiospores were cultured for 7 days on YPD agar before use. Conidiospores were suspended in autoclaved MilliQ water before aliquoting, by pipette, on to agar-based media. After 10 days, morphologies were assessed. Colonies were scored as *in vitro* pycnidia (IVP) according to three ordered stages of growth: (i) radial hyphal growth (with no sporulation), (ii) centralised melanisation and (iii) sporulation from the melanised centre. Morphological structures with two of these growth forms were scored as pseudopycnidia. Morphological structures with one of these growth forms were scored as MC, or hyphal growth, accordingly.

5.3. Results

5.3.1. *In vitro* pycnidiation (IVP) is triggered on wheat leaf agar

Pycnidiation has been previously reported on wheat leaf agar (Kema and Annone, 1991). To confirm this, wheat leaf agar (WLA) was made following the methods of Guo and Varreet (2008). Conidiospores were plated onto WLA. The developing colony morphologies were photographed every day for 10 days. Colony morphology was compared to that of colonies produced on yeast peptone dextrose (YPD) agar.

The growth form recorded on WLA (Figure 21A) was unlike that produced on any other agar tested in this thesis. For comparison, growth on YPD are shown in Figure 21B. This

growth form, which will from this point be called *in vitro* pycnidiation (IVP), is characterised by the three sequential developmental stages (Timeline is shown in Figure 21D).

(i) **Day 0 – Day 6: Radial hyphal growth, no sporulation.** Hyphal growth is radial. No conidiospores are produced Figure 21C.

(ii) **Day 7 – Day 9: Melanisation.** Occurs only centrally within colonies.

(iii) **Day 10 – onwards: Sporulation.** Occurring only within the melanised central area. The spores isolated from WLA (Figure 21G) show two phenotypes. These can be compared directly to pycnidiospores from leaf pycnidia (Figure 21G white arrows, and Figure 21E), and MC from YPD (Figure 21G black arrows, and Figure 21F).

In vitro pycnidia were found to be consistent in both their size (4 mm ± 0.5 mm), and the time taken to complete the process (~ 10 days, Figure 21D). The experiments which follow aim to identify the leaf specific trigger for this *in vitro* pycnidiation process.

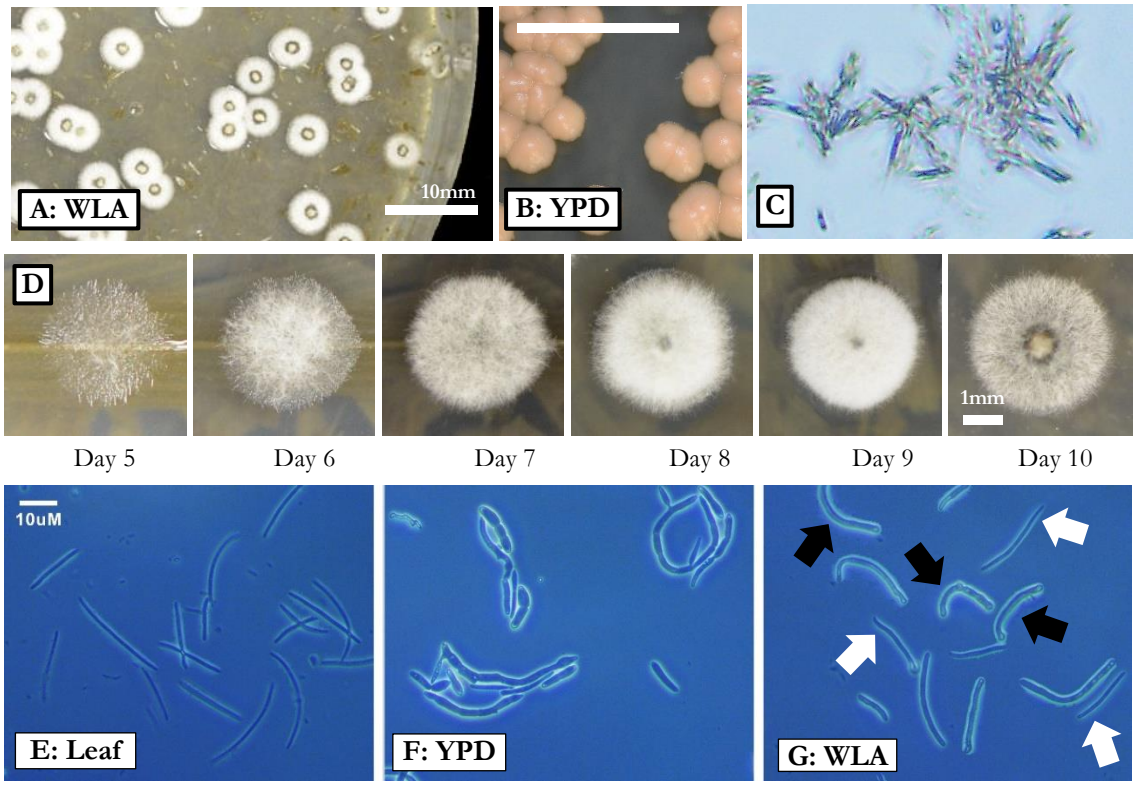

Figure 21: Assessment of colony development and sporulation on wheat leaf agar.
A: *In vitro* pycnidia on wheat leaf agar (WLA) after 10 days, compared to **B:** yeast-like growth (MC) on yeast peptone dextrose (YPD). Scale bar is 10mm. **C**: Small cells which make up the hyphal area of *in vitro* pycnidia – unlike hyphal areas on water agar, no sporulation was recorded in this area. **D:** Timeline of *in vitro* pycnidium development on WLA. The process was consistent in terms of completion time and size. **E:** Light microscopy of pycnidiospores taken from leaf pycnidia at 28 dpi, **F:** Conidiospores from YPD plates. **G:** Spores from WLA. Contains morphology of both pycnidiospores (white arrows) and conidiospores (black arrows).

5.3.2. IVP is linked to plant cell disruption

In planta, *Z. tritici* growth is reported to be initially hyphal before pycnidiation initiates (Kema *et al.*, 1996b). Plant cell disruption and the associated nutrient release occur before pycnidia are recorded (Keon *et al.*, 2007, Rudd *et al.*, 2008, Shetty *et al.*, 2003, Yang *et al.*, 2013a). However, it is not clear whether plant cell lysis occurs before, during, or after the pycnidiation process is initiated.

To assess whether plant cell disruption is necessary to trigger pycnidiation, *Z. tritici* conidiospores were cultured on wheat leaf agar with varying levels of plant leaf cell disruption. These included, both individually and collectively, the initial freezing of leaves in

liquid nitrogen, the blending of leaves in water (using a Waring blender), the grinding of frozen leaves using a pestle and mortar in liquid nitrogen, and the boiling of leaves in water for a 20-minute period. Colonies were assessed using the three sequential developmental stages which characterise *in vitro* pycnidiation outlined in Methods 5.2.2.

Results (Figure 22) shows that colony development is significantly different on all forms of WLA as compared with either nutrient rich YPD agar (Figure 22A1) or nutrient poor water agar (Figure 22A2). In all cases at least two morphologies, hyphal growth and MC, are visualised. Colony morphology on WLA also differed significantly depending on the manner and degree of plant tissue disruption used. An initial freezing of leaves before addition to water was not sufficient for IVP to be recorded (Figure 22B). On WLA made this way, only hyphal growth and MC were recorded. The subsequent fine grinding of frozen leaves (Figure 22E), or the blending of frozen leaves, initiates the three stages of IVP (Figure 22D). The act of boiling leaves is not essential for IVP development (as can be seen in Figure 22D). However, whether leaves are fresh or frozen, blended or ground, the act of boiling as a secondary means of disruption seems to increase the percentage of colonies completing IVP development (Figure 22C, E & F).

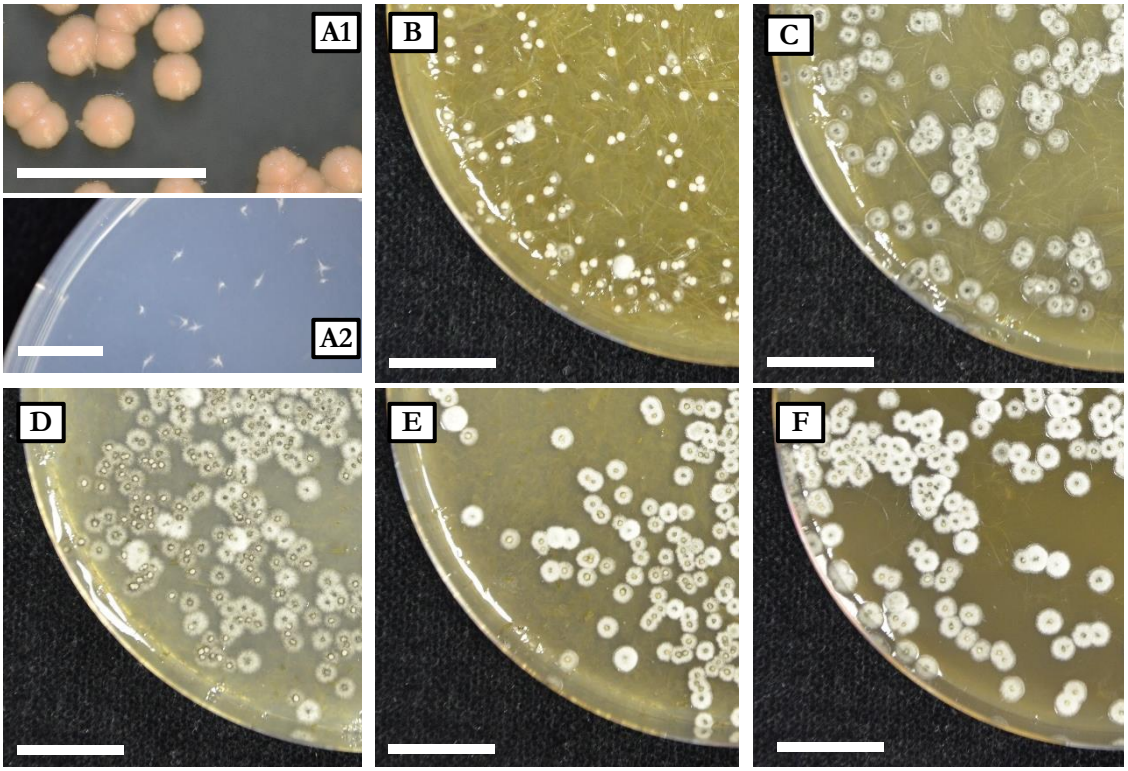

Figure 22: An assessment of colony development after 10 days on wheat leaf agar following various methods of plant leaf cell disruption.
Scale bar is 10 mm. **A1**: Colonies on YPD displaying yeast like growth via microcycle conidiation. **A2**: Hyphal colonies with microcycle conidiation on dextrose agar. **B**: WLA made with leaves frozen in liquid nitrogen then added to room temperature (RT) water. **C**: WLA made with leaves frozen in liquid nitrogen and blended in RT water and subsequently boiled for 20 minutes. **D**: WLA made with leaves frozen in liquid nitrogen and blended in RT water. **E**: WLA made with leaves frozen and ground in liquid nitrogen and boiled in water for 20 minutes. **F**: WLA made with fresh leaves blended in RT water and boiled for 20 minutes. Results suggest that an increased level of cell disruption seems to increase likelihood of IVP formation, as well as the morphological consistency of *in vitro* pycnidia. Boiled WLA increases the likelihood of *in vitro* pycnidia production (**C, E and F**). Scale bars are 10 mm.

5.3.3. IVP trigger is not wheat leaf specific

Previously, IVP has been seen to occur on agar supplemented with heavily disrupted wheat leaves (Results 5.3.1). The likely intracellular trigger for this process may not necessarily be wheat specific, but simply found in the cells of plant leaves. To test this, agar media was supplemented with leaves from 5 different plant species. Four tested belong to the monocotyledons: *Triticum aestivum* cv. Consort winter wheat, Durum wheat (*Triticum durum* cv. Volcani), barley (*Hordeum vulgare* cv. Golden Promise) and rice (*Oryza sativa* cv. Co39); and one more distantly related dicotyledon: *Arabidopsis thaliana* Col0. In all cases leaves were frozen in liquid nitrogen before adding to boiling water. Suspensions were immediately boiled for 20 minutes before autoclaving.

Results (Figure 23) show that in all leaf agars tested, the three stages of IVP were recorded – radial hyphal growth, centralised melanisation, and conidiospore production.

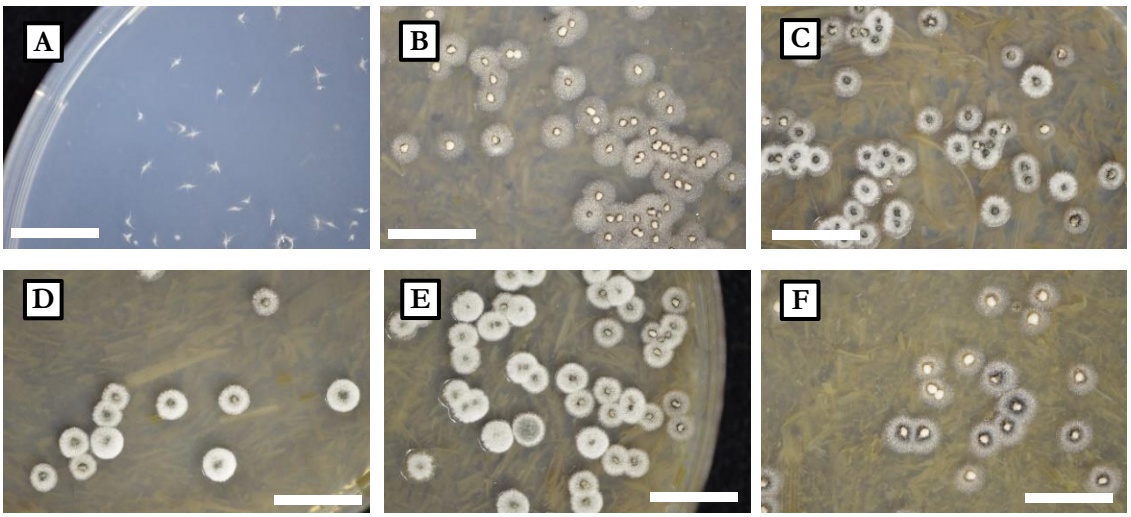

Figure 23: An assessment of i*n vitro* pycnidia development after 10 days on various leaf agars. **A:** Hyphal colonies with microcycle conidiation on dextrose agar. **B:** IVP on *Arabidopsis thaliana* (Col 0). **C:** IVP on Barley (cv. Golden Promise). **D:** IVP on Wheat (cv. Consort). **E:** IVP on Durum Wheat. **F:** IVP on Rice (CO39). In all leaf agars tested, the three stages of *in vitro* pycnidiation were recorded – radial hyphal growth, centralised melanisation, and spore production. The three stages do differ between the treatments with wheat (D) and durum wheat (E) displaying the most consistent colonies. Scale bars are 10 mm.

5.3.4. IVP requires additional sugar

The wheat leaf agar recipe used in these experiments contains 20 g/l of dextrose. To assess the importance of the sugar content, wheat leaf agars supplemented with varying levels of dextrose were compared. This revealed that in the absence of sugar MC dominates with some hyphal growth around colonies (Figure 24B). Hyphal growth is significantly less than on high sugar wheat leaf agars (Figure 24C and Figure 24D) and melanisation is not seen.

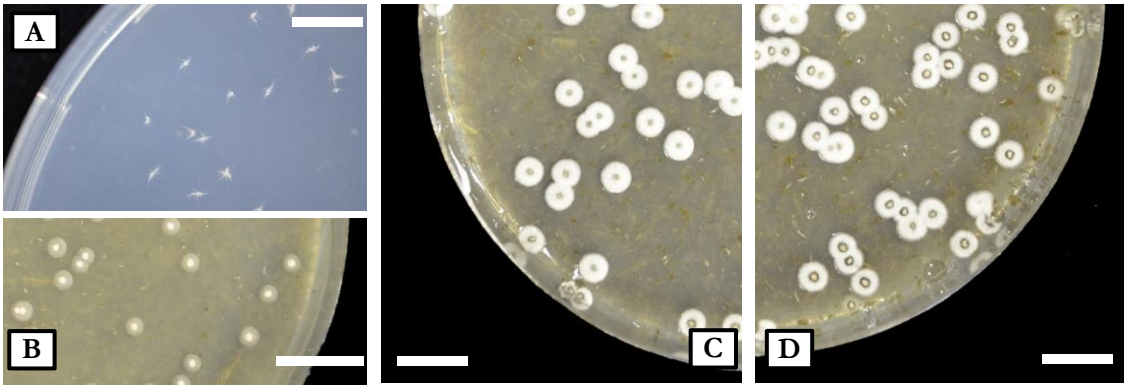

Figure 24: An assessment of IVP after 10 days on WLA supplemented with dextrose.
A: Agar supplemented with 20 g/l dextrose. Radial hyphal growth and melanisation are not recorded. **B:** WLA with no additional dextrose. Colonies are smaller and lack melanisation when no glucose is added. **C**: WLA with 15 g/l dextrose. The addition of 15 g/l dextrose is enough to increase hyphal growth and trigger melanisation, thus completing the *in vitro* pycnidiation process. **D:** WLA with 20 g/l dextrose. Additional sugar seems to only effect the final stage of the process, spore production. Scale bars are 10 mm.

5.3.5. The trigger for IVP is transient at room temperature

The evidence collected so far indicates that plant leaf cell disruption is an important factor in triggering IVP in *Z. tritici*. This disruption lyses the cells thus bringing together the extra- and intra-cellular components. Additionally, substances only found in certain subcellular compartments, or usually sequestered in the vacuole, would be brought into contact with each other, as the intracellular membranes and tonoplast will also be disrupted. The lysed cell contents would likely contain enzymes still in their active form. The disruption of cells would therefore allow some enzymes, usually separated from potential extracellular, compartmentalised or sequestered substrates, to more freely interact, thus potentially altering

the molecular composition of the plant cell lysate over time. The boiling of WLA post-disruption would negate this process. As such, when WLA is boiled more extensively, IVP can be recorded. To assess if the trigger for pycnidiation is affected by enzymatic action, an experiment was conducted which compared wheat leaf solutions left at room temperature for 10 minutes before boiling, with wheat leaf solutions that had been immediately boiled. Both solutions were subsequently autoclaved.

Results (Figure 25) show significant differences between wheat leaf solutions which are immediately boiled after cell disruption (A1 and B1), and leaf based solutions left at room temperature for 10 minutes before boiling (A2 and B2). Only in the agar media which were immediately boiled upon preparation was IVP recorded. This effect can be seen independent of the mode of cell disruption: Grinding (A) and blending (B). In non-boiled versions, one of the three stages of IVP is either missing or incomplete. In the ground but non-boiled media, some colonies seem to be missing melanisation. Hyphal growth is also reduced. In the non-boiled version of blended media, melanisation is not recorded and growth is almost purely MC.

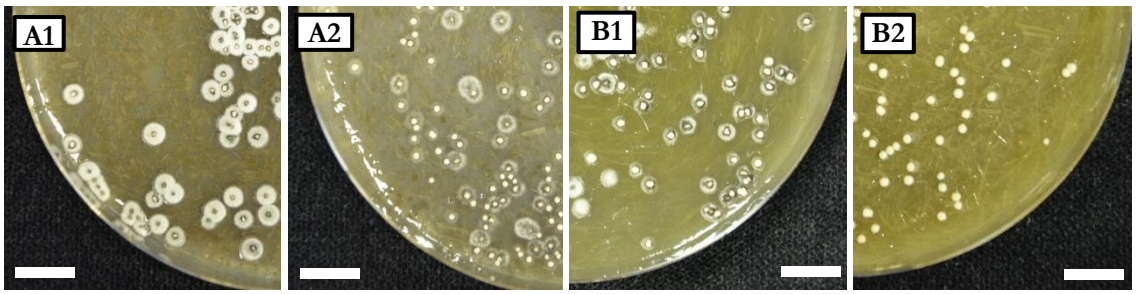

Figure 25: An assessment of enzyme activity on the trigger for IVP (cultured for 10 days).
A: Ground and **B:** blended wheat leaf agar. In **A1** and **B1** leaf tissue has been boiled for 20 minutes after adding to water. In **A2** and **B2,** leaf tissue has been suspended for 10 minutes at RT before boiling for 20 minutes. In both ground and boiled agars, the ability for *Z. tritici* conidiospores to undergo *in vitro* pycnidiation is significantly diminished if not boiled immediately. These results suggest that boiling with WLA before autoclaving preserves the presence of the trigger for IVP. Scale bar is 10 mm.

5.3.6. The trigger for IVP is transient, even after boiling

In vitro pycnidia do not appear when the WLA agar solution is not immediately boiled after plant leaf cell disruption (Results 5.3.5). Boiling the disrupted media would likely remove any residual enzyme activity in the plant leaf extracts, thus maintaining potential triggers over extended periods. To assess this, WLA plates were left at room temperature under a long day cycle (18 hours light, 6 hours dark) for 7 days. Conidiospores were plated on freshly poured WLA plates and compared to 7-day old plates. Colony morphologies were assessed after 10 days.

Results (Figure 26) show that *Z. tritici* conidiospores do not form *in vitro* pycnidia if plated onto WLA that has been stored for 7 days. Colony morphology on stored plates is primarily one of MC, with radial hyphal growth occurring secondarily. This is the reverse order of IVP, in which colonies do not produce spores until hyphal growth and melanisation have occurred.

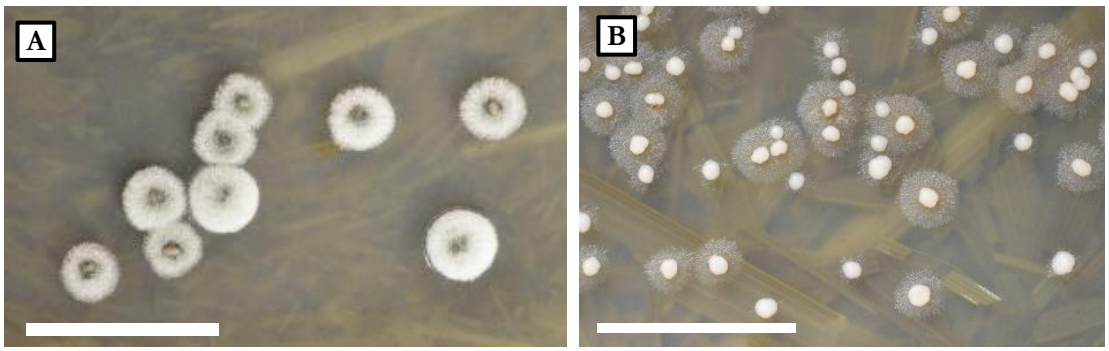

Figure 26: An assessment of colony growth after 10 days on fresh or stored WLA.
A Development of conidiospores on WLA plated on the same day as the agar was made – *in vitro* pycnidiospores dominate after 10 days growth. **B** Development of conidiospores on WLA that has been left for 7 days under laboratory conditions before inoculation. Microcycle conidiation dominates. Hyphal growth also occurs secondarily in colony development. Melanisation is not recorded. Scale bar is 10 mm.

5.3.7. The trigger for IVP is small and diffusible

The experiments carried out so far in this chapter have all contained leaf pieces. It has been reported previously that pycnidiation depends on physical contact with leaf pieces (Kema and Annone, 1991). To assess this, an experiment was conducted to restrict physical contact with leaf pieces. This was achieved using a cellophane membrane to cover the surface of the WLA. The membrane is not impermeable, therefore diffusible molecules can travel between the agar medium and the surface. Membrane covered plates were assessed against standard wheat leaf agar plates.

Results (Figure 27) show that there is no significant difference in the development of *in vitro* pycnidia on media with or without a cellophane covering. The colonies on both treatments went through each of the three sequential stages of IVP: hyphal growth, melanisation and spore production.

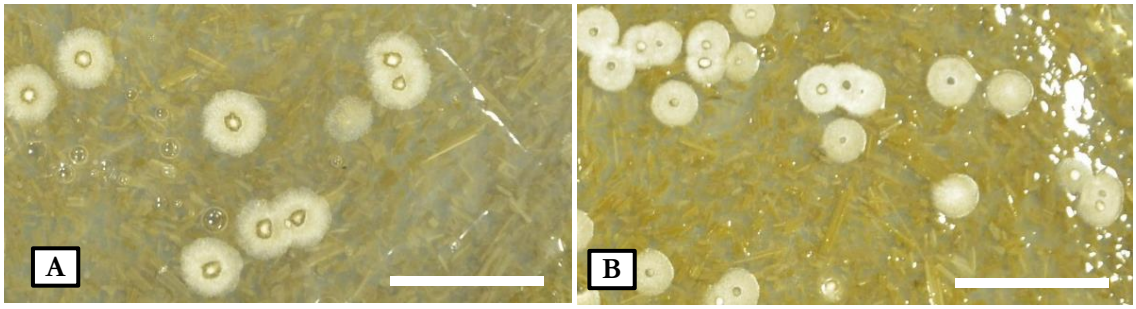

Figure 27: An assessment of *in vitro* pycnidiation on WLA with a cellophane covering
A: WLA with a cellophane membrane covering. Colonies show no obvious morphological differences when compared with WLA without a cellophane membrane covering (B). Both treatments trigger all three sequential stages of *in vitro* pycnidium development – radial hyphal growth, melanisation, and centralised conidiospore production. This result indicates that physical contact with the leaf is not necessary for *in vitro* pycnidiation. Results also suggest that any *in vitro* pycnidiation trigger is small and diffusible, thus it can diffuse through a cellophane membrane. Scale bar is 10 mm.

5.3.8. IVP is not linked to apoplastic or cytoplasmic pH

The apoplast is more acidic, pH < 5.9, than the cytoplasm, pH > 7.0 (Kulichikhin *et al.*, 2008, Ehlert *et al.*, 2011). Thus, post-plant cell disruption, hyphae within the plant apoplast may experience an increase in pH. The trigger for pycnidiation in a leaf may be linked to this

increase. To test this, wheat leaf agar was adjusted to 4 different pH levels from pH 6.2 (unadjusted) and pH 5, 6 and 7.

Results (Figure 28) showed no obvious difference in colony morphology between the 4 treatments confirming that the IVP morphology is maintained at higher (cytoplasmic) or lower (apoplastic) pH levels. This result implies that any changes in apoplastic pH which occur during plant cell lysis are not likely to be a major trigger for pycnidiation. Additionally, no significant differences were recorded with regards total spore production within a 10-day period (One-way ANOVA, p-value = 0.051, n = 2. Data were normally distributed). This result confirms that the IVP morphology is not pH dependent.

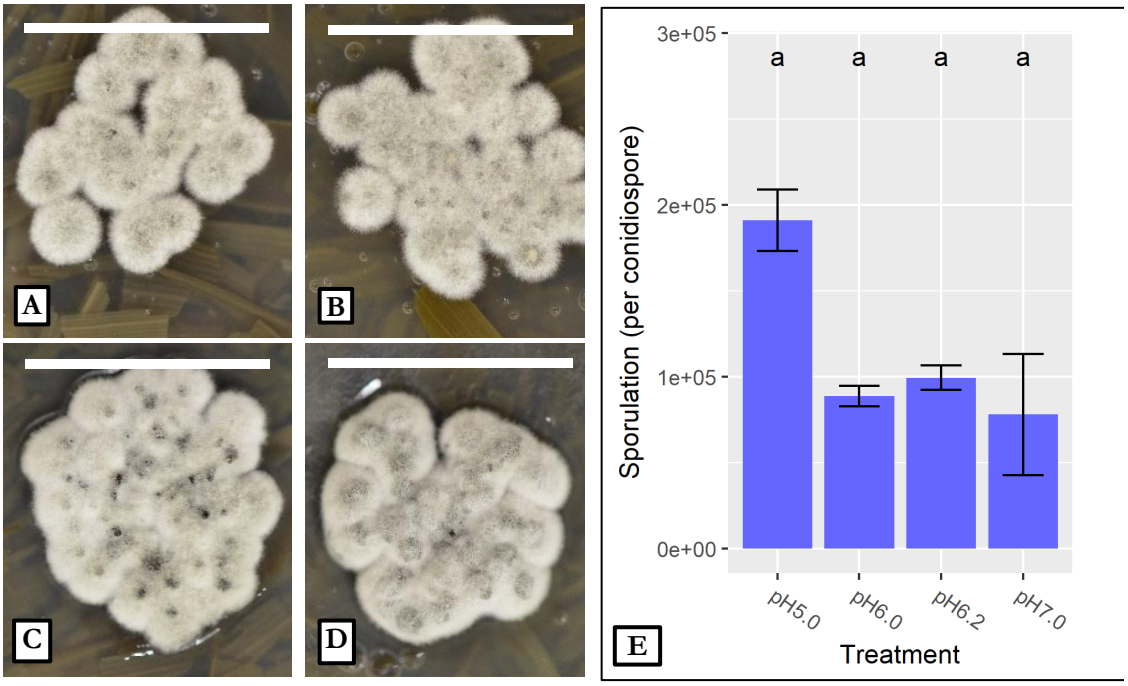

Figure 28: An assessment of sporulation on WLA with different pH.
A: pH 5.0. **B:** pH 6.0. **C:** pH 6.2. **D:** pH 7.0. There are no significant morphological differences between colonies grown on WLA with different pH levels. **C:** Sporulation, although numerically higher on WLA with a pH of 5.0, was not found to be significantly different at any pH tested (One-way ANOVA, p-value = 0.051, n = 2. Data were normally distributed). Data are mean spore counts from 2 independent experiments, each containing 3 replicate plates. Error bars show SE. Scale bars are 10 mm.

5.3.9. IVP is maintained under high nutrient conditions

The addition of high levels of nutrients to agars almost entirely eliminates hyphal growth in favour of MC. For this reason, high nutrient media (such as YPD and PD) are used in laboratory settings to generate inoculum (conidiospores) for further experiments. It can be hypothesised, therefore, that if nutritional resources were high enough, *in vitro* pycnidium development may be lost in favour of MC. To test this, WLA was supplemented with 20 g/l bacteriological peptone. Peptone contains a high peptide and amino acid content, as well as nitrogen in a readily available form (Sigma, 73049-73-7).

Figure 29A show hyphal growth on water agar. Figure 29E shows MC on peptone supplemented agar. This confirms the preference for MC when nutrients are available. On WLA (Figure 29B) IVP can be visualised. On peptone supplemented WLA, conidiospores still form colonies which complete the three stages of IVP. However, colonies with and without peptone differed phenotypically. Peptone supplemented agar colonies (Figure 29C) were significantly larger and more melanised than colonies on non-supplemented WLA (Figure 29B). Colonies on peptone supplemented WLA also complete IVP in half the time (5 days instead of 10 days). Additionally, conidiospore production was significantly higher after 10 days when compared to WLA alone (Figure 29D, $t = -12.255$, $df = 5.0896$, p-value = 5.688e-05).

In conclusion, supplementing water agar with peptone favours MC. However, when adding an equal amount of peptone to WLA, sporulation is not recorded until after the first two stages of IVP (hyphal growth and centralised melanisation) have been recorded. This result suggests that, once triggered, the three stages of IVP continue sequentially. This result also implies that IVP is a developmental process, with all three stages regulated sequentially. Finally, the increased presence of nutrition does significantly affect the final stage of IVP, that is, sporulation.

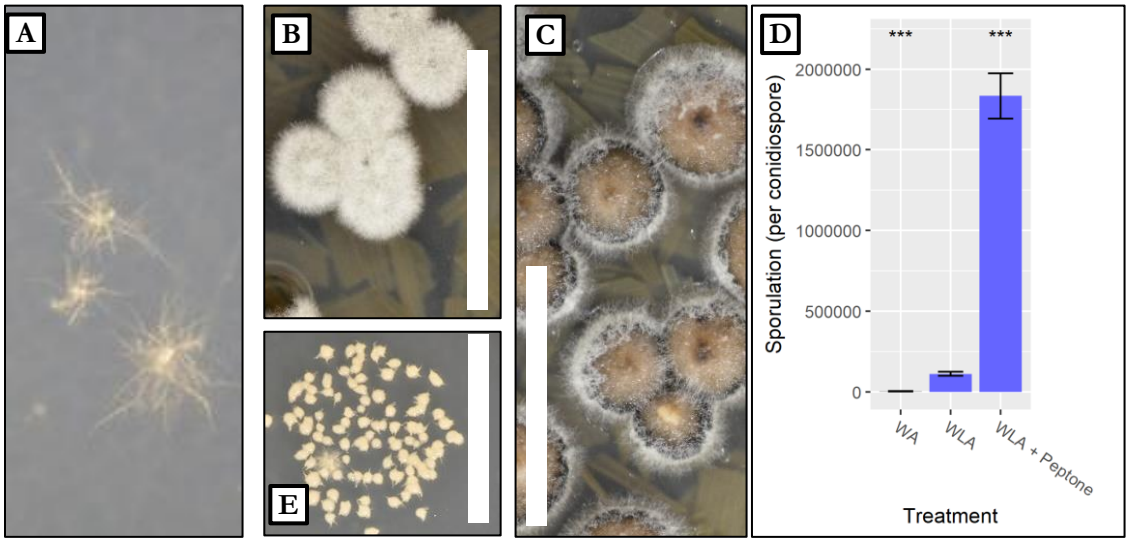

Figure 29: An assessment of growth and sporulation on WLA supplemented with peptone. Images are 10 dpi. Scale bar is 10 mm. **A**: Water agar. **B**: WLA. **C**: WLA supplemented with 20 g/l peptone. **E**: Peptone supplemented agar. The addition of peptone to WLA leads to completion of *in vitro* pycnidiation within 5 days rather than 10 days. Melanisation is also increased. **D**: Significant differences were recorded with regards total spore production after 10 days when compared to WLA (Figure 27D, Students *t*-test: t = -12.255, df = 5.0896, p-value = 5.688e-05). Data are mean total spore counts of 2 independent experiments, each containing 3 replicates (plates). Error bars show SE. Scale bars are 10 mm.

5.3.10. IVP is affected by competition for space

In planta, pycnidia size is restricted by the stomatal cavity in which they develop. A single pycnidium has been measured at 36 µM x 22.5 µM (Fones and Gurr, 2015). In comparison, the size of the individual *in vitro* pycnidia, 4 mm ± 0.5 mm, are considerably larger. Their size is likely to restrict pycnidial growth, and thus spore production. Potentially then, *in vivo/planta* pycnidia would be much larger if so too were stomatal cavities. Additionally, due to the separation of colonies on agar, the IVP shown in these experiments are free from competition from other colonies. To assess if the size of an IVP is reduced in the presence of competition, or by a reduction in free space, conidiospores were plated onto WLA at higher densities.

Results (Figure 30) shows that all plates completed the three stages of IVP formation: hyphal growth, centralised melanisation and spore production. The amount of time taken to complete these stages differs. Higher plated conidiospore counts, and therefore closer proximity between neighbouring colonies, led to reduced time in stage 1 and thus reduced

hyphal growth before the beginning of melanisation. On high concentrations (10^6 conidiospores per ml is shown here, Figure 30C) melanisation was witnessed as soon as day 3. In low concentrations where individual colonies were further separated, melanisation would not be recorded until around day 7 (Figure 30B). In vitro pycnidia, in the proximity of neighbouring colonies, therefore mature within half the time as separated colonies.

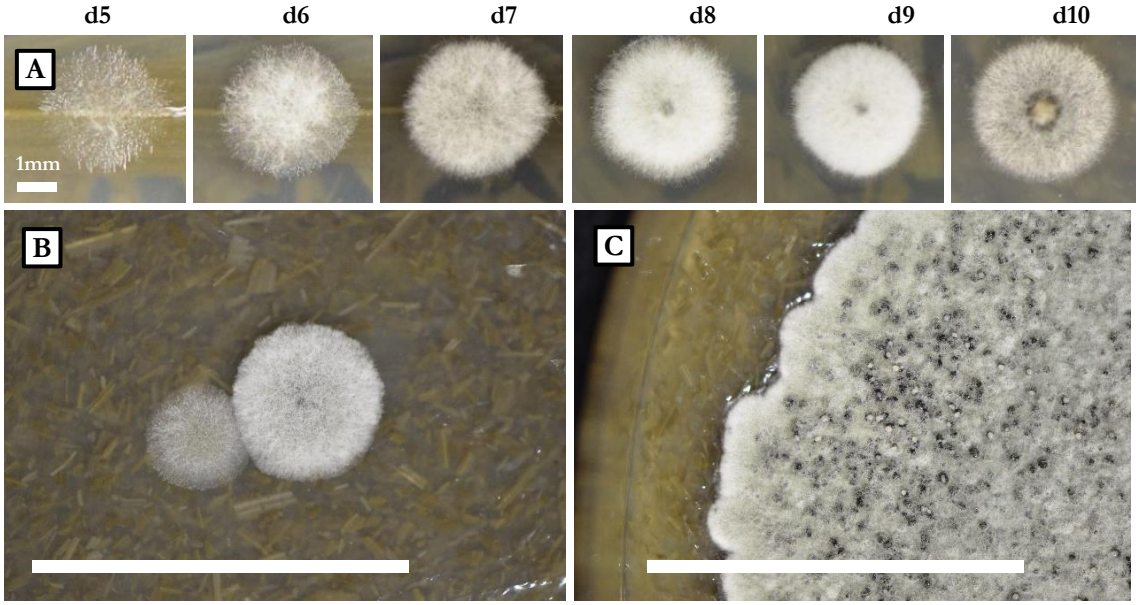

Figure 30: An assessment of IVP on WLA with differing colony separation (competition). **A:** A timeline of IVP development from a single conidiospore on WLA. **B:** IVP after 7 days growth showing the first signs of central melanisation (stage 2 in the IVP development process). **C:** Mature sporulating IVP on WLA at just 4 dpi. Results show that under increased levels of competition, or reduced physical space, the IVP process can be completed in less than 4 days.

5.4. Discussion

5.4.1. Growth on wheat leaf agar (WLA) mirrors pycnidiation

In the field, pycnidiation occurs only within the stomatal cavities of wheat leaves. *In vitro*, the same process has also been documented using wheat leaf agar, although photographic or microscopic evidence has not been published (Kema and Annone, 1991). This chapter has confirmed that when plating conidiospores onto wheat leaf agar (WLA), a morphology is

produced which is unlike any produced on other agars tested in this thesis. These structures can be defined by three sequential stages of development: (i) An initial period of hyphal growth with the absence of sporulation (ii) the subsequent melanisation of a centralised area within colonies, and (iii) sporulation from the melanised centre.

(i) Radial hyphal growth in the absence of sporulation

In planta, before appearance of pycnidia, the growth of *Z. tritici* is recorded as hyphal. This growth is initially epiphytic and subsequently apoplastic (Orton *et al.*, 2011, Fones *et al.*, 2017b). Rapid increases in hyphal growth are combined with extensive branching immediately before pycnidiation begins (Shetty *et al.*, 2003). No evidence of sporulation during this apoplastic growth stage has been published.

Colonies recorded on WLA are also entirely hyphal, with no sporulation recorded. This is particularly interesting as on all other agars assessed in this thesis, including water agar, sporulation also occurs during early colony development. The lack of sporulation indicates a different developmental programme to 'normal' colony development.

(ii) Centralised melanisation of colonies

Melanisation is an important step in pycnidium development. The reasons for this are unclear but likely surround both protection from plant defences, lysed cell contents and abiotic stresses, as well as allowing a build-up of turgor for when pycnidiospores mature and ooze onto the leaf surface (Missall *et al.*, 2004, Howard *et al.*, 1991).

In colonies on WLA, melanisation also occurs only centrally within colonies (Figure 21). These structures are small and circular and thus can be easily compared to the spore containing part of true pycnidia.

(iii) Sporulation from the melanised centre of colonies

During maturation, the leaf tissue surrounding pycnidia becomes necrotic. The subsequent uptake of water allows the spores within pycnidia to swell and ooze out onto the leaf surface (Kema and Annone, 1991).

In the WLA colonies recorded here, spores are also produced only from within the melanised centre (Figure 21C). One significant difference, however, is that when these spores are assessed, both pycnidiospore-like spores and conidiospore-like spores can be seen (Figure 21G). The reason for this difference is unknown, but it can be argued that pycnidiospores are produced initially within *in vitro pycnidia* and only after the bursting of these melanised spheres does conidiation occur. Indeed, conidiation on pycnidial structures has been reported previously (Suffert *et al.*, 2011). Assessment earlier in pycnidial development is needed (before the melanised centre bursts) to confirm this.

In conclusion, the morphologies which develop on WLA share many morphological traits with true leaf-based pycnidia. As such, these structures will be named *in vitro* pycnidia (IVP).

5.4.2. Triggers for *in vitro* pycnidiation (IVP)

In planta, pycnidiation is reported to occur after the so-called necrotrophic switch. At this point, plant cells have lysed their contents into the apoplast, thus releasing high levels of nutrients and metabolites. The protocol to produce WLA similarly entails leaf-cell disruption through the freezing, grinding, boiling and autoclaving of leaf suspensions. In both *in planta* and *in vitro* growth conditions, cell lysate is in direct contact with an individual colony/mycelium. The presence of pycnidia-like structures in both environments allows for the hypothesis that lysate contact may be linked to initiation of pycnidiation.

To assess the importance of plant-cell lysate in triggering IVP, WLA was made with many levels of leaf-cell disruption. Results showed that with increasing disruption, an increase in the number of colonies scored as IVP was also recorded (Results 5.3.2). This result provides evidence that an internal component/s of the leaf is directly related to triggering pycnidiation. During disruption, this trigger is released into the agar solution.

Further work on plant cell lysate has shown that the pycnidiation trigger contained in the leaf-cell lysate is likely small and diffusible. This was shown by plating conidiospores onto WLA covered with a cellophane membrane (Results 0). The continued presence of IVP in these conditions suggests that the trigger can diffuse through the membrane to the colony above. It also infers that pycnidiation is not dependent on physical contact with leaf pieces

as previously stated (Kema and Annone, 1991). This result is arguable, however, as hyphae could be growing through the membrane thus making physical contact with the leaf pieces below.

Two further experiments show that the trigger for IVP is transient, potentially affected by both enzyme activity and light. This has been demonstrated in two ways. Firstly, the IVP morphology is not recorded if conidiospores are plated onto WLA in which the leaves have not been boiled (Results 5.3.5). The lysis of cells caused by the initial freezing and grinding stages of WLA preparation brings together the extra- and intra-cellular components of the cell, including those usually sequestered within subcellular compartments. Active enzymes usually separated from possible substrates, are brought into contact with these. Over time, the molecular composition of the plant cell lysate therefore changes. The boiling of leaf suspensions would likely denature these active enzymes and negate this process. As such, the act of boiling potentially allows the trigger for IVP to be preserved.

However, even after boiling, the presence of this unknown trigger is not maintained for extended periods. If agar normally conducive to IVP is left at laboratory conditions after pouring (20 °C, 18:6 light cycle), the subsequent plating of conidiospores does not lead to the development of IVP (Results 0). This indicates either that the trigger is broken down by light, is chemically unstable over longer periods, is volatile, or that some heat stable enzymes remain in solution after boiling and thus continue to degrade the trigger even when in suspended agar form.

Finally, and perhaps most interestingly, the IVP morphology was not found to be specific to WLA but also occurred on agars supplemented with the leaves of rice, barley or *Arabidopsis* (Results 0). This indicates that the host specificity of *Z. tritici* to wheat is not based around the trigger for pycnidiation, but rather linked to one of the many earlier steps in the infection process. Host specificity is likely complex and multifaceted and its full elucidation is beyond the scope of the current work.

5.4.3. Pycnidiation can be a rapid process

So far in this chapter, experiments have shown us that the lysate within plant leaf-cells seems to contain a trigger for the development of pycnidia. This trigger is transient, small, and probably diffusible. It is present in all plants leaves tested, and is degraded by both enzymatic activity and, potentially, light. Once triggered, IVP development is sequential, completing hyphal growth, melanisation and sporulation in around 9 to 10 days. It could be hypothesised that this period mirrors *in planta* results where pycnidia are visible after at least 14 days.

To assess if physical space or competition may be factors in determining this time-period, an experiment was set up which plated higher conidiospore densities onto WLA. Interestingly, competition from other developing conidiospores significantly reduced the time needed to complete IVP development (Results 5.3.10). The presence of neighbouring colonies reduces the time to complete the 3 stages of in vitro pycnidiation by half, completing within around 4 days.

There are two most likely explanations for this. In a plant leaf, other individuals compete for space and resources. Indeed, a study on plant leaves within the field shows the presence of multiple strains within lesions (Linde *et al.*, 2002), meaning that conspecific competitors are not necessarily asexual clones. Strains are known to specialise in their infection cycle. For example, Suffert *et al.* (2015) showed that the success of strains is seasonally dependent with greater sporulating strains favoured during winter conditions, and rapidly infecting strains favoured in spring. It can also be argued, that due to *in planta* competition for space, the speed at which pycnidiation can be completed is important in enabling the continued success of a strain. Direct recognition of competition may therefore trigger a cessation of hyphal growth/nutrient scavenging, and an advancement of melanisation and sporulation. By doing so the stomatal cavity will be filled before a competitor can enter. This competition would select for strains which can pycnidiate more quickly and may have shaped the evolution of *Z. tritici*.

A second explanation is that the increased rapidity of IVP surrounds physical space. The apoplast in which *Z. tritici* hyphae grow prior to pycnidiation has limited space compared to an agar plate. Additionally, the stomatal cavity is also space limited when compared to agar.

The addition of multiple conidiospores (and thus colonies) to a WLA agar plate goes some way to restrict the physical space afforded for colony growth. Along with this reduced space comes a reduction in nutrient availability. As such, colony development may be advanced due to the lack of benefit to continued environmental nutrient scavenging.

From these results, it can be concluded that the extended period of hyphal growth recorded which precedes melanisation on WLA is not necessary for pycnidium maturation. Potentially, the hyphal growth stage on WLA may be longer simply due to the unusual amounts of space accorded to each developing colony in this environment. Pycnidiation *in planta* has been assessed by Dr Helen Fones using confocal microscopy (unpublished). Pycnidium development can be assumed to begin when the stomatal space begins to be colonised. It can be said to complete when pycnidiospores exude from the stomatal opening. An assessment of confocal images (Figure 31) implies that, *in planta,* pycnidium development indeed takes somewhere between 3 and 5 days. This seems to correlate with the reduced time seen on WLA with high population densities and implies that once triggered, pycnidiation is a rapid process.

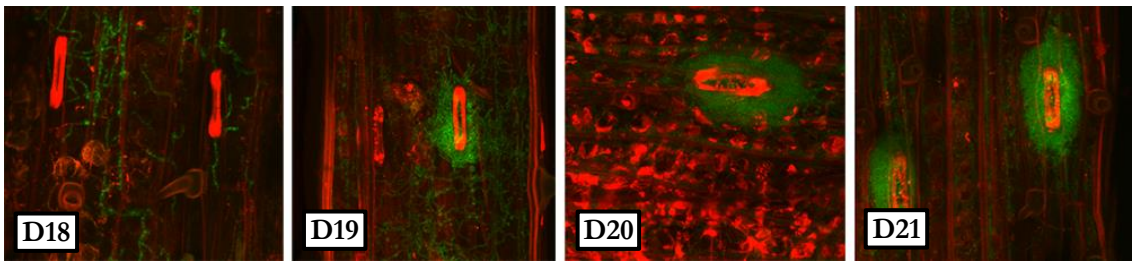

Figure 31: Representative images of pycnidia development in a leaf from days 18 – 21.
At 18 days growth is mainly apoplastic but quickly starts to group around stomatal openings. Development is subsequently rapid. Within 3 days stomatal cavity is full and signs of oozing are apparent. Confocal images courtesy of Dr Helen Fones. A cytoplasmic GFP-tagged variant of the Dutch model strain of *Z. tritici*, *Z. tritici* IPO323 was used. An argon laser with emission at 500 nm was used to excite GFP fluorescence and chloroplast autofluorescence, detected at 510-530 and 600-630 nm, respectively.

More work would be needed to confirm whether pycnidiation times are dependent on space or the recognition of competitors. However, one study which assessed disease after applying multiple spore densities to leaves (as low as 10 spores per leaf), reported no significant alterations in pycnidiation time (Fones *et al.*, 2015). Further work is needed to assess why, if pycnidiation can be so quick, it takes at least 14 days to complete after inoculation.

5.4.4. Pycnidiation is a developmental process

In all examples on WLA, these 3 stages of development occur sequentially. When not in the presence of competing colonies, these three stages also seem to be tightly temporally regulated, completing in around 9 or 10 days. Together, these results suggest that IVP is a developmental process which, once triggered, continues until completion.

To assess this further, an experiment was set up which aimed to halt the development of IVP in favour of yeast like development. This was done by supplementing WLA with bacteriological peptone, a rich source of nitrogen and amino acids which would usually promote MC over hyphal growth (Results 5.3.9).

When not in the presence of wheat leaf lysate, *Z. tritici* is predominantly hyphal on low nutrient agars. Hyphal growth is superseded by MC on high nutrient agars such as YPD, PDB and peptone supplemented agar. Supplementing WLA with peptone could therefore be hypothesised to promote sporulation in favour of hyphal growth. Interestingly, this was not found to be the case. On peptone-supplemented WLA, colony development continues to be initially hyphal, subsequently completing the IVP process through melanisation and sporulation. These results imply that once triggered, *in vitro* pycnidiation continues to completion rather than being terminated in favour of sporulation and can thus be hypothesised as a deterministic developmental process. To confirm this more conditions could be tested such as the addition of fungicides to developing colonies or the addition of other nutrients.

5.4.5. Conclusions

A study by Kema and Annone (1991) used 26 wild strains of *Z. tritici* to assess the method of inoculum production on agar-based media. They found that growth, for many strains, was not dependent on the medium on which they were cultured. Instead growth was, in most cases, strain dependent, with yeast-like phenotypes not able to produce pycnidia on any media tested. Conversely, strains which pycnidiate readily on wheat leaf agar were found to do so even on V8 agar and PDA. This study concluded that growth type manipulation is only possible to a limited degree.

This paper therefore implies that *Z. tritici* has little capacity to recognise its surroundings and grow accordingly. This chapter has only assessed one strain: *Z. tritici* IPO323. However, the growth form of this strain is highly dependent on nutritional availability, thus suggesting recognition of its surroundings. Most importantly, pycnidiation has only been found to occur in the presence of wheat-leaf cell lysate, further strengthening the idea of specific environmental cues.

If pycnidiation was to be triggered somewhere other than the leaf, the process may not compete efficiently, and sporulation may be significantly affected. Ascertaining this pycnidiation trigger may therefore be useful, as it may allow for the process to be triggered early, i.e. even when epiphytic, thus reducing subsequent disease.

6. *Ex planta* survival of *Z. tritici* conidiospores

6.1. Introduction

Strains of *Zymoseptoria tritici*, the causal agent of Septoria tritici blotch (STB) of wheat, have evolved resistance to all major commercialised antifungal chemistries (Dooley, 2015). The speed at which this has occurred, and the associated expense in controlling this fungus, has elevated the status of *Z. tritici* to the most economically important pathogen of wheat in temperate climates. *Z. tritici* now accounts for up to 70 % of EU spending on fungicides (Fones and Gurr, 2015, Torriani *et al.*, 2015).

A vast diversity of strains can be found when isolating from wheat fields (Banke and McDonald, 2005, McDonald *et al.*, 1999, Linde *et al.*, 2002, Zhan *et al.*, 2003, Wittenberg *et al.*, 2009, McDonald *et al.*, 2015). These strains have varied levels of virulence. As such many will be unlikely to complete an asexual reproductive cycle. The continued presence of these strains throughout a season implies that asexual spores may be able to survive for periods without further reproduction.

Z. tritici IPO323 has been shown to survive on the leaf surface for periods of up to 18 days (Fones *et al.*, 2017b). Survival on the non-host plant tobacco has also been documented up to 8 days when the experiment was stopped (Kettles *et al.*, 2016). Results in this thesis have shown that a protracted period of epiphytic growth may even be of benefit to the fungus, due to the ability to continue sporulation even in low nutrients conditions (Results 4.3.1).

After rain-splash, many conidiospores will not reach a host leaf, and will instead be spread to other areas, such as the surrounding soil. To date, there have been no studies of environmental survival without host tissue. The role of environmental spores in disease outbreaks is therefore unknown. Potentially, free living/autonomous spores could remain present in the environment for entire growing seasons. These spores may even survive between seasons, maintaining a source of inoculum for a newly planted crop. Using culturing and confocal microscopy, this chapter assesses the ability for *Z. tritici* conidiospore survival

in several environmental conditions. Changes in the virulence of free living conidiospore populations over time is also assessed.

6.2. Methods

6.2.1. Conidiospore survival on agar

To assess conidiospore survival on a semi-solid surface, water ager was used. Autoclaved MilliQ water was supplemented with bacteriological agar at a rate of 1.5 % agar w/v before autoclaving for 15 minutes at 121 °C. Before assessment *Z. tritici* conidiospores were grown on YPD agar for 7 days. Conidiospores were then suspended in autoclaved MilliQ water and suspension density estimated using a haemocytometer before plating ~ 1000 spores onto water agar plates. Plates were sealed with Parafilm® to prevent desiccation. Every 7 days, up to 49 days, a single plate was flooded with 2 ml autoclaved MilliQ water. A sterile spreader was then used to scrape conidiospores from plates thus forming a suspension. A small volume of this suspension was then aliquoted onto YPD plates and incubated at 20 °C under a long-day light cycle. Survival was qualified as any amount of colony growth after 7 days.

6.2.2. Conidiospore survival in dry conditions (Petri dishes)

To assess the ability of conidiospores to survive periods of drought, dry Petri dishes were used. Before assessment, *Z. tritici* conidiospores were grown on YPD agar for 7 days. Conidiospores were then suspended in autoclaved MilliQ water and suspension density estimated using a haemocytometer before plating ~ 1000 spores onto the dry Petri dishes. Plates were dried for 60 minutes in a Class II Cabinet before sealing with Parafilm®. Individual plates were re-hydrated every 7 days for a 49-day period by flooding with 2 ml of autoclaved MilliQ water. A sterile spreader was then used to suspend conidiospores in solution before a small amount was aliquoted onto YPD and incubated at 20 °C under a long-day light cycle. Survival was qualified by assessing plates for *Z. tritici* growth after 7 days.

6.2.3. Conidiospore survival in soil

For experiments concerning conidiospore survival in soil, 25 g of autoclaved John Innes No. 2 soil was added to a petri dish and flooded with 5 ml of autoclaved MilliQ water. Before assessment, *Z. tritici* conidiospores were grown on YPD agar for 7 days. Conidiospores were then suspended in autoclaved MilliQ water, estimated using a haemocytometer, and pipetted into soil at a rate of 5 ml of 1 x 10^6 conidiospores. Soil plates were sealed with Parafilm® and incubated under standard growth cabinet conditions (Methods 2.3). Every 7 days, a sterile spreader was placed into the wet soil of an individual plate and spread onto a fresh YPD plate. Plates were incubated at 20 °C under a long-day light cycle. Survival was qualified by assessing plates for *Z. tritici* growth after 7 days.

6.2.4. Conidiospore survival in water

Conidiospores were harvested from a 7-day old YPD plate and suspended in a 50 ml Falcon tube of MilliQ water. Spores were adjusted to a concentration of 1 x 10^7 conidiospores/ml using a haemocytometer. Tubes were then stored for 49 days at 20 °C under a long-day light cycle and shaken, by hand, for 10 seconds each day to maintain oxygenation. Viable conidiospores were estimated at regular timepoints by diluting an aliquot by 10^{-3} using MilliQ water and spreading 100 µl onto a YPD plate. Plates were incubated for 7 days at 20 °C under a long-day light cycle before assessment of growth by colony counting.

6.2.5. Conidiospore analysis: Live/dead staining using propidium iodide

For cell-death quantification by confocal microscopy, propidium iodide (PI) was used. PI was stored in MilliQ water at -20 °C at a concentration of 1 mg/ml and used at a final concentration of 1 µl/ml. After the addition of PI by pipette, conidiospore suspensions were used within 30 minutes. Confocal images (Methods 2.7) were taken using excitation/emission maxima of wavelength 493/636 nm. Cells were scored as dead/non-

viable if PI was found to have completely stained the cytoplasm. The number of cells per conidiospore over time was also assessed from these images.

6.2.6. Conidiospore analysis: Lipid staining

BODIPY® 493/503 (4,4-difluoro-1,3,5,7,8-pentamethyl-4-bora-3a,4a-diaza-s-indacene, Thermo Fisher, Catalogue number: D3922) was used for the staining of lipid granules. BODIPY® was stored in DMSO at -20 °C at a concentration of 1 mg/ml and used at a final concentration of 10 µM. After the addition of BODIPY® by pipette, conidiospore suspensions were used within 30 minutes. Confocal images were taken using excitation/emission maxima of wavelength 493/503 nm. As the membrane GFP signal from the *Z. tritici* strain Sso1 was too weak in comparison to the BODIPY® fluorescence, PI was often added to help visualise conidiospores. Lipids were assessed within the population by calculating: (total area of the image made up of lipid granules / total area of a single image containing fungal tissue) x 100.

6.2.7. DNA: Extractions

After 49 days, *Z. tritici* conidiospores cultures were plated onto a YPD agar plate. After 7 days, 3 single colonies per plate were combined into 500 µl MilliQ water and vortexed well to mix. 500 µl of 25:24:1 phenol: chloroform-isoamyl alcohol, was added to each tube before vortexing for 60 seconds and centrifuging for 10 minutes at 16,000 g. The top layer (~ 350 µl) was pipetted to a new 1.5 ml Eppendorf and mixed with 28 µl cold 7.5 M ammonium and 204 µl cold isopropanol. Solutions were mixed well and incubated at -20 °C for 60 minutes. The solution was then centrifuged for 3 min at 16,000 g before discarding the supernatant, and washing by adding 700 µl of cold 70 % ethanol, inverting once to mix and centrifuging for 1 min at 16,000 g. A final wash stage added 700 µl of cold 100 % ethanol before inverting to mix, centrifuging for 1 min at 16,000 g, and again discarding the supernatant and air drying for 60 minutes at room temperature. Finally, the DNA pellet was resuspended in 100 µl MilliQ water and incubated at 55 °C for 60 minutes. DNA was subsequently stored at -80 °C.

6.2.8. DNA: PCR and agarose gel electrophoresis

PCR Mixes were made up in micro centrifuge tubes as so: 1ul of 200 ng/µl DNA template, 10 µl 2 * GoTaq® hot start master mix (Promega), 7 µl sterile distilled water, 1 µl – Forward primer (Sep-67), 1 µl – Reverse primer (Sep-68). Primers amplify ATG8 - A gene which encodes a ubiquitin-like autophagosomal protein in *Z. tritici* (Kilaru *et al.*, 2017). Thermal cycling conditions were as follows: (1) Initial denaturation – 2 min at 95 °C (2) Denaturation – 30 s at 94 °C. (3) Annealing – 30 s at 60 °C. (4) Extension – 1 min at 72 °C. Repeat steps 2-4 x 34. (5) Final extension – 5 minutes at 72 °C. (6) Hold – 10 °C until electrophoresis.

DNA was fractionated and visualised using agarose gel electrophoresis. Samples were placed inside the wells of a 0.8 % (w/v) agarose gel matrices in 1 x TBE buffer (TBE: 0.09 M Tris-borate, 0.002 M EDTA) containing 0.5 µg/ml ethidium bromide. An Invitrogen® 1 kb plus ladder was used to assess DNA fragment size. Gels were run at 120 V for 90 minutes and photographed using a gel documentation system (Image Master VDS) with a Fujifilm® Imaging system (FTI-500, Pharmacia Biotech).

6.3. Results

6.3.1. Conidiospore survival on water agar and sterilised soil

Conidiospores can grow and sporulate on water agar for at least 28 days (Results 4.3.1). To assess further time-points, colonies were incubated on water agar plates for periods of up to 180 days. At regular intervals during this period, growth was transferred using a sterile spreader, onto fresh YPD plates. *Z. tritici* survival was qualified by colony growth on YPD. Growth was recorded at all time-points tested until the experiment was stopped.

6.3.2. Conidiospore survival in sterilised soil

Conidiospore survival was also assessed in sterilised soil. At regular intervals over a 49-day period, growth was transferred from soil onto fresh YPD plates using a sterile spreader. *Z.*

tritici survival was qualified by colony growth on YPD after 7 days. Growth was recorded at all time-points tested until the experiment was stopped.

6.3.3. Conidiospores can survive in water for at least 49 days

The previous results have shown that conidiospores can live autonomously on both water agar and in soil for extended periods. To facilitate in depth microscopic analysis of spore populations during extended survival without exogenous nutrients, conidiospores were maintained in autoclaved MilliQ water. PI dye was used to assess cell-death within conidiospores. If the cell cytoplasm was flooded with dye (fluorescent cytoplasm), cells were scored as non-viable.

Results in Figure 32 show that there is no significant decrease in the number of viable conidiospores during the first 4 days after suspending spores (Students t-tests. df = 5, Bonferroni corrected p-values = 1.0, 0.85, 0.05, 0.09 respectively) A significant difference can be seen from day 7 onwards. After this point, a rapid loss of viability is witnessed. After 14 days, the rate of decline in viability reduces. A small population of conidiospores remaining viable even after 49 days in water. This experiment confirms that around 10 % of conidiospores remain viable in nutrient-free water cultures for at least 49 days.

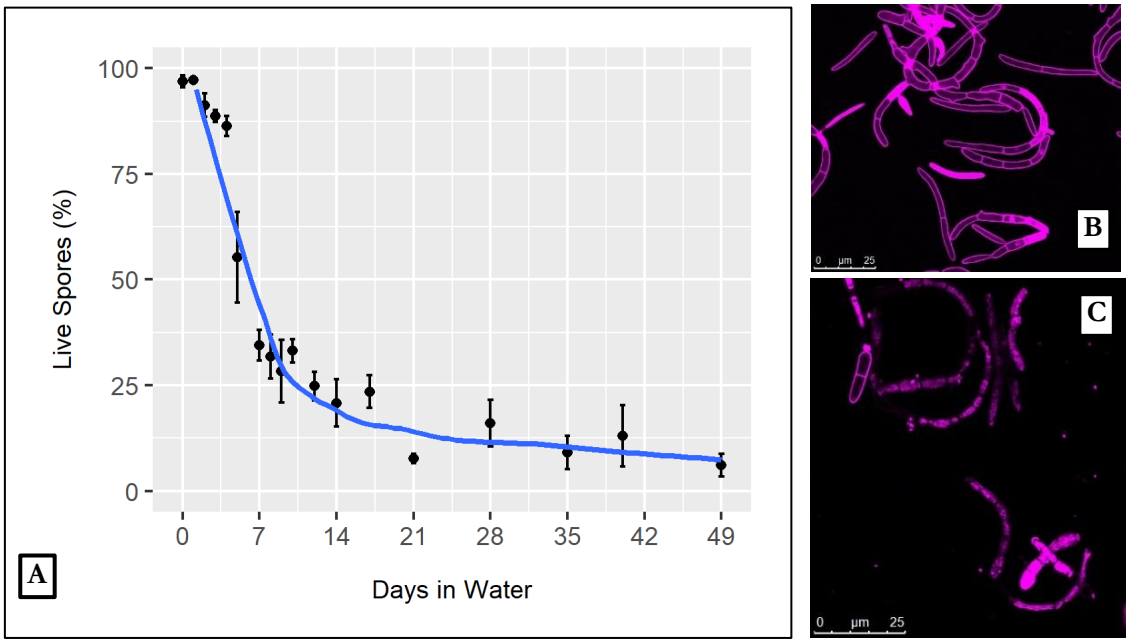

Figure 32: An assessment of *Z. tritici* conidiospore viability over 49 days in water cultures. **A:** Viable conidiospores (spores with at least one live cell) over time. Data are means of counts from 4 independent experiments, each containing at least 4 confocal images. Error bars show SE. Lines of best fit are moving averages based on the LOESS function in R. **B and C:** Representative confocal microscopy images showing conidiospores stained with propidium iodide to visualise cell-death: Day 0 (**B**) and day 49 (**C**). Dead cells have completely stained cytoplasm. Results on day 0 show a high percentage of viable conidiospores (spores with at least one unstained cell) when compared to day 49. Day 49 displays a mostly 'flattened' phenotype suggesting death and lysis of cell contents.

6.3.4. Conidiospores retain culturability after long term water storage

Conidiospores remain viable for at least 49 days in water cultures (Results 6.3.3). It is unknown however, if these viable conidiospores also retain their ability to germinate and form colonies (culturability). To test this, an experiment was conducted in which the same volume, 0.1 µl of initially 1 x 10^7 conidiospore/ml cultures, was plated onto YPD agar (1000 spores on day 0). An equal volume of conidiospore suspensions was plated at multiple time-points over a 49-day period. Culturability was assessed by counting resulting colonies after 7 days on agar.

Results of 4 experimental repeats (Figure 33) show that the culturability of the population (the number of colonies formed) did not significantly fall, relative to day 0, for the first 13 days. In fact, the number of culturable cells in the same volume of suspension increased during this period. After 21-days, culturability consistently declined until day 49. However, colonies were still recorded at day-49 – a colony number of approximately 10 % when compared to day 0.

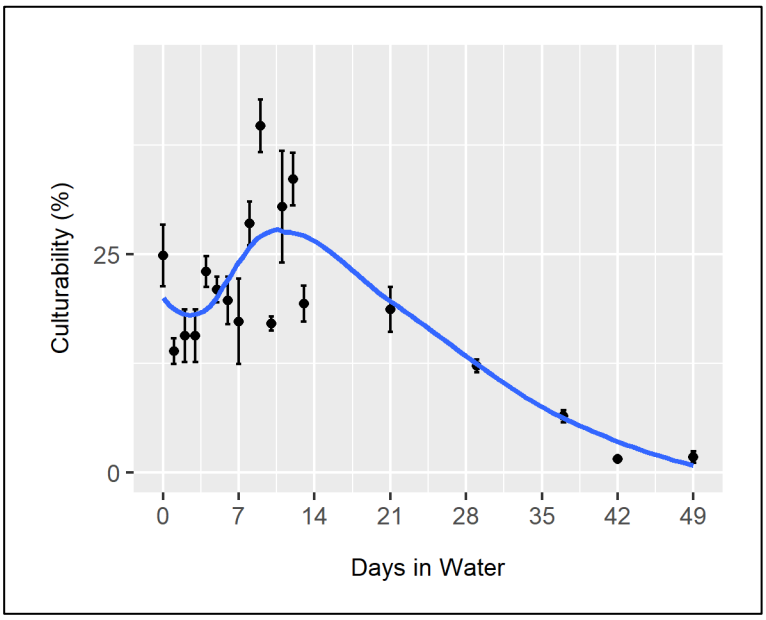

Figure 33: An assessment of suspended *Z. tritici* conidiospore culturability 49 days.
Data are the number of CFUs from plating 100ul of a 10^{-3} dilution of original 1 x 10^{7} conidiospore suspensions over 49-days of storage in water. Data are mean colony counts from 4 independent experiments, each containing at least 3 plates. Error bars show SE. Lines of best fit are moving averages based on the LOESS function in R. Results show that the culturability of the conidiospore suspension is initially low (250 of 1000 spores plated) but then increases over the first 10 days of suspension in water. After day 13 the number of culturable cells reduces steadily for the remaining time. After 49 days, culturable conidiospores remain in the population.

6.3.5. Conidiospore population size initially increases in water cultures

The observed increase in culturability (Results 6.3.4) may be linked to either (i) the recovery of previously non-culturable spores or (ii) an increase in the total spore number in the suspension. To investigate this, conidiospores were counted over time using a haemocytometer and bright field microscopy. Results (Figure 34A), show that the total number of conidiospores increases significantly over the first 7 days in water. This suggests sporulation (MC) is occurring during this period. At later time-points, due to difficulties in conidiospore visualisation, phase contrast microscopy was used. Many conidiospores took on a 'flattened' phenotype (Figure 34B2 and B3). It can be hypothesised that cell-death is occurring in populations, thus leading to cell lysis. This results in cells with a 'flattened' appearance.

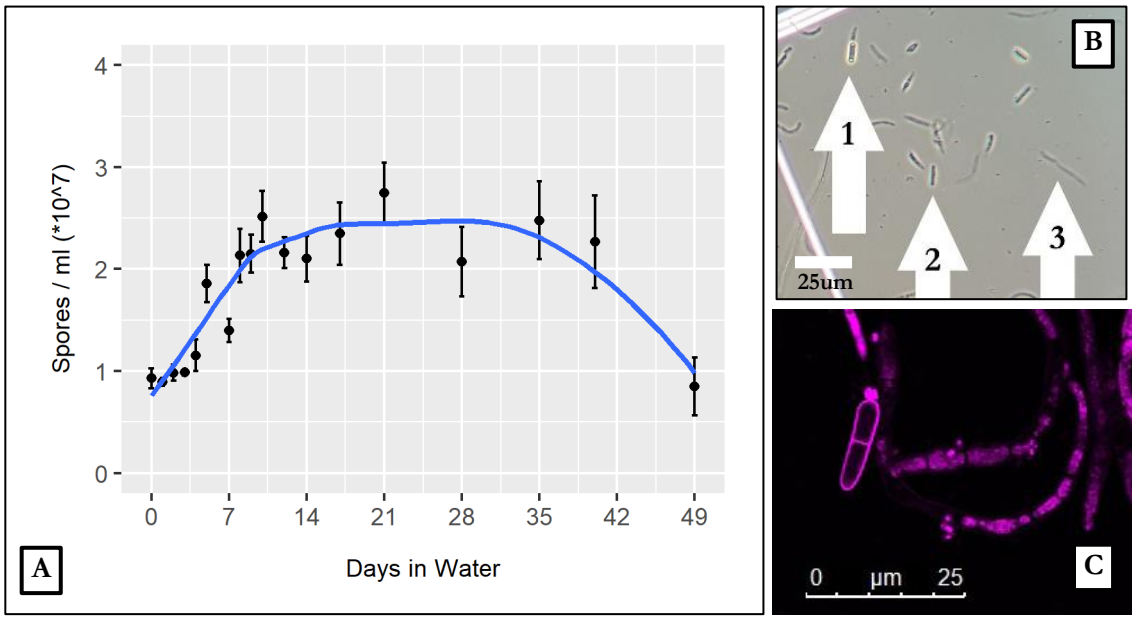

Figure 34: An assessment of suspended *Z. tritici* conidiospore population size over 49 days. **A:** Total conidiospore count of populations over time using a haemocytometer. The number of individual conidiospores in water culture populations increase for the first 8 days. Conidiospore numbers are subsequently maintained until day 49. Data are means of haemocytometer counts from 4 independent experiments. Error bars show SE. Lines of best fit are moving averages based on the LOESS function in R. **B and C**: Morphologically different cells when viewed under phase contrast microscopy and confocal microscopy. Cells take on either a 3-dimensional (intact) shape (**B1**) or varying levels of a flattened appearance (from **B2** to **B3**), suggesting death and probable lysis of cell contents. **C**: Confocal microscopy image of conidiospores after 28 days suspended in water. Spores show clearly flattened phenotype suggesting lysis of cell contents. Spores are stained with propidium iodide.

6.3.6. Microcycle conidiation maintains population size

It has been shown that total conidiospore counts increase over the first 21 days suspended in water (Results 0). *Z. tritici* is known to produce spores via MC. It is likely therefore, that this is occurring within the suspended spore population. To assess this, an experiment was carried out to assess mean conidiospore size (number of cells per spore) over time. At multiple time-points over 49 days, an aliquot of conidiospores from suspended populations was stained using PI and visualised by confocal microscopy.

Results (Figure 35A) show that the number of cells per conidiospore drops significantly over time. Confocal microscopy images reveal two processes which may be responsible for this. Yellow arrows in Figure 35B shows evidence for MC, leading to the production of new, single celled spores, is provided by the presence of budding cells. White arrows from Figure 35B provide evidence for the fragmentation of larger spores into multiple smaller entities.

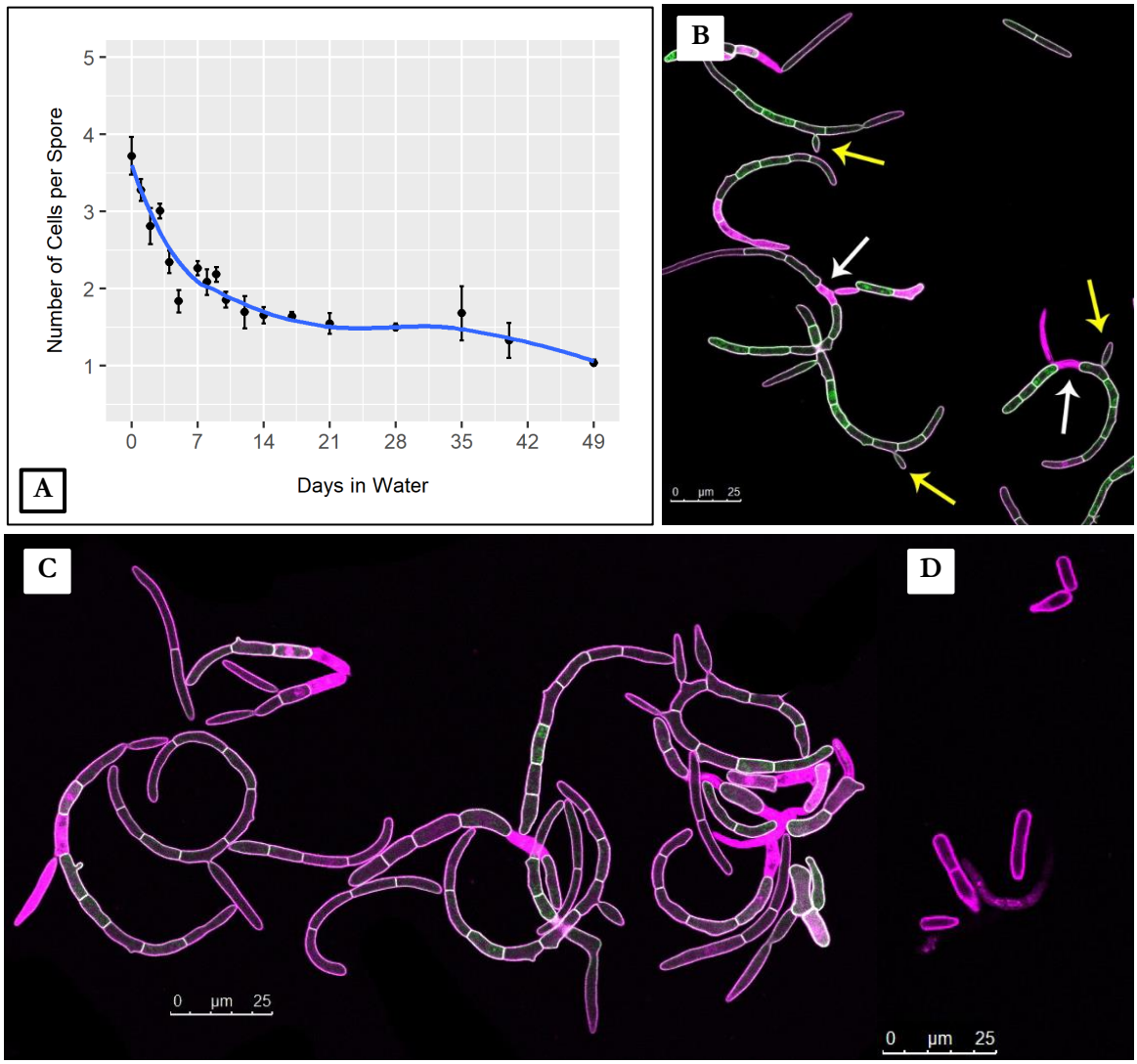

Figure 35: An assessment of conidiospore size (cell number) over 49 d suspended in water. **A:** The mean size of an individual conidiospore significantly reduces over time from a mean of around 4 cells per conidiospore at day 0, to a mean of around 1 cell per conidiospore at day 49. No conidiospores larger than 2 cells can be found at day 49. Data are means of counts from 4 independent experiments, each containing at least 4 confocal images. Error bars show SE. Lines of best fit are moving averages based on the LOESS function in R. **B**: Conidiospores showing cell-death (white arrows) and microcycle conidiation (yellow arrows). Cells are stained using propidium iodide to highlight live (unstained cytoplasm) and dead (stained cytoplasm) cells. Image shows a non-end cell (white horizontal arrows) which has died, potentially leading to splitting. **C**: A representative confocal image of conidiospores taken from a 2-day old water culture at a concentration of 1 x 10^7 conidiospores/ml. Conidiospores are variable in size from single celled to multi-celled spores. **D**: Conidiospores after 28 days in water. A confocal image of live conidiospores taken from a 10^7 conidiospores/ml population of *Z. tritici* that had been stored in water for 28 days. Conidiospores are almost exclusively single celled with some double celled conidiospores remaining.

6.3.7. DNA confirmation of *Z. tritici* from 49-day old water cultures

As a confirmation that water suspensions contained *Z. tritici* and not a contaminant, DNA extractions were carried out (Methods 6.2.7). PCR was carried out using *Z. tritici* specific primers which amplify ATG8: a 0.5 Kb gene which codes a ubiquitin-like autophagosomal protein in *Z. tritici* (Kilaru *et al.*, 2017). Primers were kindly provided by Dr S Kilaru and Prof. G Steinberg. PCR products were analysed using agarose gel electrophoresis, shown in Figure 36. Results confirmed that all 6 biological replicates contained *Z. tritici*, however this test does not confirm a lack of contamination as a PCR needs only a very small amount of DNA to amplify. No contamination was found when plating the samples onto YPD. To assess true starting concentrations and contamination between samples, real time PCR could be used to monitor samples between each duplication cycle

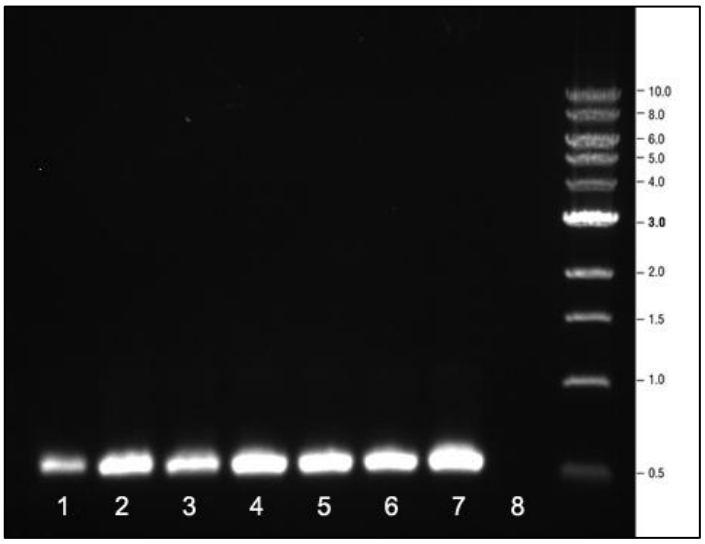

Figure 36: Gel electrophoresis of PCR products from suspended *Z. tritici* populations
Primers were specific to the *Z. tritici* ATG8 gene region (0.5 Kb), a ubiquitin-like autophagosomal protein in *Z. tritici* (Kilaru et al., 2017). Concentrations and purity of DNA were determined post-extraction using the Thermo Scientific NanoDrop™ 1000 Spectrophotometer. DNA was diluted to 200 ng/µl before PCR was undertaken. Extracted DNA was run using agarose gel electrophoresis, using TAE buffer in a 0.8 % agarose gel. Lanes 1-6: 6 Biological reps of 49-day old *Z. tritici* cultures. Lane 7: *Z. tritici IPO323* from freezer stock (+ve control). Lane 8: no DNA added to reaction mixture (-ve control). Lane 9: 1kb Invitrogen ladder.

6.3.8. Neutral lipids as a potential source of nutrition

Z. tritici conidiospores can survive for at least 49 days in water cultures without external sources of nutrition (Results 6.3.3). Previous evidence has suggested that the use of lipid stores by *Z. tritici* may play an important role in infection (discussed in chapter 1). For this reason, lipid content was assessed throughout the 49-day starvation period. To determine whether lipid reserves were initially present and if they decreased over time (indicating their degradation), conidiospore populations were stained BODIPY® 493/503. BODIPY® binds to lipids such as those known to be contained in internal lipid stores. PI was also used as a counterstain to aid visualisation of conidiospores. Lipid content was calculated (percentage of the image filled with green fluorescence / percentage of the image filled fungal tissue) x 100.

Figure 37 shows that the lipid content of fungal populations drops from around 50 % at day 0 (Figure 37B) to around 5 % after 21 days in water cultures. The lipid content of conidiospores remains low up to day 49. Small lipid granules do remain in the population and can still be visualised in live conidiospores at day 49 (Figure 37C, white arrows).

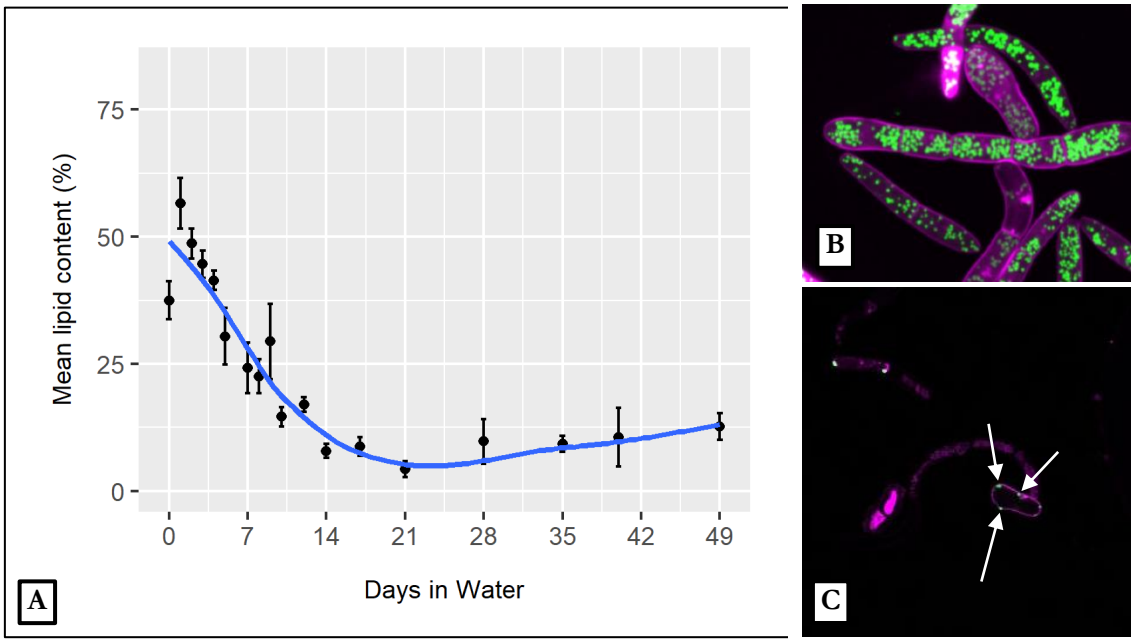

Figure 37: Lipid content (estimated from images) of a conidiospore population over time. **A:** Lipid granules stained using BODIPY® 493/503 and propidium iodide. Lipid content of fungal conidiospore population is calculated as % of image filled with green fluorescence (lipid granules), divided by % of image filled fungal tissue. Data are means of assessments from 4 independent experiments, each containing at least 4 confocal images. Error bars show SE. Lines of best fit are moving averages based on the LOESS function in R. **B**: Day 0 representative image showing extensive lipid granules in conidiospore population. **C**: Day 49 representative image showing significantly depleted lipid granules in conidiospore population. Lipid granules can still be visualised (white arrows).

6.3.9. Conidiospores retain virulence for extended periods

Conidiospores of *Z. tritici* are capable of survival for periods of at least 49 days when stored either in sterile nutrient-free water (Results 6.3.3), water agar, or in damp soil (Results 6.3.1). Survival in water is accompanied by a transient rise and subsequent loss in spore numbers (Results 0) but with a culturable population of spores surviving up to 49 days (Results 6.3.4). Over this period, conidiospores become smaller, in terms of the number of constituent cells (Results 6.3.6). The internal lipid content of spores also diminishes significantly (estimated from confocal images, Results 6.3.8). The role of a *Z. tritici* asexual spore is for the spread of disease between hosts. The continued viability of these spores, therefore, implies that pathogenicity is also retained over these extended periods.

To test this, both 'fresh' (suspended from a 7-day old YPD plate) and 'stored' (taken from 49-day old water suspensions) conidiospore populations were inoculated onto the leaves of 14-day old Consort wheat plants using the paintbrush method (Methods 2.4). These plants were then treated under standard growth cabinet conditions (Methods 2.3) and assessed for disease by counting pycnidia on leaves after 28 days. For these trials, 'stored' conidiospores were tested against 'fresh' in two ways: (i) by inoculating using equivalent 'total' conidiospores counted using a haemocytometer, and (ii) adjusting conidiospore counts according to the culturability of the population. These two methods are designed to contrast the infectivity of the ~ 10 % of conidiospores which retain their culturability and viability after 49-days in H_2O (as shown in Figure 38B and Figure 38C).

These results (Figure 38), show that when comparing equal numbers of conidiospores (estimated on a haemocytometer), 'stored' conidiospore populations (those taken from 49-day old water cultures) have significantly reduced virulence when compared to 'fresh' inoculum. This is not surprising as by day 49, 90% of conidiospores within the stored water suspension populations are no longer culturable (tested by plating onto nutrient rich agar and counting colonies: Figure 38B). If 'stored' conidiospore inoculum is adjusted to match the culturability of 'fresh' conidiospores (concentrated by 10-fold), no significant differences are observed in disease after 28 days on plants (Figure 38, Bar 3 compared to Bar 5, or Bar 2 compared to Bar 4).

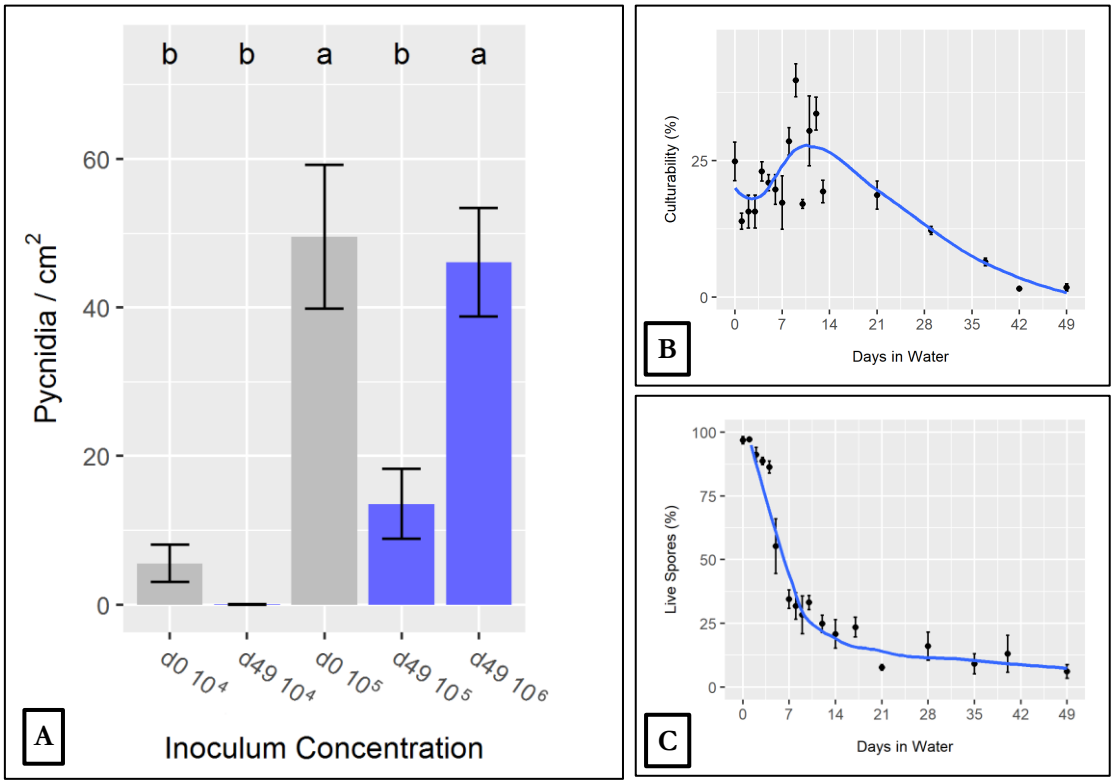

Figure 38: An assessment of *Z. tritici* conidiospore virulence after 49 days in water cultures. **A:** Disease trials comparing the virulence of 'fresh' conidiospores (suspended in water immediately before application), against 'stored' conidiospores (suspended in water for 49 days). Graph shows that when comparing equal numbers of conidiospores (estimated with a haemocytometer), 'stored' conidiospore populations (Bar 4), produce significantly reduced numbers of pycnidia compared to fresh populations (Bar 3) (One-way ANOVA, p-value = 6.9e-43, Letters indicate significant differences between treatment groups as determined by post-hoc Tukey test; p-value < 0.05) – this can be accounted for by showing that at day 49, relative to fresh inoculum, only around 10 % of 'stored' conidiospores remain culturable **(B)** and viable **(C)**. If inoculum concentrations are adjusted for equal conidiospore culturability, no significant differences in disease are observed. Data are means of CFU counts from 4 independent experiments. Error bars show SE. Lines of best fit are moving averages based on the LOESS function in R.

6.3.10. Conidiospore survival under dry conditions

Conidiospores can survive immersed in water and on water agar for more than 49 days. Neither environment is moisture limited. However, in agricultural systems, free living *Z. tritici* conidiospores might need to survive protracted periods of drought if they are to overwinter between growing seasons. To assess moisture-limited survival, conidiospores from water suspensions were spread thinly in empty petri dishes. Plates were dried in a class II cabinet before being covered with lids, sealed with parafilm, and stored for 56 days. Populations

from 28-day old plates were recovered for further analysis by adding 2 ml of water to plates and transferring suspended conidiospores into Eppendorfs. These populations were then compared to day zero populations for cell-death and lipid content using confocal microscopy imaging with PI and BODIPY®, respectively.

Results show that conidiospores from moisture-limited conditions remained culturable up to 56 days, when the experiment was stopped. Analysis of conidiospores at 28 days show that spores from moisture limited conditions suffered significant losses in viability over this period (Figure 39A, One-way ANOVA, p-value = 3.4e-35. Letters indicate significant differences between treatment groups as determined by post-hoc Tukey test; p-value < 0.05). However, the viability of spores from dry plates was not significantly different than that for conidiospores from suspended water cultures. The lipid content of conidiospores in dry conditions was also depleted compared to day 0. However, when compared to conidiospores stored in water cultures, lipid depletion was reduced, indicating that lipid stores are conserved in moisture limited conditions.

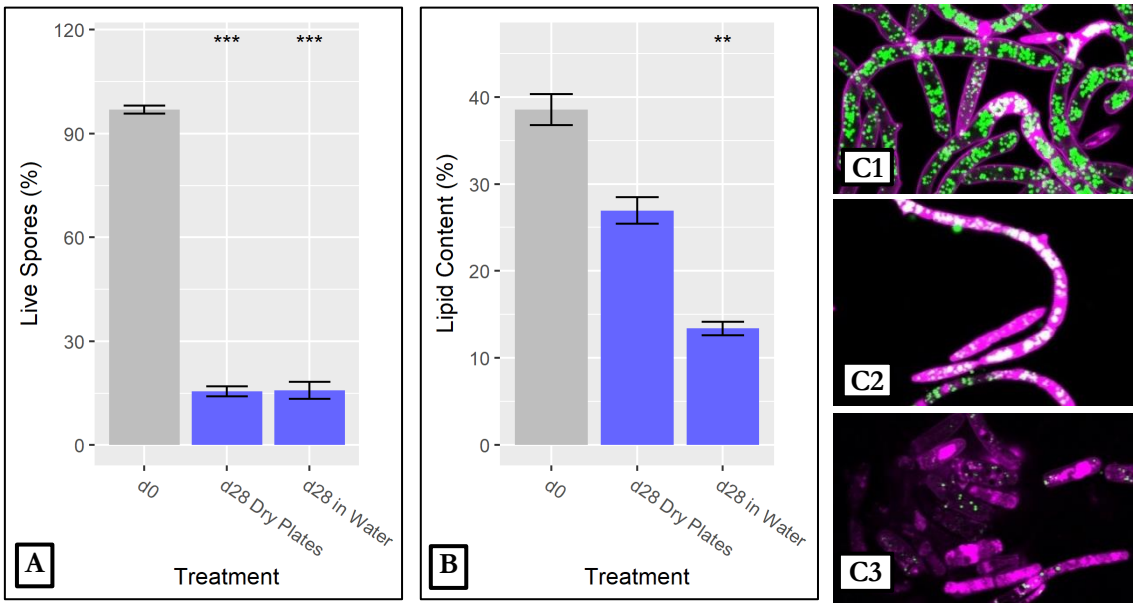

Figure 39: *Z. tritici* conidiospores after 28 days in moisture limited conditions.
A: Spores stained with propidium iodide. Graph shows percentage of conidiospores stained using propidium iodide with at least 1 live (unstained cytoplasm) cell. Data are means of counts from 4 independent experiments, each containing at least 4 confocal images. Error bars show SE. Results show a highly significant drop in % of live conidiospores in both dry (Students *t*-test, t = 28.215, df = 3, p-value = 9.774e-05) and water cultures (Students *t*-test, t = 12.082, df = 5, p-value = 6.859e-05) after 28 days. However, there is no significant difference between survival rates in these conditions, suggesting that water is not a necessary resource for *ex planta* conidiospore survival. B: Lipid granules stained using BODIPY® 493/503 passive lipid stain and Propidium Iodide. Lipid content of fungal conidiospore population is calculated as % of image filled with green fluorescence (lipid granules), divided by % of image filled fungal tissue. Data are means of assessments from 4 independent experiments, each containing at least 4 confocal images. Error bars show SE. Lipid content of conidiospores from moisture limited conditions is significantly higher than those from water tubes (Students *t*-test, t = -4.8558, df = 5, p-value = 0.00465), suggesting a decrease in lipid catabolism in desiccated conditions. **C1:** Day 0 conidiospores; **C2:** Day 28 conidiospores from dry plates showing reduced lipid content (white and green); **C3:** Day 28 conidiospores from water culture showing low lipid content (green fluorescence. Lipids in dead cells appear white, due to overlay of pink and green fluorescent signals).

6.4. Discussion

Due to the polycyclic nature of *Z. tritici*, asexual reproduction occurs throughout a growing season. The leaves on which these asexual spores are produced, subsequently become areas for sexual reproduction (Suffert and Sache, 2011, Steinberg, 2015). The associated recombination leads to a shuffling of gene combinations (Zhan *et al.*, 2003). This has consequences for the disease-causing potential of populations. Particularly, due to the gain

and loss of genes coding for proteins involved in virulence (Hartmann *et al.*, 2017, Selin *et al.*, 2016). Recombination therefore, leads to the maintenance of highly variable populations. As such, a wide range of strains can be found at all scales from within a single lesion to between continents, with particularly high genetic variance found within a field (Linde *et al.*, 2002).

The high-planting-density monocultures associated with wheat farming mean that traits associated with high virulence are rapidly selected for (McDonald and Stukenbrock, 2016, Suffert *et al.*, 2015). This would suggest that highly virulent strains would quickly outcompete low- or non-virulent strains due to their ability to successfully infect and reproduce. This, however, is not necessarily found to be the case, as strains with both high and low virulence are often isolated from wheat fields (Banke and McDonald, 2005, McDonald *et al.*, 1999, Linde *et al.*, 2002, Zhan *et al.*, 2003, Wittenberg *et al.*, 2009, McDonald *et al.*, 2015).

A potential reason for such varied strain presence is that, post-sexual reproduction, strains survive within plant tissue. This could occur both on live leaves during a growing season, and on post-harvest stubble between seasons. Indeed, there is evidence for this, including the finding that pycnidiospores, when held within the leaf in the cirrus-containing-pycnidium, can persist for up to 132 days when humidity outside the leaf is below 35 % (Gough and Lee, 1985).

Additionally, extended *ex planta* survival may also be possible. Asexual spores are produced in high numbers, reported to be between 5 and 10 x 10^3 pycnidiospores for every pycnidium produced (Eyal, 1971). Over a growing season this form of sporulation alone is estimated to account for up to 10^{11} conidiospores per hectare (Fones and Gurr, 2015). The spread of these spores, via rain-splash, leads to the colonisation of new host plants. Many spores, however, will not splash onto a host, thus becoming dispersed environmentally such as in the soil. Reports of *ex planta* survival are mostly limited to laboratory storage situations such as cold, sterilised soil storage and freezer stocks (Shearer *et al.*, 1974). Such conditions are not designed to assess environmental survival.

6.4.1. Long term survival of conidiospores

Firstly, this chapter showed that conidiospores can survive in moisture-rich environments such as in soil (> 49 days) and on water agar (> 180 days). Drought tolerance was also confirmed by showing that conidiospores can also survive in moisture limited conditions (dry Petri dishes stored at < 50 % rH).

To better assess the "behaviour" of conidiospores during periods of extended survival, conidiospore suspensions were chosen as a medium for study. Conidiospore suspensions have many benefits over other culture methods. They can remain sterile for extended periods, they are completely nutrient free unlike either soil or agar-based media, and they are also free from other particles such as soil which may hinder observations. During a 7-week period, cell-death, spore death, spore size, culturability, lipid content and virulence were all assessed.

Results show that suspended conidiospores can survive the full 7-week period with no external source of nutrition (Results 6.3.3). However, cell-death, assessed using propidium iodide and confocal microscopy, does occur, although initially quite slowly – around 10 % of cells are dead within the first 5 days, before falling much more rapidly. After 14 days, the number of conidiospores with no live cells rises to around 75 %.

Interestingly, the ability of spores to form colonies or rich agar (culturability) does not follow the same pattern. Conidiospore culturability drops rapidly, with only 15 % of spores producing a colony on YPD agar within 24 hours of suspension in water. The number of colonies does again rise, more than doubling between day 1 and day 10 (Results 6.3.4). An explanation for this increase is that the actual population size is increasing. Supporting evidence for this comes from haemocytometer estimations. The population roughly doubles in numbers between day 0 and day 14, therefore matching the increase in culturability (Results 6.3.3). There is also visual evidence of MC in suspended populations throughout the time course (Figure 34). In populations cultured on rich agar, MC leads to a wide variety of conidiospore sizes, ranging from single celled to large, sometimes branched networks. At day 0, spore population have an average size of 4 cells per spore. In suspended populations, the mean number of cells per conidiospore drops to around 1 cell by day 49 (Results 6.3.6). This

indicates that conidiospores continue to replicate by conidiation even in these low nutrient submerged conditions. This result mimics the result seen on water agar (Results 4.3.1), where populations assessed after 14 days were made up of almost exclusively single-celled conidiospores.

The relevance of this population growth in the field is unknown. However, the finding that a population can increase even when on/in environments which offer no external nutrition is striking. It can be hypothesised that during periods of epiphytic growth or autonomous survival, *Z. tritici* populations have the capacity to not only maintain, but increase in numbers. The recently reported persistence of epiphytic growth by *Z. tritici* (Fones *et al.*, 2017b) shows that the fungus is likely to remain epiphytic for extended periods before entering a host leaf. The ability of *Z. tritici* to reproduce by MC during this period may not just be an artefact of survival, but an evolved behaviour to build a population.

6.4.2. Lipids as an internal source of nutrition

The number of viable cells in suspended populations drops over time (Results 6.3.4). After 49-days the number of viable conidiospores is around 10 % compared with populations plated immediately after suspension in water. As this fall in viability remains steady, this suggests that dormancy does not occur, but rather, that survival depends on endogenous energy reserves.

Lipid granules are reported to be synthesised under high nutrient (particularly carbon rich) conditions (Aguilar *et al.*, 2017). Indeed, in *Z. tritici*, growing on rich agar media (YPD) leads almost all available space within a cell to be filled with lipid granules (Figure 37).

Evidence for the importance of lipids to *Z. tritici* during early (therefore likely epiphytic) infection has been shown using RNAseq analysis, where the upregulation of several genes involved in fatty acid generation from lipid granules, as well as their subsequent transport and β-oxidation, have been reported (Rudd *et al.*, 2015, Palma-Guerrero *et al.*, 2015, M'Barek *et al.*, 2015, Keon *et al.*, 2007). Lipids are stored in fungal cells as, for example, triacylglycerols and steryl esters. They are present as distinct droplets, or granules. They have dual roles, the first of which is as a store for the synthesis of membrane phospholipids during hyphal

extension. The second role is to be a source of internal energy during periods of starvation (Thiam *et al.*, 2013, Steinberg, 2007, Singh *et al.*, 2009).

Active growth by hyphal extension was not observed in suspended cultures. In these nutrient-free conditions, however, lipid granules may be used as an energy source for the cellular homeostasis associated with survival and the synthesis of the membranes of budding conidiospores. To assess this, conidiospores from suspended populations were stained with BODIPY® 493/503 – a dye which binds to passive lipids such as those found in lipid granules.

Results show that lipid granule depletion occurs over time (Figure 37), falling steadily over the first 21 days of suspension in water. Interestingly, 21 days is also co-incident with the time-point where the number of living/viable conidiospores in suspended populations also reaches its minimum level. Indeed, correlation analysis of these two factors over the time course gives a positive correlation of 0.79 (t = 5.23, df = 16, p-value = 8.114e-05, Figure 40). This indicates a relationship between the presence of lipids in cells and their ability to survive in nutrient-free conditions.

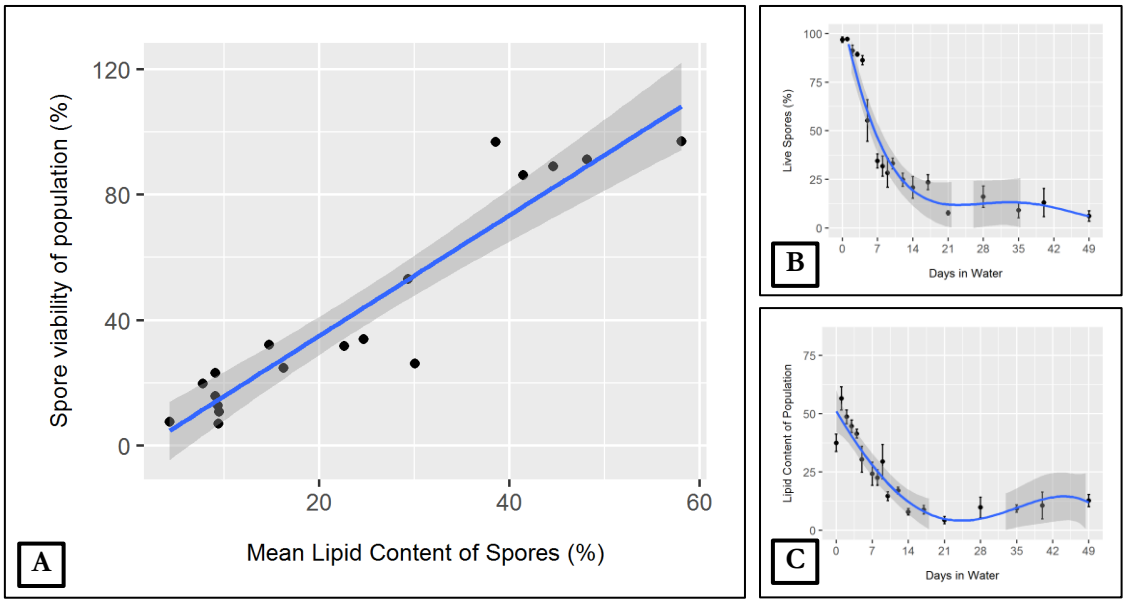

Figure 40: A correlation between the mean lipid content of *Z. tritici* conidiospores, and the mean viability of the conidiospore population.
A: A positive correlation of 0.94 (Pearson's product-moment correlation, t = 5.23, df = 16, p-value = 8.114e-05) suggests that the presence of lipid granules within spores may be linked with spore viability. Experimental data taken from a 49-day time course using data from Results 6.3.3 (**B**) and 6.3.4 (**C**).

Within each time-point post-21 days, viable conidiospores can be seen containing lipid granules. However, these granules become smaller and harder to visualise as time goes on. What differentiates the subpopulation of conidiospores that remain viable after 21 days is unknown. These conidiospores may simply start with more lipids, or they may use them more slowly. Spores may also be reliant on other sources of internal nutrition such as arabitol, mannitol and trehalose - metabolites detected at very high concentrations in fungal spores (Rudd *et al.*, 2015).

It should be mentioned that results attained from suspended, nutrient free cultures may not be environmentally relevant. Studies of population genetics in bacteria have shown that in pure cultures there is a tendency for specific mutations to arise. These mutants, terms 'growth advantage in stationary phase' (GASP) mutants have distinct mutations have been found in genes involved in amino acid uptake and catabolism. Uptake of environmental nutrients allows entry into an extended stationary phase (Zinser and Kolter, 1999, Navarro Llorens *et al.*, 2010). If genetic mutations within the *Z. tritici* conidiospore populations are occurring which are beneficial to a surviving sub-population, we might expect to witness a subsequent population increase. In this thesis chapter, however, viable conidiospore numbers drop consistently as lipids deplete – this implies that viability is closely linked with nutrient depletion. Whole genome sequencing would be needed to elucidate this.

6.4.3. Pathogenicity of long-lived conidiospores

Experiments have confirmed that water suspended conidiospores are able to: (i) remain viable, (ii) remain culturable, and (iii) continue to sporulate via MC over a 49-day period. An interesting question is whether these small, mostly single-celled and lipid-depleted conidiospores retain their virulence.

An experiment was carried out comparing the virulence of conidiospore populations taken directly from nutrient rich (YPD) agar plates, to those from 49-day old water cultures. Firstly, results showed that 49-day suspended conidiospore populations (when using equal total conidiospore counts) are less virulent than freshly made conidiospore suspensions. This is an expected result due to 90 % of conidiospores no longer being viable. However, if equal numbers of culturable conidiospores are used, population virulence is not significantly

different. This result was found at both 10^4 and 10^5 conidiospores/ml, ruling out the possibility that all apoplastic space for fungal invaders was already filled (disease saturation). This second result confirms that individual single celled, lipid-depleted conidiospores remain equally infectious, even when no external source of nutrition is available. Starvation therefore, at least in a surviving sub-population of conidiospores, does not reduce fitness.

At day 49 few detectable lipid stores remain in conidiospores (Results 6.3.8). This implies that external sources of nutrition may play a subsequent role in infection. No biotrophic feeding structures have been reported in *Z. tritici*, however there is evidence for the secretion of enzymes involved in the liberation of host nutrients early in infection (Palma-Guerrero *et al.*, 2015, Yang *et al.*, 2015, Goodwin *et al.*, 2011). These enzymes, which include cutinases, lipases, cellulases, hemicellulases and xylanases, may in fact be sufficient to acquire all the nutrients needed for infection on a host plant. Lipid stores may be used simply for survival – more work is needed to assess relative differences in lipid granule depletion between spores and hyphae. It is also possible that internal and external sources may be used in parallel. More work, such as comparative transcriptomics, is needed to assess internal lipid levels in fungal individuals in various points of the infection process to find out the relative importance of internal and external nutrition for survival and growth.

6.4.4. Conidiospores: A source of primary and secondary inoculum

Sexual conidiospores (ascospores), spread by wind from infected stubble, are thought to be the source of primary inoculum in a newly-sown wheat field (Suffert *et al.*, 2011). However, there is conflicting evidence. One example is a recent study showing that the number of ascospores trapped in fields not does not correlate with early onset disease (Morais *et al.*, 2016). This either indicates most ascospores are not pathogenic, or that other sources of inoculum are present in fields during early growth.

The results in this chapter suggest that long-lived (49 days) asexual conidiospores have equal capacity to cause disease as conidiospores taken straight from rich agar plates. This indicates that conidiospores have enough internal resources to remain infectious between seasons – a period often less than 2 months (Sylvester-Bradley *et al.*, 2008). Subsequently, only rain-splash is needed to spread spores onto plants and re-start the infection cycle. Indeed, disease is

known to occur as soon as seedlings emerge (Suffert and Sache, 2011). If the results presented here are relevant to a field scenario then the primary inoculum may not only be ascospores, but conidiospores surviving autonomously between seasons. The ability of soil-borne spores to splash and infect a host during rain will be assessed in the next chapter (7: Control of *Zymoseptoria tritici*).

6.4.5. Behaviour of surviving population

Results in earlier chapters showed that sporulation via MC occurs extensively on water agar (Results 4.3.1). The extent of sporulation increases significantly on agar supplemented with carbon and/or nitrogen (Results 4.3.2 & 4.3.3). Disease was also shown to be slightly increased if foliar fertiliser applications were applied post-infection. This may suggest that epiphytic *Z. tritici* is able to use nutrient sources to promote population growth (Results 4.3.4). By combining these findings with the results in this chapter it can be hypothesised that populations may not simply survive, but increase in numbers. With multiple annual fertilisation events, a pool of conidiospores could be available indefinitely.

Fertilisation has been previously linked to increased disease. One study concluded increased leaf size and density post fertilisation leads to more spores landing on leaves (Lovell *et al.*, 1997). Another concluded that the post-uptake N concentration in leaves was a major factor (Leitch and Jenkins, 1995). A third study offered no mechanism but confirmed a link between disease and the quantity and timing of N applications (Simon *et al.*, 2003). The results presented in this chapter show that population sizes may be significantly affected by N availability. These results offer an alternative mechanism for fertilisation increasing disease.

The prevalence of surviving conidiospores also provides a pool of strains constantly available for sexual reproduction events. Classically, sexual events in *Z. tritici* are thought to occur in stubble post-harvest. However, there are many examples which show this not to be exclusively true. For example, Zhan *et al.* (1998) applied in-field inoculations with ten strains of *Z. tritici* and found that, by the end of the growing season, 24 % of flag leaf infections arose from sexual progeny. If sexual recombination indeed occurs throughout the season, then the presence of even non-virulent strains poses a threat due to the shuffling of virulence genes.

Additionally, genes such as those involved in fungicide resistance, would likely be able to sweep to fixation in such a highly competitive environment. It may therefore be the case that current methods of control are not adequate to deal with the ever-present conidiospore populations. This will be explored in chapter 7: Control of *Zymoseptoria tritici*.

7. Control of *Zymoseptoria tritici*

7.1. Introduction

7.1.1. Control of *Zymoseptoria tritici*

Disease control methods are tailored to the life-cycle of the pathogen which they are targeting. Accurate information about the pathogen in question is therefore very important if control is to be successful. For *Zymoseptoria tritici,* the extended period of concealed/latent/asymptomatic growth makes tracking fungal development difficult. As such, fungicide sprays are recommended to be used before the onset of visual disease symptoms (AHDB, 2016b). Sprays are instead targeted to key stages of crop development, rather than key stages of fungal development. The 4 stages chosen for applications can be seen in Figure 41.

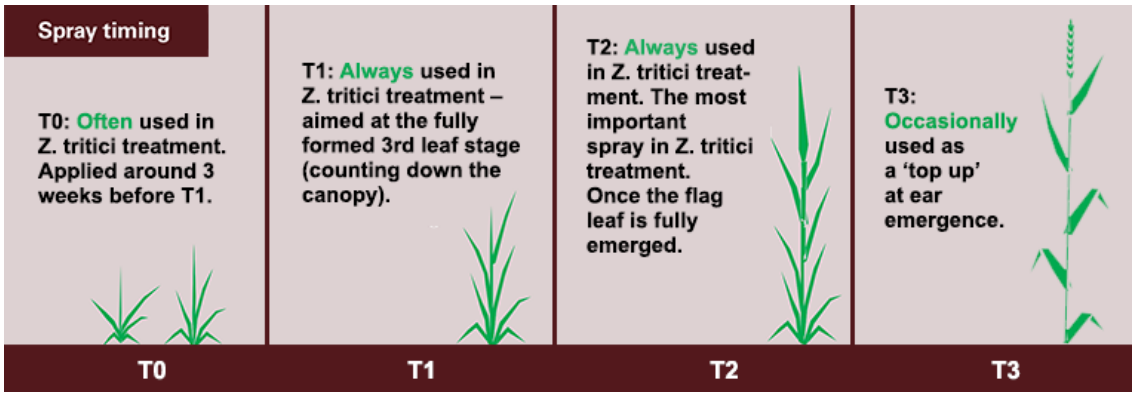

Figure 41: Fungicide spray timings for *Z. tritici* control recomended by HGCA (2010).

The number of fungicide sprays applied to wheat fields in a growing season is optimised using two major aims. The first is to minimise disease. However, spraying to excess can lead to potential harmful ecological effects, including the increased likelihood of fungicide resistant isolates emerging. Therefore, the second aim is to minimise these risks. Recent modelling has suggested that *Z. tritici* can be controlled with a single, maximum dose fungicide application, but only if the timing is "perfect" (van den Berg *et al.*, 2016). Away

from simulated models however, the number of sprays used depends on both the characteristics of the crop, and the prevalence of the fungus during a given season. As shown in Figure 41, the recommended number for *Z. tritici* is 3 sprays a season. This is much higher than the modelled ideal, but is necessary because once a fungal population has established, eradication is seemingly impossible. The prevalence of *Z. tritici* has led to this single pathogen accounting for around 70 % of the European fungicide market (Torriani *et al.*, 2015). Clearly, any reduction in fungicide use would be a great boon for wheat agriculture.

7.1.2. Fungicide chemistries and resistance

The fungicides most commonly used to control *Z. tritici* have a single (rather than multi) target site mode of action. These have a high risk of pathogens developing resistance (van den Berg *et al.*, 2016). The major single site chemistries used include azoles, succinate dehydrogenase inhibitors (SDHIs) and quinone outside inhibitors (QoIs), all of which have resistance events documented.

Resistance to QoI fungicides such as the strobilurins (which disrupt metabolism by inhibiting electron transfer in mitochondria) is reported to be common amongst strains of *Z. tritici* in Europe, North America, and Africa (Heick *et al.*, 2017, Fraaije *et al.*, 2005, Torriani *et al.*, 2009, Estep *et al.*, 2014, Allioui *et al.*, 2016). Resistance has been documented to have evolved independently in several lineages of *Z. tritici* (Estep *et al.*, 2015). The wind-based spread of *Z. tritici* ascospores means that resistance can spread easily. Indeed, resistant strains have been isolated from fields which have received no strobilurin applications (Stewart *et al.*, 2016b). Resistance to azoles is reported to have been more gradual, with some, such as epoxiconazole and prothioconazole, remaining relatively effective (AHDB, 2016a). Sterol Demethylation Inhibitors (SDHIs or DMIs), a relatively recent addition to the *Z. tritici* arsenal introduced around 2005 (Fraaije *et al.*, 2012), remain successful, but resistance has been documented in a laboratory mutagenesis study as well as in the field (Dooley *et al.*, 2016a).

The chemistries discussed here are traditionally used individually. However, more recently, combinations are now recommended (Bounds *et al.*, 2016). Choosing which chemistries work well together is an important step to avoid issues with uptake, efficacy and continued resistance (Dooley *et al.*, 2016b, Hayes *et al.*, 2015).

7.1.3. Exploring reasons for rapid fungicide resistance

To plan new fungicide programmes, it is important to explore and understand the reasons for resistance emerging. In *Z. tritici*, major factors have been described that are expected to speed up the evolution of fungicide resistance…

7.1.3.1. Complex genome architecture

The genome of *Z. tritici* is relatively large for a plant pathogen. It consists of 39.7 Million base-pairs, coding for around 11,000 genes. *Z. tritici* has 13 core chromosomes (CCs). These are found in all known *Z. tritici* strains. CCs have a high amount of proportion sequences (18.6%), and many gene-rich areas interspersed with transposable elements (Schotanus et al., 2015, Plissonneau et al., 2016, Hartmann et al., 2017).

Z. tritici also has accessory chromosomes (ACs). These are thought to have evolved from ancient core chromosomes. The reference strain *Z. tritici* IPO323 has eight ACs, the highest number reported so far for filamentous fungi. Also termed 'dispensable chromosomes', ACs are frequently lost from the genome. However, no strains have been documented with no ACs present. The spread of ACs between strains, in terms of their presence and sizes, seems to be well correlated with the level of geographic separation. Compared to CCs, ACs are small (ranging in size from 0.39 Mb to 0.77 Mb). They carry half as many genes per Mb of DNA as CCs, but, due to evolving under less selective constraints (low rates of recombination), ACs have proportionally more unique genes than CCs. (Wittenberg *et al.*, 2009, McDonald *et al.*, 2015).

7.1.3.2. Genome plasticity allows for high genomic diversity

Z. tritici populations undergo regular sexual reproduction (Zhan *et al.*, 2003). Thus recombination rates within a population are exceptionally high on CCs, particular on the smaller chromosomes (Möller and Stukenbrock, 2017). Recombination leads to offspring with varied genetic traits, and as such leads to highly mixed populations. This constant shuffling of alleles between strains is particularly important for mixing genes with a role in virulence such as effectors. Effectors are proteins involved in avoiding or overcoming host

plant defences. However, due to the co-evolution of the host-pathogen relationship, some effectors are recognised by specific receptors in plants (R-proteins). Recognition leads to avirulence. As such, the presence or absence of a single effector gene can be the difference between a pathogenic (compatible) and non-pathogenic (non-compatible) *Z. tritici* strain.

This gene-for-gene relationship is termed 'qualitative resistance' and has been confirmed to exist in the in wheat-*Z. tritici* pathosystem only with the *Stb6* gene (Zhong *et al.*, 2017). In fact, *Z. tritici* virulence seems to work in a 'quantitative' manner with 167 quantitative trait loci (QTLs) involved in causing disease (Brown *et al.*, 2015, Stewart *et al.*, 2016a). Recombination, particularly the loss of previously recognised effector genes, is therefore very important for the development of mixed populations and the maintenance of virulence within them (Hartmann *et al.*, 2017, Selin *et al.*, 2016).

Accessory chromosomes also display very low rates of recombination and as such are thought contribute to high rates of adaptive evolution (Croll *et al.*, 2013, Hartmann *et al.*, 2017). ACs often exist in the genome without a homologue. ACs are lost and gained readily (even *de-novo*) during sexual and asexual reproduction (Wittenberg *et al.*, 2009, Croll *et al.*, 2013, Möller and Stukenbrock, 2017). When recombination occurs between ACs, nonsynonymous substitution can introduce further variation (Stukenbrock *et al.*, 2011). Like the ACs the CCs also have areas of low combination termed 'accessory compartments'. These are rich with putative effector genes, thought to have evolved through extensive chromosome rearrangements. Accessory compartments are not found to be transcribed but are instead linked to increased mutation and higher rates of evolution. The rapidly evolving ACs, combined with the less rapidly evolving core chromosomes, allows *Z. tritici* to be defined as having a 'two-speed genome' (Plissonneau *et al.*, 2016, Rudd *et al.*, 2015, Dong *et al.*, 2015).

Both ACs and CCs have unusually high numbers of transposable elements (TEs). Transposable elements are small repeated regions of the genome. They are flanked with (often inverted) regions of repetitive DNA and as such are thought to move throughout the genome without RNA intermediates. These have been found particularly surrounding gene dense regions (Plissonneau *et al.*, 2016). The movement of TEs leads to a constant shuffling of genes throughout the genome.

7.1.3.3. Large effective population size

Wheat is grown in vast areas of monoculture, with tight planting densities. This means that transmission of strains is relatively unhindered and thus outbreaks can rapidly spread. Increased virulence is subsequently selected for by conditions where multiple strains, and even other pathogens, are co-infecting the same host plants/leaves and thus increasing competition (McDonald and Stukenbrock, 2016).

The rapid spread of strains through a field means local gene variation increases easily (Linde *et al.*, 2002). The high variability of strains, coupled with sexual reproduction, leads to a large population with the potential to contribute to the next generation – this is termed the effective population size. A high effective population means more recombination, more mutation, and less loss of alleles by random genetic drift. The outcome is the maintenance of varied populations with mixed levels of virulence (Stukenbrock *et al.*, 2011, Plissonneau *et al.*, 2016). Indeed, confirming this theoretical suggestion, genetically and phenotypically diverse strains, showing a range of virulence, are indeed often isolated from wheat fields (Banke and McDonald, 2005, McDonald *et al.*, 1999, Linde *et al.*, 2002, Zhan *et al.*, 2003, Wittenberg *et al.*, 2009, McDonald *et al.*, 2015).

Due to these virulence differences, some strains will be more successful than others *in planta*. However, the potential for pycnidiospore production is very high. The number of asexual spores produced by just a single strain was recently estimated to be around $10^{10} – 10^{11}$ spores per hectare over a growing season (Fones and Gurr, 2015). However, this estimate was based on how many spores could fit within a pyncidium at any one time, 300, rather than the amount produced by that pycnidium over its life span. This number has been estimated to be between 5 and 10×10^3 (Eyal, 1971) thus increasing the potential levels of inoculum considerably. Additionally, this thesis has shown that sporulation by microcycle conidiation occurs readily (10^3 spores per spore), even when no external nutrition is available (Results 4.3.1). The presence of agricultural fertilisers further increases the potential for sporulation by at least 10-fold more (Results 4.3.2). Mathematically, this implies a maximum spore pool over a whole season of more than 10^{18}. This is unrealistically high, but demonstrates the multiple ways that a population can grow.

In terms of population maintenance, wheat is grown all year round in temperate zones, thus host tissue on which to replicate is constantly available (Fones *et al.*, 2017a). Additionally, in periods where no tissue is available, this thesis (chapter 6) has shown that asexual spores have an extensive capacity for survival *ex planta*. These results suggest that infectious propagules can remain available throughout the growing season.

7.1.4. Current and novel methods of control

Foliar fungicide applications are currently the main method of control for *Z. tritici*. However, these treatments never completely eradicate the fungus. To combat disease, multiple sprays are applied during a growing season (HGCA, 2010). The reliance on chemical forms of control provides a strong selective pressure for fungicide resistant strains to emerge. Indeed, there have been strains isolated which display resistance to all widely-used commercial chemistries (Heick *et al.*, 2017, Fraaije *et al.*, 2005, Torriani *et al.*, 2009, Estep *et al.*, 2015, Allioui *et al.*, 2016, Fraaije *et al.*, 2012, Dooley *et al.*, 2016a).

To better control the fungus, and to avoid issues with resistance, *Z. tritici* is now often treated with multiple fungicides, either at different times, or together in a 'stacked' fashion (Dooley *et al.*, 2016b). However, the cost of this control is reportedly now responsible for up to 70 % of fungicide spending in the EU (Torriani *et al.*, 2015, Fones and Gurr, 2015) – clearly, any reduction in the number of sprays would be both financially and environmentally beneficial (Torriani *et al.*, 2015, Fones and Gurr, 2015).

New single and multi-site fungicides, combinatorial chemistries, breeding programs, biological controls, improved stubble management, increased crop rotation, early disease warning systems, and the spraying of crops with plant defence priming elicitors (Arraiano and Brown, 2016, Lynch *et al.*, 2016, Bektas and Eulgem, 2014, Linde *et al.*, 2002), are all disease control strategies which may help to alleviate future losses. However, results in this chapter suggest that future control successes may not be reliant on new products or technologies.

As discussed in the thesis introduction (chapter 1), it is commonly believed that new disease enters a field mainly via wind-blown ascospores. Therefore, disease can only begin when new

wheat leaf tissue becomes available. This may not, in-fact, be accurate. Results in chapter 5.4 showed that asexual conidiospores can survive, either freely in soil or water, or under dry conditions, for periods long enough to span between wheat growing seasons. Therefore, a source of primary inoculum, the asexual conidiospores, may be ubiquitous within fields, making the 'primary' contribution from ascospores non-essential. This may be of particular note when the next season's crop is planted within a relatively short period, as with winter wheat. If this is the case, then early rainfall will be sufficient to re-start the disease cycle.

Supporting evidence of the importance of asexual conidiospores as the primary inoculum has recently been demonstrated by showing that the presence of ascospores, found using air traps, does not correlate with the onset of disease epidemics (Morais *et al.*, 2016). Further evidence comes from a study on crop rotation where neighbouring fields were sown with wheat with different yearly cycles. Results showed that a year's rotation was enough to significantly reduce disease suggesting that in-field (i.e. not wind-blown) inoculum plays a role in primary infections (Pedersen, 1992).

If this hypothesis is accurate, we would expect that during rain-fall events, asexual conidiospores persisting within the soil would splash onto seedlings causing infections early in the growing season. This would suggest that the current foliar spray regimes may simply be timed too late to have a significant preventative effect on controlling the fungal population, as it only takes one conidiospore to splash onto a leaf to begin the in-field disease cycle again (Fones *et al.*, 2015). Confirmation of the importance of early control, has been shown by using triazole seed treatments. If used, disease reduction persists many months into the growing season, well after the fungicide has lost its efficacy (Parker and Lovell, 2001, Sutton, 1985).

An alternative solution to the control of in-field primary inoculum may be to alter the T0 spray (currently targeted to leaf 3 counting down the canopy) to an earlier time-point. Here it may be possible to treat the soil instead of, or as well as the leaves, therefore controlling conidiospores before spread (via rain-splash) onto seedlings. If successful, these sprays would alleviate the requirement for new products or technologies, and may lead to a reduction in the number of sprays or seed treatments needed over a growing season.

7.1.5. Phosphite

Phosphite (PO_3^{2-} or Phi), is already used within small scale agriculture, primarily as a fertiliser. It is widely reported to have antimicrobial properties affecting conidiospore germination, mycelial growth and lesion development (Massoud *et al.*, 2012, Dalio *et al.*, 2014, Hofgaard *et al.*, 2010b, Amiri and Bompeix, 2011, Hofgaard *et al.*, 2010a, Eshraghi *et al.*, 2011, Lobato *et al.*, 2011). Most evidence comes from research with oomycetes, particularly in *Phytophthora* spp. (Burra *et al.*, 2014, Dalio *et al.*, 2014, Eshraghi *et al.*, 2014a, Smillie *et al.*, 1989, Rebollar-Alviter *et al.*, 2007, Massoud *et al.*, 2012, Abbasi and Lazarovits, 2006). However, although there is no direct evidence of *Z. tritici* control with Phi, antifungal properties are widely reported in crop pathogens such as *Metarhizium majus, Fusarium culmorum* and *Magnaporthe grisea* (Hofgaard *et al.*, 2010b, Pagani *et al.*, 2014, Davis *et al.*, 1994, Lobato *et al.*, 2011, Amiri and Bompeix, 2011, Mayton *et al.*, 2008).

Moreover, Phi has additional features which make it interesting as a potential antifungal product. It is fast-acting and long-lasting (Rebollar-Alviter *et al.*, 2007, Burra *et al.*, 2014), systemic (Hardy *et al.*, 2001, Lovatt and Mikkelsen, 2006, Danova *et al.*, 2008), it accumulates within plant tissues thereby potentially acting as a protectant (Smillie *et al.*, 1989, Burra *et al.*, 2014). It also acts an elicitor of features of plant defence such as cell wall strengthening (callose deposition), hydrogen peroxide production (H_2O_2), pectin deposition, defensive hormone biosynthesis and increases in other defensive molecules such as chitinases, phytoalexins and proteinase inhibitors (Eshraghi *et al.*, 2011, Olivieri *et al.*, 2012, Liu *et al.*, 2016, Lobato *et al.*, 2011, Burra *et al.*, 2014).

7.1.6. Glyphosate

Another alternative fungicidal control is the globally used broad-spectrum herbicide glyphosate. Originally developed by Monsanto™ under the trade name Roundup®, this chemical is predominately applied before the sowing of crops to remove weeds which have appeared between growing seasons – pre-planting. It is also used after sowing but before the new plants emerge – post-planting pre-emergence (Glyphosate.eu, 2013).

The mechanism for glyphosate activity is the inhibition of an enzyme involved in the synthesis of 3 aromatic amino acids: phenylalanine, tyrosine, and tryptophan. This enzyme, 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), is primarily found in plants and thus its use is mostly as foliar applications on target weeds. However, as well as being found in plants, EPSPS is found in many microorganisms, thus treatment with glyphosate has also been found to be fungicidal, with organisms tested including *Cryptococcus neoformans, Pyrenophora tritici-repentis* and *Puccinia psidii* (Tanney and Hutchison, 2010, Nosanchuk *et al.*, 2001, Sharma *et al.*, 1989, Feng *et al.*, 2005, Tuffi Santos *et al.*, 2011). Although there is no evidence that glyphosate is toxic to *Z. tritici* specifically, an effect can be seen in many fungi in pure culture where high concentrations are used (> 1000 mg/g) (Franz *et al.*, 1997).

7.1.7. Carbendazim

Carbendazim, a benzimidazole fungicide, targets the fungal cytoskeleton by inhibition of ß-tubulin polymerisation (FRAC, 2017). In sensitive fungi, this causes abnormalities in areas such as germ tube elongation, conidiospore germination, mycelial growth and cellular multiplication (FRAC, 2017). Benzimidazoles were widely used, either singly or in combination, to treat *Z. tritici* since the 1970s. However, the widespread evolution of resistance means this group is now less widely used. For example, a 1985 paper showed that 9 of 21 *Z. tritici* isolates from English crops were resistant to benomyl, a very similar benzimidazole fungicide (Griffin and Fisher, 1985). Benzimidazoles have also now been linked with disrupting human and animal hormones and thus will be banned from both sale and use by August 2017 (HSE).

7.1.8. Future control methods

Building on the data that *Z. tritici* conidiospores can survive for extended periods in water and soil, this chapter first assesses if conidiospores can infect plants from soil by rain-splash alone. *Z. tritici* isolates will be tested for sensitivity to carbendazim, which will subsequently be used as a positive experimental control to compare sensitive isolate treatment against both glyphosate and Phi. These chemicals will be assessed firstly using agar to gauge active concentrations, and subsequently, in soil and foliar treatments.

7.2. Methods

7.2.1. Assessment of antifungal chemicals (agar-based)

For all agar-based experiments, spread plates were made by plating ~ 200 *Z. tritici* conidiospores. Colony number and colony size were assessed after 8 days incubation at 20 °C in the light.

7.2.1.1. Minimal media agar (MM)

MM agar was made as per Methods 2.2.

7.2.1.2. Glyphosate agar

For agar-based glyphosate tests, Roundup Optima+ (170 g/l glyphosate acid) was diluted to a concentration of 10 g/l. The resulting solution was adjusted to pH 6 and autoclaved at 121 °C for 15 minutes. To assess fungal growth and development, glyphosate was then added to minimal media agar and wheat leaf agar at final concentrations of 1000, 500, 250, 100, 50, 10, 5, 2 and 1 mg/l. In each experiment, control plates of water agar and YPD were also made for comparison.

7.2.1.3. Phosphite agar

Phosphorus acid (Phi) (Sigma, 13598-36-2), was diluted in sterile distilled water (H_2PHO_3 or Phi), adjusted to pH 6, autoclaved at 121 °C for 15 minutes, and stored at an initial concentration of 100 mM. For agar experiments this solution was added to minimal media agar as a serial dilution to 5 final concentrations: 10 μM, 100 μM, 1 mM, 10 mM and 100 mM. In each experiment, control plates of water agar and YPD was also plated.

7.2.1.4. Carbendazim agar

An initial carbendazim stock solution of 100 μM was made up with sterile distilled water before adjusting to pH 6 and autoclaving at 121 °C for 15 minutes. The resulting solution

was added to minimal media agar to final concentrations of 10 nM, 100 nM, 1 μM and 10 μM. In each experiment, control plates of water agar and YPD was also plated.

7.2.2. Disease trials: Soil-based methods

7.2.2.1. Soil inoculations for rain-splash experiments

Five ml of a 10^6 /ml conidiospore suspension was pipetted into each cell of a 24-cell plant tray, each containing two 14-day old wheat plants. Conidiospores were left to penetrate soil completely for 10 minutes. To mimic rainfall, trays were watered from a height of 2 metres at a rate of 4 litres of sterile distilled water per 24-cell tray from a Haws No.14 medium rose head watering can. Disease was assessed as pycnidia per cm^2 of leaf after 28 days incubation. The cotyledon and two true leaves were assessed. Three controls were conducted: (i) soil with no conidiospores added, (ii) plants grown in conidiospore inoculated soil without the rain-splash event, and (iii) leaves inoculated by the standard paint brush method.

7.2.2.2. Soil applications for fungicide trials

For soil-based antifungal trials, soil was first inoculated (via pipette) with a 10^6 conidiospore solution at a rate of 5 ml per cell of a 24-cell plant tray containing 2 plants per cell. After 24 hours, the soil within each cell was sprayed with 1 ml of antifungal product using an airbrush with fine mist spray (Iwata Revolution SAR airbrush). Based on agar fungitoxicity trials, the concentrations used for carbendazim, glyphosate and Phi were 100 μM, 6 mM (1 g/litre) and 100 mM respectively. Controls were water only sprays. Soil trays were subsequently stored at standard growth cabinet conditions (Methods 2.2) for 48 hours, before two seeds were sown into each cell – soil moisture was maintained throughout. After 14 days growth, trays were watered from a height of 2 metres to mimic rainfall, at a rate of 4 litres of sterile distilled water per 24-cell tray, from a Haws No.14 medium rose head watering can. After 28 days, disease was assessed as the number of pycnidia per cm^2 of leaf.

7.2.3. Disease trials: Foliar inoculations and fungicide applications

Plants were grown for 14 days under standard growth cabinet conditions (Methods 2.2) and inoculated by paintbrush at a conidiospore concentration of 1 x 10^5 conidiospores/ml (Methods 2.4). At 7, 14 or 21 dpi, fungicide sprays were applied on both the abaxial and adaxial sides of inoculated leaves using an Iwata Revolution SAR airbrush, at a rate of 1 ml per cell of a 24-cell plant tray (2 plants per cell, avoiding run off). Concentrations used for carbendazim and Phi were 100 μM and 100 mM respectively. Controls consisted of either no fungicide application, or a mock treatment with water only. Disease was quantified as pycnidia/cm^2 of leaf after 28 days.

7.2.4. Vernalisation trials

Consort winter wheat plants were grown from seed in Sanyo growth cabinets set to a short day (8:16 light:dark) cycle at a constant temperature of 8 °C. After 3, 6 or 9 weeks, wheat plants were moved to the University of Exeter greenhouse and grown on a long day (16:8 light:dark) cycle at 20 °C for the remainder of their life cycle. Flowering (GS61) was recorded when at least 5 flowers were present on the head. Heads were harvested and weighed when tissue was completely dry.

7.3. Results

7.3.1. Conidiospores can infect plants via rain-splash from soil

Following the finding that *Z. tritici* conidiospores stored in nutrient-free water for 49 days suffer no significant loss in virulence (chapter 6), a further experiment was conducted to test whether long-lived conidiospores have the potential to infect leaves from the soil via rain-splash alone. This experiment was designed to mimic "in field" conditions. For this experiment, 5 ml of 1 x 10^6 /ml *Z. tritici* conidiospore solution were inoculated into soil by pipette, immediately before seeds were sown. Plants were left to grow in the inoculated soil for 14 days, before rain was mimicked using a Haws No.14 medium rose head watering can. Treatments were also set up where (i) disease was assessed without rain-splash (to assess if conidiospores could infect cotyledons), and (ii) where conidiospores were added only on the day where rain was mimicked, as opposed to 14 days before. As a positive control, the previously used method of painting the leaves with a conidiospore solution in 0.01 % Silwet L77 (v/v) was also carried out.

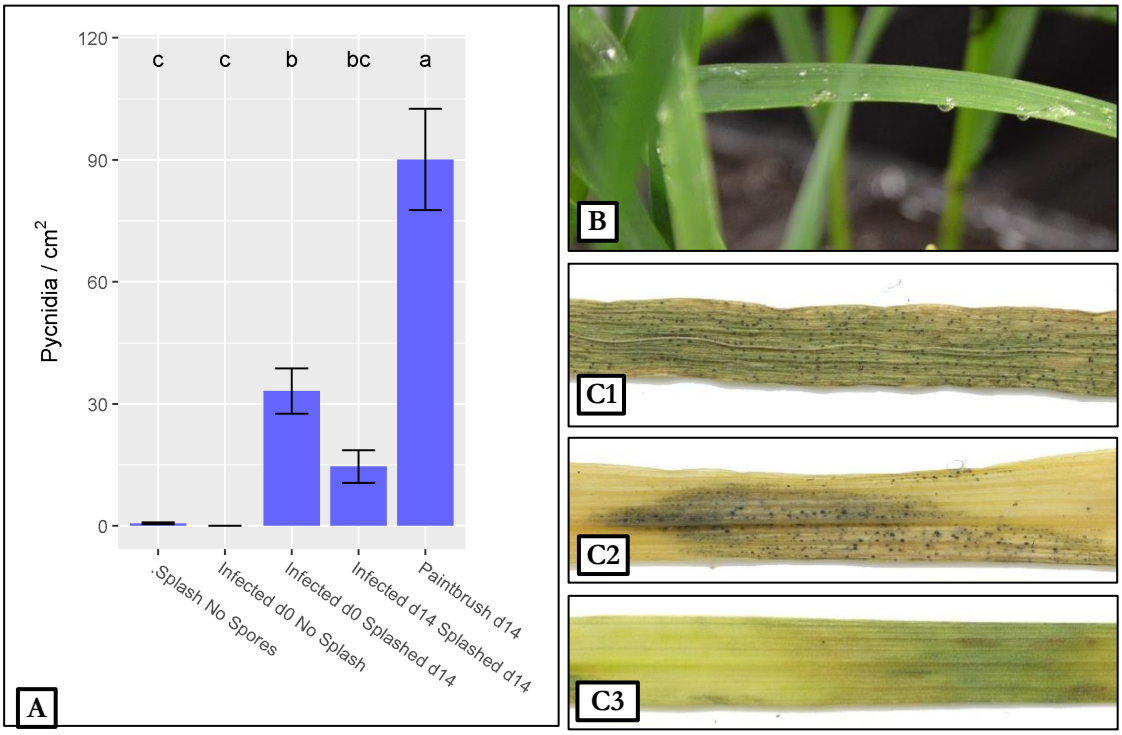

Figure 42: Assessment of *Z. tritici* disease (pycnidia counts) on wheat leaves grown in spore-inoculated soil. A rain-splash event was mimicked at 14 days.
Leaves were assessed for pycnidia after 28 days **A:** Result shows that both 14-day old soil-borne conidiospores and freshly added soil-borne conidiospores can infect plants via rain-splash alone (Bars 3 and 4 respectively), (One-way ANOVA, p-value = 3.7e-35. Letters indicate significant differences between treatment groups as determined by post-hoc Tukey test; p-value < 0.05). Results also show that growing plants through infected soil (without rain-splash) is insufficient to cause pycnidiation (Bar 2), yet leaves appear chlorotic (**C3**). **B**: Example leaf after rain-splash event confirming spread of droplets. **C:** Examples of leaves from 3 conditions: **C1**: Paintbrush inoculation method; **C2**: Rain-splash of fresh conidiospores; and **C3**: Plants sown into infected soil without a subsequent rain-splash event.

Firstly, as seen in Figure 42B, the method of mimicking rain-splash was successful; water droplets can clearly be seen on the leaves directly after treatment. Secondly, it can be seen, by running a post-hoc Tukey test between all treatments, that there are significant differences in levels of disease between plants infected by rain-splash, compared to plants inoculated by paintbrush (Figure 42A: One-way ANOVA, p-value = 3.7e-35, Letters indicate significant differences between treatment groups as determined by post-hoc Tukey test; p-value < 0.05). An additional result that this experiment reveals, is that seeds being sown directly into soil containing conidiospores (Figure 42A: Bar 2), is not enough to result in infection without an additional rain-splash event (Figure 42C3).

7.3.2. Assessment of antifungal chemicals (agar)

Before assessing whether soil-based or foliar based treatments are effective against *Z. tritici*, agar experiments were conducted to assess potential antifungal chemistries. In all experiments ~ 200 conidiospores were plated per 9 cm Petri dish and colony number and area was assessed after 8 days in the light at 20 °C.

7.3.2.1. Carbendazim agar

To assess the efficacy of antifungal compounds in controlling *Z. tritici*, carbendazim was chosen as positive control. An experiment was conducted to assess the concentration necessary for controlling colony formation and growth of *Z. tritici* on minimal medium agar. Results in Figure 43, show that carbendazim completely repressed fungal colony formation at 10 µM. Reductions in growth, measured by colony area after 8 days, can also be seen from as little as 10 nM (IC50 = 1.5 µM). This indicates that carbendazim affects the rate of growth, as opposed to conidiospore germination.

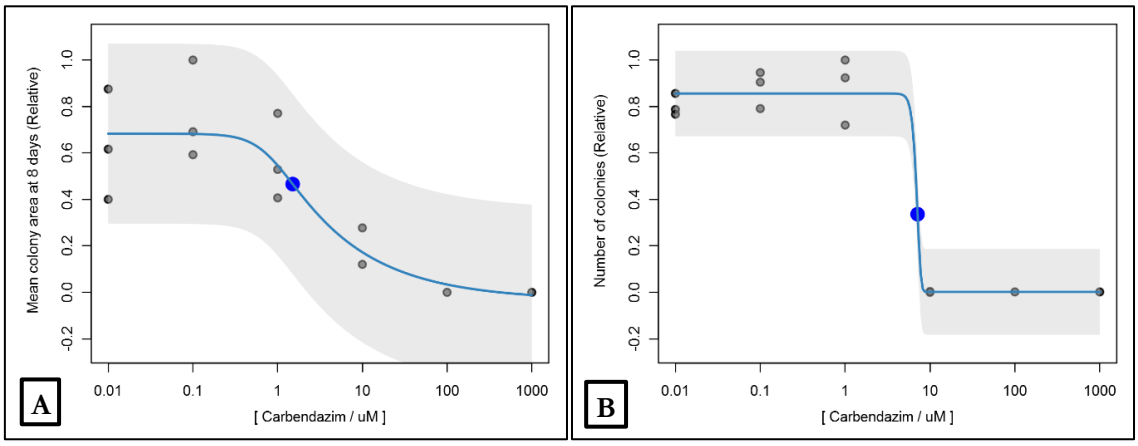

Figure 43: The effect of carbendazim on colony development on minimal medium agar. ~200 conidiospores were plated onto minimal medium agar and assessed after 8-days growth in the light at 20 °C. Reduction in the number of colonies formed (B) is not seen until complete cessation of growth at 100 µM. Colony growth rate is affected from around 1 µM (A). Using the colony area data, IC50 (50 % inhibitory concentration) was found to be 1.5 µM (n = 3). Fitted values estimated from NPLR analysis in R. Linear regression lines are indicated in colour, grey polygons indicate 95% confidence intervals of regression lines, and coloured points indicate actual results.

7.3.2.2. Glyphosate agar

It has been previously reported that glyphosate, Roundup™, has negative effects on the growth and development of fungi as well as plants. This is due to the target enzyme, EPSPS, being present in many fungi and bacteria. To test if glyphosate displays fungicidal effects against *Z. tritici*, agar experiments were carried out on minimal medium agar supplemented with varying concentrations glyphosate acid. Results (Figure 44) show that the rate of colony growth is significantly affected at around 10 mg/ml. However, the number of colonies formed on the agar, each resulting from an initial conidiospore germination event, is not affected until glyphosate concentrations reach 500 mg/ml. These results show that germination/yeast like growth can still proceed under relatively high concentrations of glyphosate (< 500 mg/ml), but rate of colony growth is affected at much lower concentrations. No conidiospore germination was recorded at 1000 mg/ml (0.59 mM).

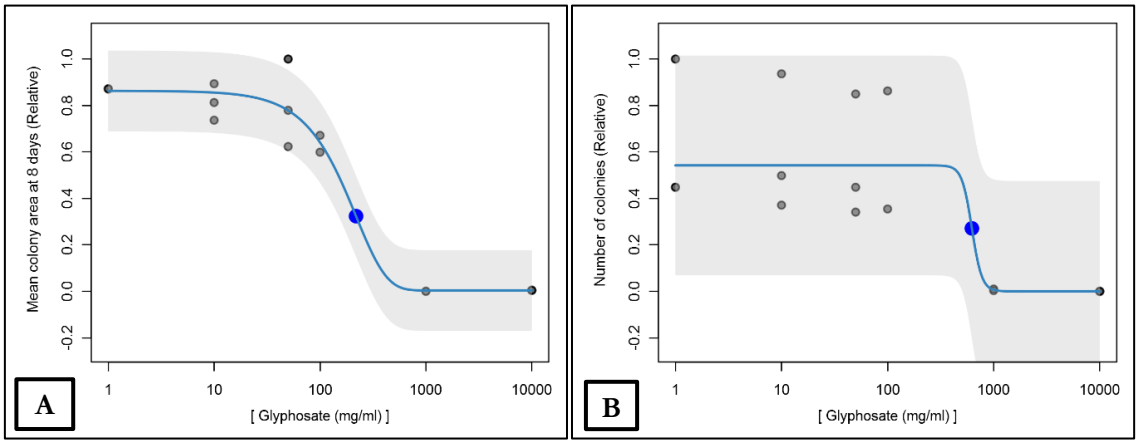

Figure 44: The effect of glyphosate colony development on minimal medium agar.
~200 conidiospores were plated onto minimal medium agar and assessed after 8-days growth in the light at 20 °C. Reduction in the number of colonies formed (**B**) is not seen until complete cessation of growth at around 1000 mg/ml. Colony growth rate is affected from around 50 mg/ml (**A**). Using the colony size data, IC50 was found to be 219 mg / ml (n = 3). Fitted values estimated from NPLR analysis in R. Linear regression lines are indicated in colour, grey polygons indicate 95 % confidence intervals of regression lines, and coloured points indicate actual results.

7.3.2.3. Phosphite (Phi) agar

Phi has been reported to have direct antifungal effects on many fungi, thus an experiment was conducted to examine the possibility that *Z. tritici* is also affected. To do this, varying concentrations of Phi were added to a minimal medium agar before plating ~ 1000 conidiospores and incubating for 8 days. Results shown in Figure 45A indicate that until complete cessation of growth, at around 10 mM Phi, there are no negative effects on colony formation/germination. However, the colonies growth rate, measured by total colony area at 8 days (Figure 45B), is significantly reduced at concentrations of just 1 µM, showing significant antifungal effects at micromolar concentrations.

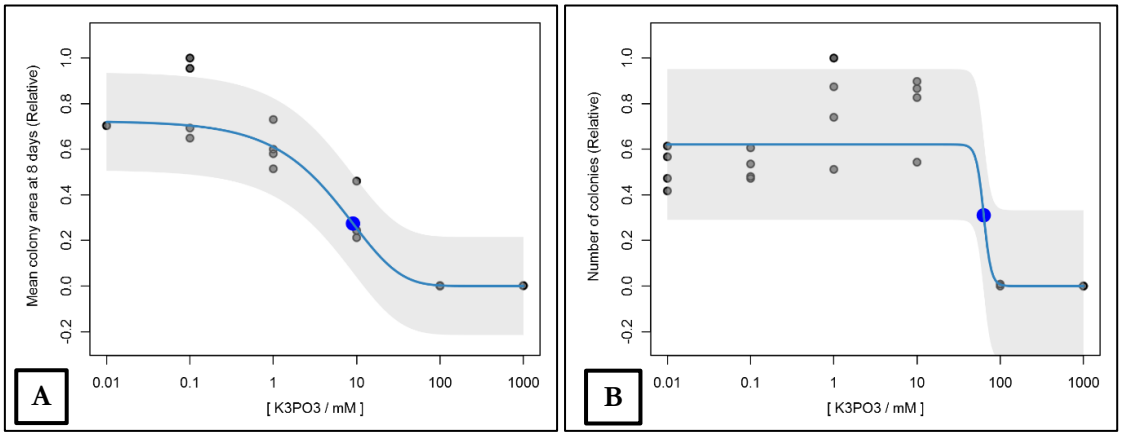

Figure 45: The effect of Phi on colony development on minimal medium agar.
~200 conidiospores were plated onto minimal medium agar and assessed after 8-days growth in the light at 20 °C. Reduction in the number of colonies formed (**B**) is not seen until complete cessation of growth at around 100 mM. Colony growth rate is affected from around 1 mM (**A**). Using the colony size data, IC50 was found to be 8.9 mM (n=4). Fitted values estimated from NPLR analysis in R. Linear regression lines are indicated in colour, grey polygons indicate 95 % confidence intervals of regression lines, and coloured points indicate actual results.

7.3.3. Controlling *Z. tritici* using soil applications

All three chemistries tested (carbendazim, glyphosate and Phi) are detrimental to *Z. tritici* colony growth on agar. To test if these chemistries could also control soil-borne spores, an experiment was carried out where sprays were applied to conidiospores suspended in soil. Fungicidal sprays were applied 24 hours after conidiospore inoculation. The concentrations used were based on the minimum concentration at which *Z. tritici* growth was completely restricted on agar. Seeds were then planted 48 hours later. Seedlings were left to grow for 14 days before rain-splash was mimicked. After 28 days, leaves were assessed for disease by counting pycnidia. Results (Figure 46) showed that the treatment of soil borne spores with carbendazim was not sufficient to significantly reduce disease after a subsequent rain-splash event. However, both glyphosate and Phi application were found to reduce the incidence of disease (One-way ANOVA, p-value = 0.00067, Letters indicate significant differences between treatment groups as determined by post-hoc Tukey test; p-value < 0.05, n = 3).

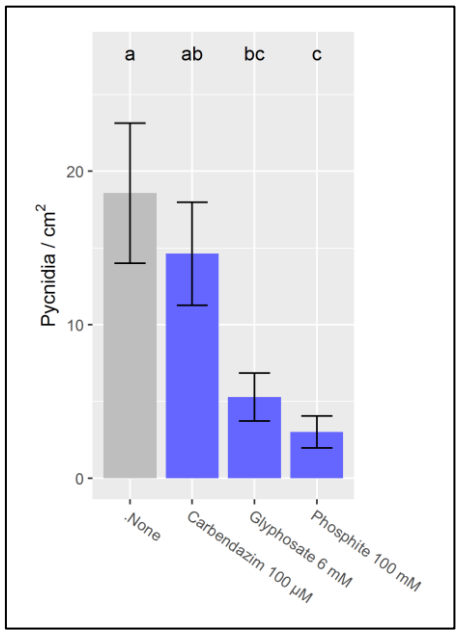

Figure 46: Pycnidia counts 28 days after a single rain-splash event onto infected soil.
Soil had been treated with fungicidal chemicals 24 hours after inoculation with *Z. tritici* conidiospores. Seedlings were planted 48 hours after fungicde treatment and allowed to grow for 14 days before a rain-splash event was mimicked. Fungicide concentrations used were chosen on the minimum concentration at which *Z. tritici* growth was completely restricted on minimal medium agar. Effect of fungicides was significant (One-way ANOVA, p-value = 0.00067, Letters indicate significant differences between treatment groups as determined by post-hoc Tukey test; p-value < 0.05, n = 3). Carbendazim treated soil shows no sinigificant reduction in disease when compared to untreated.

7.3.4. Controlling *Z. tritici* using foliar sprays

Based upon the finding that Phi and carbendazim are directly antifungal to *Z. tritici* on agar, it was decided that the efficacy of these chemistries as foliar control should be investigated. Plants were grown for 14 days before being inoculated, by paintbrush, with a 10^5 /ml conidiospore suspension in 0.01 % Silwet L77 (v/v). Plants were grown under standard growth cabinet conditions. At 7, 14 and 21 dpi, plants were treated with foliar sprays of either water, carbendazim or Phi.

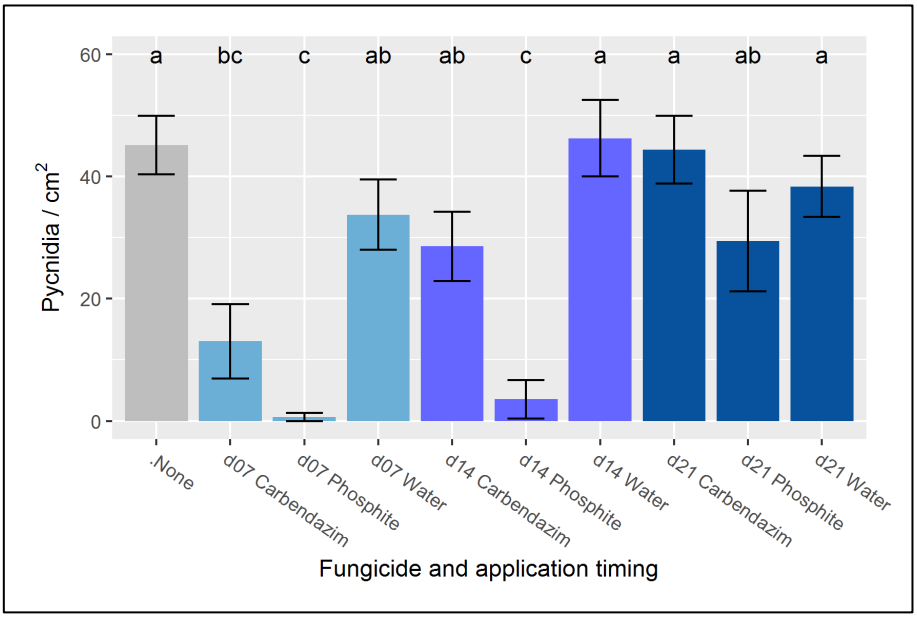

Figure 47: Pycnidia counts after various fungicidal foliar spray (28 dpi).
Results show that foliar sprays of both carbendazim and Phi significantly reduce disease if applied within 7 days of fungal inoculation (One-way ANOVA, p-value = 3.7e-15, Letters indicate significant differences between treatment groups as determined by post-hoc Tukey test; p-value < 0.05. n = 2). Carbendazim has no significant effect on disease if sprayed on day 14, yet Phi continues to significantly reduce disease. Phi application on day 21 no longer significantly affects disease development.

Results shown in Figure 47, confirm that both chemistries tested significantly reduce disease if applied to leaves within 7 days of fungal inoculations. However, if sprayed at 14 days, only Phi foliar sprays reduce disease – this difference is even stronger if spraying on 21 dpi. As shown in agar experiments, carbendazim and Phi are both directly antifungal, therefore, this result indicates that *Z. tritici* individuals are accessible to the fungicides at 7 dpi.

7.3.5. An assessment of vernalisation in Consort winter wheat

Fungicides are used to control *Z. tritici* at an average of 3 foliar sprays per season (HGCA, 2010). It is known that post-germination, winter wheat plants need a period of sustained low temperature to progress effectively from the vegetative to the reproductive stage of development (Sylvester-Bradley *et al.*, 2008). This chapter initially aimed to assess the control of *Z. tritici* on the flag leaf of wheat plants. To achieve this, a protocol was developed to maxmimise the speed at which Consort could be grown in greenhouse conditions.

Results showed that time to GS61 (flowering) is significantly dependent on vernalisation time (Figure 48A). A period of at least 6 weeks at temperatures below 8 ºC was required to minimise flowering time to a mean of 148 days post planting. Additionally, the head weight at harvest was also significantly dependent on vernalisation. Head weight was at its maximum if plants had been held at below 8 ºC for 9 weeks post planting (Figure 48B).

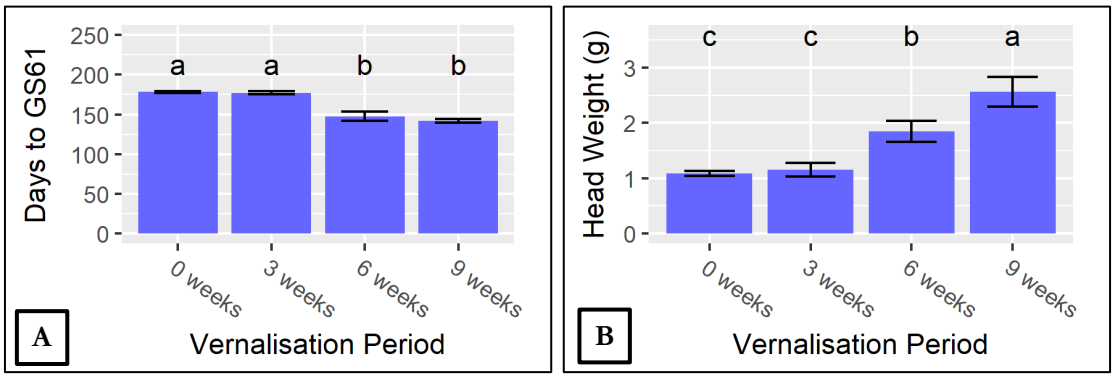

Figure 48: An assessment of vernalisation on flowering time and head weight at harvest. **A:** The effect of vernalisation time on days to GS61 was significant: One-way ANOVA, p-value = 7.6e-23, n = 82. Letters indicate significant differences between treatment groups as determined by post-hoc Tukey test; p-value < 0.05. **B:** The effect of vernalisation time on Head Weight (g) was significant: One-way ANOVA, F=29.61, MSQ=10.35, p-value < 0.001. Letters indicate significant differences between treatment groups as determined by post-hoc Tukey test; p-value < 0.05. Data are means of at least 20 plants.

7.4. Discussion

Earlier in this thesis (chapter 6), it was demonstrated that the conidiospores of *Z. tritici* can survive autonomously for extended periods in the absence of nutrients. This was shown in sterilised water, sterilised soil, and on dry Petri dishes. From these results, it can be suggested that the main source of primary inoculum in a new season may not purely be wind-blown ascospores (as is the current dogma), but also asexual conidiospores (pycnidiospores and conidiospores) surviving environmentally. If this is correct, then the current control practices for *Z. tritici* may not be optimal. Foliar sprays will not control soil-borne conidiospores, leaving them to infect plants via rain-splash both during the current season, and into the next.

Importantly, long-lived conidiospores can only contribute to the field inoculum pool if their pathogenicity is retained. It has been previously demonstrated that conidiospores that have been stored long term (24 months) in soil at 4 °C in the dark retain their pathogenicity. However, the study in which this was demonstrated assessed conidiospores only after re-isolating on rich agar, rather than applying aged populations directly (Shearer *et al.*, 1974). In this chapter, disease trials were conducted using 'fresh' conidiospores (taken from a rich agar plate) and 'stored' conidiospores (taken directly from long term water cultures). Plants were inoculated at two comparative concentrations: (i) 'total' conidiospore number (estimated using a haemocytometer; conidiospores were simply counted if they were visible structures under a light microscope, no matter their size, shape or culturability) and (ii) 'culturable' conidiospore number – the number of those conidiospores which formed colonies on YPD.

Using total conidiospore counts, 'stored' populations had significantly reduced virulence when compared to fresh conidiospores (Figure 38A). However, if using only culturable conidiospores number to compare the virulence of the populations, then no significant difference can be found. The difference between these two experimental results can be explained by the reduced culturability of stored conidiospore populations. At 49-days, around 10 % of conidiospores in stored cultures retain their ability to form colonies on rich agar (Figure 38-B). If the conidiospore concentrations used for infection are increased by 10x to account for this, no significant difference in disease is recorded when compared to

fresh conidiospores. This result demonstrates that those stored conidiospores which retain culturability do not lose virulence.

From this experiment, we can make two major conclusions: Firstly, that populations lose culturability over time; and secondly, that among individual 'stored' conidiospores, the sub-population of around 10 % that are able to survive for extended periods in nutrient-limited conditions do not display attenuated virulence after this 'storage', when compared to 'fresh' conidiospores. As discussed in this chapter introduction, in-field asexual conidiospore numbers may reach above 10^{17} per hectare during a growing season. Even if just 10 % of this population survived, as shown in the experiment outlined earlier in this thesis (Results 6.3.1), this will be sufficient to restart the disease cycle on a new crop. It is unknown how, nutritionally, these mostly single-celled (Figure 35), lipid-depleted (Figure 37) conidiospores survive and retain enough energy for hyphal extension into the plant to cause disease.

In an agricultural context, it can be hypothesised that *Z. tritici* conidiospores are able to scavenge nutrients available within soil. However, the competitive abilities of conidiospores in soil, even when remaining in necrotic cop tissue, is reported to be limited (Shearer *et al.*, 1974). There are no reports of nutrient acquisition in soil. However, all nutrient containing agar media tested in this thesis increased sporulation, confirming that environmental nutrition, if available, can be utilised (see chapter 4). It can also be hypothesised that enzymes which have been found to be released during early epiphytic growth such as cutinases, peptidases, cellulases, hemicellulases and xylanases, may be serving to liberate sources of nutrition once on either wheat leaves or secondary host plants (Rudd *et al.*, 2015, Palma-Guerrero *et al.*, 2015, Brunner *et al.*, 2013, Goodwin *et al.*, 2011, Yang *et al.*, 2015).

If infection is to occur from long-lived soil-borne conidiospores, the spread from soil to freshly emerged leaf tissue, is as important as the maintenance of pathogenicity. This has not been previously documented from free-living soil-borne conidiospores. However, it has been shown using conidiospore containing agar pieces within soil (Bannon and Cooke, 1998) and with water filled trays of infected straw (Brennan *et al.*, 1985). To further test soil-leaf spread, rain-splash conditions were mimicked using 14-day old wheat plants growing in conidiospore supplemented soil. Results (Figure 42), recorded 28 days later, show that a single rain-splash

event was enough to initiate disease. This confirms that soil-borne conidiospores would likely be able to contribute to early infection.

An additional interesting result is that conidiospores that had resided in soil for 14 days before rain-splash (compared to those added immediately before rain-splash) caused more disease. Although the difference was not statistically significant, this result suggests two things: Firstly, *Z. tritici* conidiospores do not lose virulence over a 14-day period in soil. This supports the previous result showing no loss in virulence in conidiospores suspended in water (Results 6.3.9).

Secondly, this result could be due to an increase in conidiospore populations over this 14-day period, through microcycle conidiation, as has already been shown to occur on water agar in Results 4.3.1. If this result were confirmed by counting the conidiospores in soil (not tested here), then it could be argued that a period residing in soil (or on leaves) may, in fact, be beneficial to *Z. tritici* populations. The ability to increase the population size before leaf infection would be a highly advantageous life history trait, and may go some way to explaining why fungal growth on a leaf has been found to be both slow, and untargeted (Fones *et al.*, 2017b, Kema *et al.*, 1996b, Yang *et al.*, 2013a).

In conclusion, the findings that: (i) conidiospores can survive for extended periods with no nutrition; (ii) that pathogenicity is maintained over these periods and; (iii) rain-splash is sufficient to spread conidiospores from soil to leaves suggest that the spread of long-lived, soil-borne conidiospores may be a sufficient method of infection for *Z. tritici* between growing seasons. From this we can hypothesise that current control methods are not necessarily fit-for-purpose, and new methods are needed to completely eradicate in-field inoculum.

7.4.1. Improved control of *Z. tritici*

To assess whether fungicidal soil treatments could form an alternative, or additional, technique to current foliar spray regimes, this chapter assessed three reportedly antifungal products. These were carbendazim, a previously widely-used wide-spectrum fungicide;

phosphorous acid (Phi), widely reported to have antimicrobial properties; and glyphosate, a globally used herbicide, also been reported to have antifungal properties.

Initial agar-based experiments confirmed the antifungal properties of all three chemicals, by showing complete cessation of *Z. tritici* growth on agar at various concentrations (Results 0). However, when conidiospore infected soils were treated with sprays, only Phi and glyphosate accomplished a significant reduction in disease (Results 0).

Glyphosate is used globally as a broad-spectrum herbicide. In wheat agriculture, it is used primarily as a weed treatment and is used when a field has been left fallow between wheat growing seasons. Its additional function as a antimicrobial is not one that is taken advantage of commercially, although there is evidence, both in this chapter and elsewhere, that it does have antifungal activity (Tanney and Hutchison, 2010, Nosanchuk *et al.*, 2001, Sharma *et al.*, 1989, Feng *et al.*, 2005, Tuffi Santos *et al.*, 2011). Glyphosate is not used as a soil treatment *per se* (there will likely be some run off from foliar applications), but its reported low environmental toxicity indicates that if found to be a good control agent for *Z. tritici* in further field trials, its use could potentially be extended to a dual herbicide-fungicide role.

The treatment of soil-borne conidiospores with a single spray of Phi was also sufficient to significantly reduce disease in wheat plants. This result supports many other papers which have found that Phi has antifungal properties. Phi has already been trialled as a fungicide in crops including potatoes, stone fruits, cereals and *Brassicaceae* spp. (Amiri and Bompeix, 2011, Heaton and Dullahide, 1990, Oka *et al.*, 2007, Mayton *et al.*, 2008, Hofgaard *et al.*, 2010b, Pagani *et al.*, 2014, Abbasi and Lazarovits, 2006, Kromann *et al.*, 2012). However, Phi is mostly sold as a fertiliser, and is not used at all in large-scale agricultural systems such as wheat. The application of Phi as a soil-based fungicide has not hitherto been tested, however, applications of Phi on to golf course turfgrass have been found to significantly reduce *Microdochium* patch occurrence – no observations of unforeseen ecological toxicity were reported (Dempsey, 2009).

Leading on from the soil-based experiments, this chapter also assessed the timing of foliar sprays. Until recently, it was widely believed that, post-conidiospore germination, *Z. tritici* rapidly entered the leaf apoplast (Kema *et al.*, 1996b, Duncan and Howard, 2000, Orton *et al.*, 2011). However, recent research has shown that epiphytic survival may be a much larger

part of the life cycle than previously assumed. *Z. tritici* individuals of the model strain *Z. tritici* IPO323 have been shown to undergo slow epiphytic growth, achieving penetration after a 10 day period (Fones *et al.*, 2017b). For this reason, 3 time-points were chosen for foliar sprays: 7 days, 14 days and 21 days, post leaf inoculation. Two products already assessed on agar for their fungicidal properties were used: carbendazim and phosphite.

Results show that, if sprayed on day 7, both Phi and carbendazim significantly reduced disease. This finding indicates that *Z. tritici* cells are accessible to the fungicides at 7 dpi confirming the findings reported by Fones *et al.* (2017b), that a significant fraction of the fungus remains external at this time-point. Additionally, only Phi significantly controlled *Z. tritici* when sprayed at 14 dpi. Since 14 dpi is after most reported penetration for *Z. tritici* IPO323 on Consort winter wheat (Fones *et al.*, 2017b), it can be suggested that carbendazim may only able to control epiphytic, and not apoplastic, individuals, while Phi is a more adept systemic fungicide.

Alternatively, it can also be argued that Phi is able to upregulate the plant defence system. More experiments would be necessary to confirm the secondary mode of action of Phi in this pathosystem, however, many physiological observations linked to the active plant defence system have been reported elsewhere after Phi treatment. These include rapid increases in: callose deposition; hydrogen peroxide (H_2O_2) production, pectin deposition and defensive molecules such as chitinases, phytoalexins and proteinase inhibitors (Olivieri *et al.*, 2012, Lobato *et al.*, 2011, Burra *et al.*, 2014, Eshraghi *et al.*, 2011, Berkowitz *et al.*, 2013, Massoud *et al.*, 2012). There are also instances in the literature of Phi being linked with plant defence priming – the ability of cells to respond to potential threats in a more rapid and robust manner than non-primed cells (Conrath, 2011). For example, phosphite has previously been shown to control plant disease (*Hyaloperonospora arabidopsidis* in *Arabidopsis thaliana*) at concentrations lower than have been shown to be directly fungitoxic or fungistatic (Massoud *et al.*, 2012).

How phosphite activates plant defences is still unclear. One theory is that direct effects on the microbial cell wall may release a greater number of elicitors (PAMPs), thus triggering a faster or stronger immune response in plants (Eshraghi *et al.*, 2011, Dalio *et al.*, 2014). It seems doubtful whether this is the mechanism in the wheat-*Z. tritici* relationship, as disease

is still reduced at 14 days – after fungal individuals have entered the plant. However, PAMPs would still be released from epiphytic individuals, which may be sufficient to trigger plant defences.

7.4.2. The potential of phosphite as an agricultural chemical

As the results in this chapter have shown thus far, phosphite performs well as an antifungal agent in agar, soil, and as a foliar spray. There are, however other positive and negative aspects to the use of phosphite, which should be explored before further trials. These include: (i) the potential to act as a fertiliser; (ii) its potential environmental toxicity and; (iii) the risk of *Z. tritici* developing resistance.

Fertilisation of soils is an essential element of many agricultural systems. Many soils are deficient in nutrients essential for crop growth and food production. Phosphorus, one of the 'macronutrients' alongside nitrogen, potassium, calcium, magnesium, and sulphur, is essential for myriad cellular constituents including phospholipids, nucleic acids and ATP. However, the procurement of P for fertilisers comes entirely from mined rock phosphate (PO_4^{3-} or Pi), a source suggested to be completely depleted within as little as 50 years (Gilbert, 2009). Early studies on agricultural Phi use suggested a direct fertilisation effect (Lovatt, 1990b, Lovatt, 1990a). However, more recent work has refuted this: although phosphite (Phi) is chemically similar to phosphate (Pi), the apparent lack of an enzyme in plants able to convert Phi to Pi (Phi dehydrogenase), means that phosphite cannot be directly metabolised by crops and is therefore not useful for Pi fertilisation (Thao and Yamakawa, 2009, Thao *et al.*, 2008, Ratjen and Gerendás, 2009, McDonald *et al.*, 2001). However, Phi is broken down by soil microbes which gain energy and nutrients from the biological conversion (Lovatt and Mikkelsen, 2006). While this process is reportedly slow, for example a study looking at phosphite break down after soil application found that even after 7 days only 35 % of the product has been broken down in the soil (Morton *et al.*, 2005, McDonald *et al.*, 2001), there may be some future fertilisation boosts associated.

To take better advantage of the P in phosphite for fertilisation, a feasible direction could be to engineer transgenic plants to produce the enzymes needed for the conversion of Phi to Pi. This has been trialled twice before, once in 2012 where a transgenic *Arabidopsis* plant was

engineered to produce the bacterial phosphite oxidoreductase gene (López-Arredondo and Herrera-Estrella, 2012), and again in 2016 (Manna *et al.*) where the Phi dehydrogenase (PtxD) gene was inserted and upregulated in rice plants. In both these studies, plants showed significant signs of fertilisation such as increased root growth and general improvements in physiology and overall phenotype.

Widespread use of Phi reportedly comes with potential issues, the first of which is that it has been reported to be phytotoxic. This characteristic may affect early plant growth, thus counteracting any antifungal benefits. The basis for phytotoxicity seems to surround an indirect effect of the presence of Phi: namely that Phi stimulates Pi receptors, thus potentially masking Pi deficiencies in soil and avoiding an essential starvation response. This can lead to reduced uptake and subcellular compartmentation of phosphate, causing stunted development (Berkowitz *et al.*, 2013, Varadarajan *et al.*, 2002, Eshraghi *et al.*, 2014b). It must be noted that no obvious signs of phytotoxicity were witnessed in the work reported in this thesis; however, multiple applications may lead to future issues due to accumulation. On a wider ecological scale, negative effects for non-target plants have already been reported in the Jarrah Forest in Australia (Hardy *et al.*, 2001). Here, it was found that if phosphite was used at high concentrations (~ 5 g/l), the agricultural run-off reduced the reproductive fitness of some annual and perennial understory species (Hardy *et al.*, 2001). Minor phytotoxic effects have also been reported on some conifers and woody angiosperms (Scott *et al.*, 2016). Turning to aquatic systems, potentially of great importance due to the run off associated with soil treatments, phosphite has not been found to be toxic to a range of microalgae including *Chlamydomonas reinhardtii, Botryococcus braunii* and *Ettlia oleoabundans* (Loera-Quezada *et al.*, 2015). If phosphite is to be used on such a large-scale as wheat agriculture, more extensive environmental trials would need to be carried out to assess any knock-on effects.

A third potential issue, and one shared with all fungicidal products, is the likelihood of *Z. tritici* strains developing resistance. There is documented evidence of naturally occurring Phi-resistant isolates in both oomycetes and fungi in field settings where sufficient selection pressure (heavy use of Phi fungicides) is applied (Fenn and Coffey, 1989, Vegh *et al.*, 1985, Brown *et al.*, 2004). However, the dual mechanism by which Phi seems to control *Z. tritici* (direct fungitoxicity and plant defence induction), may in fact increase the longevity of this

product, as even if individuals evolve resistance to the direct effects, the primed plant may stop the resistant strain from undergoing asexual reproduction within the plant.

7.4.3. Conclusions on control

This chapter has confirmed that conidiospores stored in sterilised water cultures, retain their pathogenicity for periods of at least 49 days. These conidiospores, if residing in soil, can splash on to leaves via a single rain-splash event, subsequently causing disease. Soil-based applications of both phosphorous acid (Phi) and glyphosate significantly reduced disease, leading to the conclusion that this method of fungicide application may have a role to play in the reduction of *Z. tritici* populations and their ability to cause disease.

Phi, unlike the herbicide glyphosate, can also be sprayed directly onto crop leaves. Here, Phi was found to control *Z. tritici* completely if sprayed up to 14-days post-fungal inoculation. Combining these application methods (soil and leaf treatments), therefore has the potential to effectively control environmental, epiphytic, and even endophytic, individuals of *Z. tritici* – particularly if used within 14-days of seed germination/appearance of the first leaf.

8. General Discussion

8.1. Spread and maintenance of disease

Current understanding is that, within a growing season, *Z. tritici* spreads to other hosts/leaf tissue via rain-splash dispersal of asexual spores (pycnidiospores). As these spores can spread relatively large distances (horizontally > 4 m, and vertically > 50 cm), it is likely that many will not find a host but instead will spread to other areas of the environment, such as the soil which surrounds plants.

Results in chapter 6, showed that, independent of the media on which spores are maintained (soil, water agar, or suspended in water), conidiospores could survive for many weeks in media with no nutritional source. Thus, if asexual spores are spread to soil, long term survival is likely. The long-term survival of asexual spores allows for the hypothesis that inoculum may even be able to survive between growing seasons. In winter wheat agriculture, the gap between harvest and the drilling of the next crop can be as short as a single month (Sylvester-Bradley *et al.*, 2008).

Additionally, spores which have survived for long periods without nutrition retain their virulence (Results 6.3.9) and have been shown to spread easily to young plants during a simulated rainfall event (Results 7.3.1). Asexual spores are already thought to be the main source of secondary inoculum. Collectively however, these results indicate that, alongside sexual spores, asexual spores may be an important source of primary inoculum, particularly as the potential for rain spread is so high during the early season due to increased rain and an undeveloped canopy.

Like most crops, wheat plants develop mostly during the summer months. As such, successful pathogens would also have some level of protection during periods of drought. The results presented here confirm that *Z. tritici* can survive for more than 28 days with no water in low humidity (Results 6.3.9). Under these conditions, spores have been shown to maintain significantly more of their lipid stores, compared to spores stored for the same length of time on water agar. Internal lipid storage correlates very highly with spore survival

(Figure 40). This implies that the maintenance of lipids during periods of drought may result in the maintenance of populations during summer months. The capacity for drought survival may be linked to the evolution of *Z. tritici* in the fertile crescent 12,000 years ago (Snape and Pánková, 2013, McDonald and Mundt, 2016).

These results do not contradict the accepted view that ascospores are the main source of primary inoculum for a wheat crop (Shaw and Royle, 1989, Suffert and Sache, 2011, Suffert *et al.*, 2011). However, due to the long-term survival capacity of asexual spores, it can be proposed that disease propagules are present in a wheat field even before a crop is sown. Additionally, as wheat fields can be used for the same crop in successive seasons, only rain-splash is needed to restart the disease cycle. Early infection means that disease is never eradicated. Additionally, early infection may even make plants more vulnerable to continued infection throughout the season.

Early infection may, therefore, be better managed by methods of control other than foliar sprays. Currently, crops are treated using foliar sprays (HGCA, 2010). These sprays only begin at the growth stage where the 3rd leaf (counting down the canopy) is present – this is usually many months into the growing season (HGCA, 2012, HGCA, 2010, Sylvester-Bradley *et al.*, 2008). The results presented here show that primary inoculum is likely to be in the soil before the new crop is sown. If this is also the case in the field, the treatment of soil pre-planting would be an important addition or alternative to the treatment regime. Chapter 7 explored soil treatments as a potential control method for soil borne spores with positive results. In particular, glyphosate, the herbicide which is already used in agriculture for weed control pre-planting (Glyphosate.eu, 2013), could be used as a dual-purpose pesticide, thus controlling soil-based *Z. tritici* spores.

In conclusion, asexual spores can (i) survive in both wet and dry conditions *ex planta*, (ii) spread from soil to plants via rain-splash and (iii) remain virulent, therefore causing disease after extended periods *ex planta*. Controlling *Z. tritici* through soil-based methods early in the season may lead to a large reduction in inoculum and a reduced need for multiple foliar sprays later in the growing season. Any reduction from the ~ 3 sprays used by farmers now could lead to a reduction in the > € billon currently spent on fungicides used to control *Z. tritici* in the EU (Torriani *et al.*, 2015, Fones and Gurr, 2015).

8.2. Germination

Leaf-based studies have shown that *Z. tritici* germination occurs at high rates on leaf surfaces (Cohen and Eyal, 1993, Kema *et al.*, 1996b, Fones *et al.*, 2017b). The leaf is a hard-hydrophobic surface composed of cutin and waxes (solvent-soluble lipids) which are known to act as germination and development cues in many fungi (Ringelmann *et al.*, 2009, Gilbert *et al.*, 1996, Uppalapati *et al.*, 2012, Zabka *et al.*, 2008). Work in this thesis used a semi-solid substrate (agar) to mimic the leaf surface and assess spore germination. This was done by plating spores onto agar and counting colonies 7-days later.

In this thesis, the number of colonies which formed has been termed a measure of 'culturability' rather than of germination, as no microscopy was used to assess if the non-culturable spores produced a germ tube. However, germination is necessary for colony formation, and thus the two processes are closely linked. Colony formation is therefore a reasonable proxy for germination.

Surprisingly, germination on water agar was found to be very low. Subsequent experiments showed that the period of submergence for a spore population correlates significantly with the culturability of said population. If spores are suspended for just a single minute, their culturability is maintained at high levels. However, after just 60 minutes in water, culturability drops by more than half (Results 3.3.3). After 24 hours, less than 10 % of conidiospores form colonies on YPD. Further studies show that populations do not recover their culturability, even over very long time-scales (Results 6.3.3).

These results are surprising as the asexual spores of *Z. tritici* are spread by rain and therefore an initial period of submergence is inevitable. A closer inspection using the cell-death stain propidium iodide (PI) showed that spores remained completely viable, at least for the first 4 days of suspension in water (Results 3.3.5). As discussed in chapter 3, this suggests that spores enter a dormant, or viable but not culturable (VBNC) state. This state may assist in the maintenance of a virulent population over space and time. Additionally, although it hasn't been documented, non-culturable spores may participate in sexual events away from a host. Strain diversity may therefore increase even when no host is available.

Based on these results, the virulence of suspended conidiospore populations was assessed over short time-scales. It was found that virulence was also lost rapidly over time, suggesting that culturability (the ability to form a colony on agar) is directly related to the ability to cause disease on a plant (Results 0). This is an interesting result as it might be expected that spores should break dormancy/VBNC as soon as a leaf-like surface is detected. The fact that *Z. tritici* spores do not do so indicates either that they are dying, or that there are other environmental cues associated with field conditions that are missing in the conditions used in this work.

A second hypothesis is that conidiospores behave differently to pycnidiospores upon submergence (not assessed here). This brings the applicability of these results to field situations into question. In either case, these results are of importance, particularly for researchers who work on *Z. tritici* in the laboratory. Most work comparing the virulence of multiple *Z. tritici* strains is carried out by suspending, quantifying, and applying conidiospores to wheat leaves (Kettles *et al.*, 2016, Fones *et al.*, 2017b, Rudd *et al.*, 2015, Yang *et al.*, 2015, Lee *et al.*, 2013). The fact that virulence is affected so significantly by this process, at such rapid time-scales, means that suspension time is a factor which must be accounted for in experimental design. Researchers should only apply spore populations to plants after precisely the same period of suspension. To assess true virulence of a spore population this period should be as short a time as possible.

8.3. Epiphytic growth and development

Growth on the leaf and on the low nutrient surfaces has consistently been reported to be hyphal (Duncan and Howard, 2000, Cousin *et al.*, 2006). In this thesis, water agar was used as a proxy for the solid, low-nutrient, leaf surface. Indeed, on water agar hyphal extension of *Z. tritici* colonies has been shown to continue for at least 21 days. This growth is initially slow, increasing consistently until growth stops at around day 21 (Results 4.3.1). As discussed in chapter 4, this result correlates with a recent study showing that hyphae enter the plant after a 10-14 day period of random epiphytic growth (Fones *et al.*, 2017b).

Interestingly, the period of slow hyphal growth during the first 7 days of colony development on water agar is combined with extensive sporulation (Results 4.3.1). This sporulation potential allows for the hypothesis that epiphytic growth stage may have evolved to be extended, so that the infective population can be increased by sporulation before infection occurs. This behaviour would allow a constant spread of spores to new hosts during the growing season. Additionally, the infective population size could be increased even if spores are not on a compatible host.

This thesis has used many agar-based media to assess *Z. tritici*. Growth form is highly nutrient dependent, with microcycle conidiation (MC) seemingly increasingly favoured over hyphal extension as nutrient availability rises. It could be hypothesised, therefore, that populations which favour MC, combined with long periods of epiphytic growth, may have evolved in response to modern agricultural practices such as fertilisation. Indeed, results in this thesis have shown that in both carbon supplemented and nitrogen supplemented agar, sporulation was increased (Results 4.3.2 and 4.3.3). Additionally, in the presence of nitrogen or phosphorous, hyphal growth is reduced thus providing evidence for the hypothesis that, in environments supplemented with fertilisers, MC increases while entry is delayed. This implies that *Z. tritici* can indeed take advantage of the increased macronutrient availability of a wheat field and continue to build populations during epiphytic growth. As discussed above, any increase in the population size would likely augment disease thus reducing yields.

Potentially then, fertilisers should be chosen while considering the ability of pathogens such as *Z. tritici* to utilise the nutrients within them. For example, *Z. tritici* grows more slowly (hyphally), and sporulates less, in the presence of ammonium ions when compared to nitrates (Results 4.3.3). Therefore, ammonium-based fertilisers may lead to less disease promotion if used instead of nitrates.

Finally, combining epiphytic sporulation potential with the capacity for long term survival under low nutrient conditions means that MC is likely occurring on host leaves, non-compatible cultivars, other non-host plants, and potentially even in soil. Some supportive evidence for this comes from rain-splash experiments where it was shown that spore populations stored in soil for 14 days before rain-splash were more virulent than spore populations added to soil on the same day as the rain-splash (Results 7.3.1). This indicates

that, as on water agar, the population had grown during this 14-day period in soil. More work is needed to confirm this, but if so, fertilisation could also positively affect soil-borne populations and subsequent disease.

8.4. Entry into the leaf

Water agar results imply that infection/leaf entry may be dependent on the nutrient availability on the leaf surface. More nutrients mean more sporulation and less hyphal growth, therefore potentially slower infection. This hypothesis has not been tested here explicitly, but supportive evidence comes from the result that disease is not increased in the presence of foliar fertilisers (Results 4.3.4). Previous papers have linked fertilisation to increased disease via enhanced leaf entry, increased apoplastic nutrients, or augmented canopy size (Simon *et al.*, 2003, Leitch and Jenkins, 1995, Lovell *et al.*, 1997). The results shown here also imply an additional role: the promotion of sporulation leading to augmented spread.

8.5. Apoplastic growth

Apoplastic growth has not been studied in this thesis. However, hypotheses can be generated from some of the work carried out here. Some work on apoplastic growth raises the possibility, due to the expression of relevant enzymes at appropriate time-points, that nutrients within the apoplast are utilised by *Z. tritici* (Keon *et al.*, 2007, Palma-Guerrero *et al.*, 2015). These nutrients may enable an extended period of growth, resulting in large scale colonisation of the apoplast. No firm evidence of apoplastic nutrient utilisation has been offered, however (Yang *et al.*, 2015, Yang *et al.*, 2013b, Keon *et al.*, 2007, Rudd *et al.*, 2015).

Results from water agar experiments (Results 4.3.1) show that exogenous nutrition is not needed for extensive hyphal extension to occur up to 21 days. This supports papers which have which have shown no evidence of apoplastic feeding. Additionally, between days 14 and 21 on water agar, extensive hyphal branching is recorded. A similar phenotype been

shown to occur just before pycnidia appear *in planta* (Shetty *et al.*, 2003). This result again provides evidence that apoplastic nutrition is not needed for growth *in planta*.

These results are particularly interesting as they suggest that there are enough internal nutrient stores in *Z. tritici* pycnidiospores / conidiospores to complete the whole life-cycle up to the onset of pycnidiation. The internal stores most likely to be utilised during this period of growth are lipid granules (M'Barek *et al.*, 2015, Keon *et al.*, 2007, Palma-Guerrero *et al.*, 2015, Rudd *et al.*, 2015). This thesis has assessed lipids in the conidiospores of *Z. tritici* and confirmed that their presence is initially very high within cells (Results 6.3.8). Additionally, lipid granules deplete over time, correlating very strongly with cell death (Figure 40). The importance of other internal sources of nutrition such as arabitol, mannitol, trehalose and stachydrine are yet to be assessed in *Z. tritici* (Rudd *et al.*, 2015). It is likely that some, or all, also play a role.

8.6. Plant cell lysis and the necrotrophic switch

The necrotrophic switch has been proposed to be initiated by effectors, small secreted peptides which can be recognised by resistance proteins in plants, thus triggering cell-death. This process is called effector triggered susceptibility (ETS) and has evolved as a mechanism by which necrotrophic pathogens initiate plant cell death and nutrient release (Stotz *et al.*, 2014, Faris *et al.*, 2010). In *Z. tritici*, 246 putative effectors have been highlighted from the genome (Goodwin *et al.*, 2011, do Amaral *et al.*, 2012, Yang *et al.*, 2013a, Rudd *et al.*, 2015, McDonald *et al.*, 2016). Many have also been shown to be secreted during infection (Rudd *et al.*, 2015, Palma-Guerrero *et al.*, 2015). A study using *Agrobacterium*-mediated transient expression was used to screen putative *Z. tritici* effector genes for recognition in *Nicotiana benthamiana*. This study tested 63 *Z. tritici* effectors and found that 14 caused a cell-death phenotype. However, injecting these 14 into the wheat apoplast did not result in cell-death. Other putative effectors have been knocked out of the *Z. tritici* genome with no reduced effect on virulence (M'Barek *et al.*, 2015).

Many secreted effectors remain untested for their defence triggering capabilities, however, the majority of these will be used for plant defence suppression rather than induction. For

example, the LysM proteins, which bind to chitin released during hyphal growth are used to supress recognition by the plants thus allowing for successful invasion and pathogenicity. Knocking out this effector leads to impaired colonisation and a cessation of asexual sporulation (Marshall *et al.*, 2011).

Additionally, plants recognise the presence of pathogens via their microbe associated molecular patterns (MAMPs) - highly conserved, often structural, molecules such as glucan, xylanase, chitin and ergosterol (Thonart *et al.*, 2012). The recognition of MAMPs leads to immune responses such as reactive oxygen species production, cell wall reinforcements and the release of systemic antimicrobial chemicals (Apel and Hirt, 2004, Chowdhury *et al.*, 2014, Ahuja *et al.*, 2012, Collinge *et al.*, 1993). These responses are characteristically not as strong as those seen in ETS. However, MAMP induced cell-death has been reported – specifically bacterial MAMPs such as the elongation factor (EF-Tu) protein (Zipfel, 2008).

This thesis has shown that cells/spores can survive for extended periods (Results 6.3.4). However, spores do lose viability with time. Results have shown that, suspended in water, cell viability drops from around day 5 onwards (Results 6.3.3). Cell-death of fungal spores leads to cell lysis, which can be visualised by light microscopy (Figure 34). The lysis of fungal cells while in the apoplastic space would lead to the release of many MAMPs and potentially, (non-secreted) effectors. *Z. tritici* cell lysates have been sprayed onto the leaf surface leading to chlorosis (Kettles *et al.*, 2016). However, there has been no study on injecting cell contents (and the associated effectors and MAMPs) into the plant apoplast.

The recognition of either MAMPs or effector could potentially trigger plant cell-death. It can be hypothesised that this unintentional triggering of the plant defence system may in fact be an evolved mechanism to trigger the necrotrophic switch. There are examples of this in other organisms, particularly bacteria. This subject was reviewed by Ackerman *et al.* (2008) who gave three examples of host cell death triggered by the spillage of pathogenic cell contents. These examples were: TcdA, a key virulence factor of *Clostridium difficile* which lacks a standard secretion signal and is released post-lysis; the toxin pneumocystis which is released from *Streptococcus pneumoniae* cells; and finally, the lysis of a small sub-population of *Salmonella typhimurium* cells, causing an inflammatory response which aids infection by the remaining

live population. The theory titled "self-destructive cooperation" is characterised by clonal populations with a mixture of phenotypes.

Subsequent work has highlighted the presence of a suicide gene in bacteria called pneumococcal zeta toxin (PezT). It has been suggested that during rapid growth, *PezT* is responsible for cell death via autolysis in a small number of cells within the population. This cell death, and the subsequent toxin release, may affect either the host or competing microbes, thus enhancing infection (Sedwick, 2011, Mutschler *et al.*, 2011).

The evolution of a cooperative population structure has not been reported in *Z. tritici*. However, it can by hypothesised that a similar strategy could evolve here. As shown in Results 6.3.3, the cells from *Z. tritici* populations survive for varying periods. Therefore, within an infecting population there is likely a constant turnover of cells occurring. If fungal cell death (and lysis) leads to recognition of cell contents by plants and the triggering of plant cell death, then any remaining viable individuals could take advantage of this. The released nutrition would allow these individuals to completing the disease cycle without the active secretion of molecules such as effectors. Unlike the case reported for bacteria, a mixed phenotype population is not necessarily required here. A single colonising fungal individual, if not taking advantage of host apoplastic nutrition, will likely have cells within its hyphal network which become unviable before others. This has been shown in water suspended pupations through staining with PI (Results 6.3.3). These cells would therefore lyse earlier than others and this might be adequate to trigger the necessary cell-death phenotype in plants.

Supportive evidence can be offered here by showing that fungal cell death correlates well with the depletion of lipids (Figure 40) By around 14 days lipid content has fallen from around 50 % to less than 7 % (Results 6.3.8). Combining this knowledge with the fact that disease symptoms occur *in planta* after a minimum period post inoculation (> 14 days), it can be suggested that plant cell-death is linked to the time when many fungal individuals have run out of internal lipid stores and start to undergo autolysis.

This hypothesis suggests that there is no necrotrophic switch, but simply an accidental triggering of the plant hypersensitive response due to fungal cell death. This could be described as 'passive necrotrophy'. To test this further, the supernatant of fungal populations which have undergone varying degrees of cell death can be injected into wheat apoplast.

8.7. Pycnidiation

This thesis has used wheat leaf agar (WLA) as a medium of growth for *Z. tritici*. On WLA, structures are produced which are very similar in structure to pycnidia. These structures which are characterised by three sequential stages of growth (described in results 5.3.1), have been called *in vitro* pycnidia (IVP) to separate them from those found *in planta*.

The IVP phenotype seems to be actively triggered by the plant cell lysate and thus it can be hypothesised that plant cell death and lysis is necessary for pycnidiation to occur. Once triggered, IVP continues until completion, even on rich-nutrient media. This is unexpected, as yeast-like growth usually dominates on rich-nutrient media. This indicates that pycnidiation is a deterministic developmental process. The knowledge of how pycnidiation is triggered, and how it can be stopped, may allow breeders to manipulate this by producing wheat with/without certain cues. Conversely, the triggering of pycnidiation before leaf entry may lead to reduced competitive ability of *Z. tritici* strains.

Pycnidiation seems to be completed within 4 days on WLA. Earlier in this thesis (Results 4.3.5) it was shown that the fungus is primarily epiphytic for between 7 and 14 days. This has also been shown elsewhere (Fones *et al.*, 2017b). *In planta*, using the combination of Consort wheat and *Z. tritici* IPO323, pycnidia are visible from around 14 days. Therefore, the life cycle of a fungal individual in a field may not have an extended period of latent, apoplastic growth, as suggested in the literature. It may instead be simpler, dominated by epiphytic growth, with the triggering of pycnidiation occurring almost immediately after stomatal infection.

8.8. Future work and overall conclusions

The aim of this thesis was to assess the asexual life cycle of the fungus *Z. tritici*. Attention has been paid to the behaviour of spores upon submergence (chapter 3), the capacity for early/epiphytic growth (chapter 4), the trigger for apoplastic pycnidiation (chapter 5), and *ex planta* spore survival (chapter 6).

Much of the work presented here has only been carried out in the laboratory and as such is a proof of concept rather than complete proof. Work such as the capacity for environmental survival in asexual spores should also be carried out in the field to confirm the relevance of this work. Additionally, in chapter 7 where soil based fungicide sprays were used to control environmental spores. Field trials are now needed to take this idea to the next stage of development.

RNA extractions have been carried out on suspended spore populations over multiple time points. RNA has also been sequenced using the Illumina MiSeq Nano protocol. It was the intention of the author to undertake a thorough assessment of transcriptome data to study two aspects of development (i) the shock response associated with leaving the pycnidium and (ii) the mechanisms of long term survival. This work will now be carried out after this thesis has been submitted.

Collectively, the results presented provide new insights to the *Z. tritici* life cycle. As such the following changes can be proposed:

1. *Z. tritici* asexual spores can survive without a host (or external sources of nutrition) for extended periods (> 49 days). Thus, spores are likely present in soil both within and between growing seasons. (chapter 6).
2. Spores can splash onto plants from soil (Results 7.3.1)
3. On the leaf, conidiation occurs readily, thus increasing population sizes during periods of epiphytic growth (Results 4.3.1).
4. On the leaf, sporulation may be increased in the presence of agricultural fertilisation (Results 4.3.3).
5. On a leaf, hyphal growth rate is initially slow but increases over time (Results 4.3.1).
6. Fungal growth is maintained using the nutrition from internal lipids granules/stores (Results 6.2.6).
7. Apoplastic entry is slow (Results 4.3.5).
8. No external nutrition is necessary for apoplastic entry (Results 4.3.1).

9. After apoplastic entry (assisted by fungal effector proteins), fungal cell death occurs, due to internal lipid depletion (Figure 40).

10. Plant cell death is subsequently triggered due to lysis of fungal cell contents and the recognition of the release of MAMPs and (non-secreted) effectors.

11. Plant cells contain trigger(s) for pycnidiation (chapter 5)

12. Therefore, pycnidiation is initiated in remaining viable fungal individuals.

13. Under competition or space restrictions, such as is seen in the leaf, pycnidiation can occur rapidly (Results 5.3.10).

14. Asexual spores are released into the environment.

15. *Z. tritici* spores released into the environment could be controlled by fungicidal soil treatments in addition to foliar sprays (chapter 7).

In conclusion, this thesis has achieved its goal of assessing the asexual life-cycle of a strain of *Z. tritici* and offers many novel insights into the behaviour of this devastating pathogen. The increased understanding achieved from this thesis will lead to improvements in both the laboratory study, and the field-based control of *Z. tritici*.

9. An updated life-cycle for *Z. tritici*

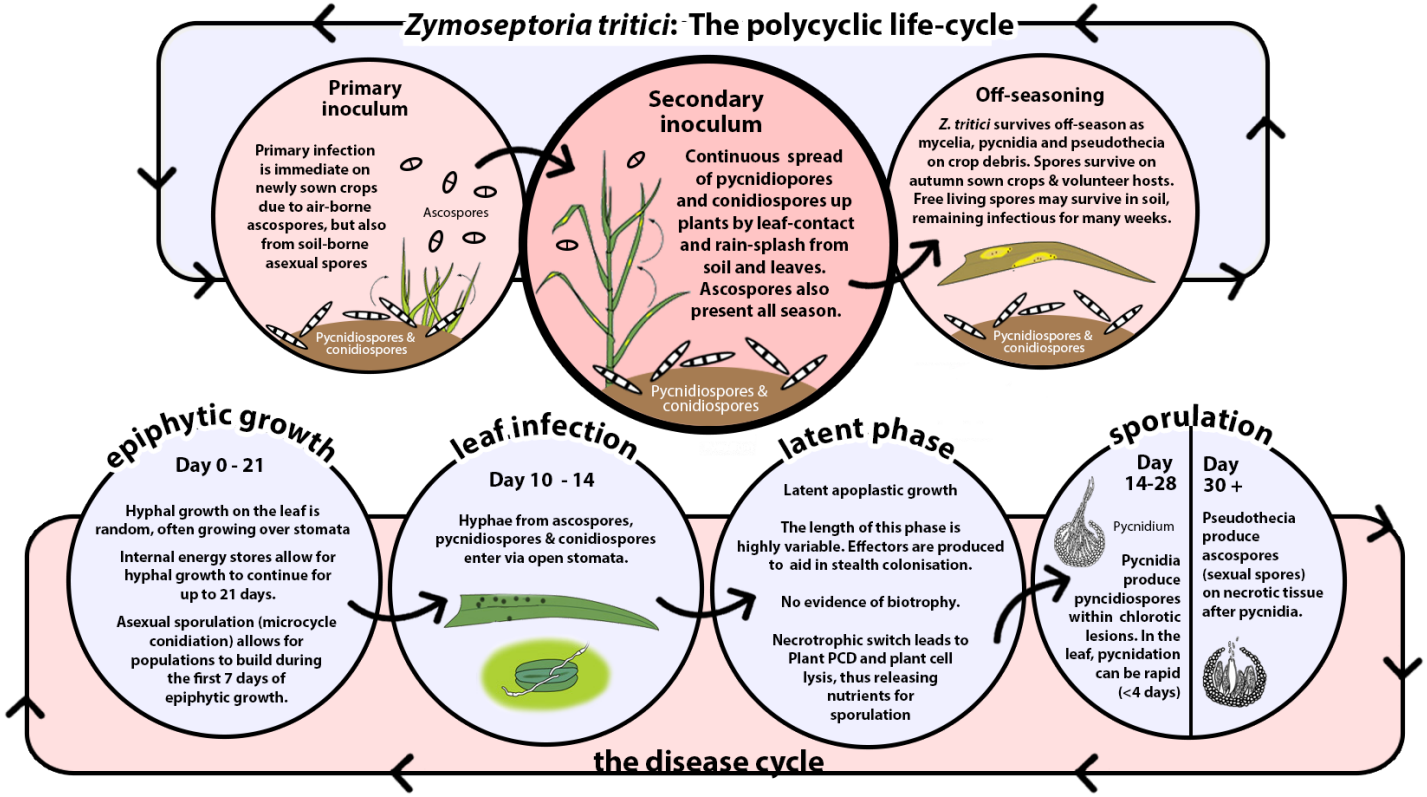

Figure 49: An updated *Z. tritici* life-cycle

Adapted from by Alisa Ponomarenko, Stephen B. Goodwin, and Gert H. J. Kema (Ponomarenko *et al.*, 2011). Life cycle has been split into two cycles representing the life cycle of the fungus and the disease cycle within a host. Cycles contain new information from this thesis and numerous other sources (Fones *et al.*, 2017b, Suffert *et al.*, 2011, Suffert and Sache, 2011, Kettles *et al.*, 2016, Lee *et al.*, 2014, Palma-Guerrero *et al.*, 2015, Rudd *et al.*, 2015).

10. Appendix 1: Scripts

10.1. ImageJ Script: Agar-based colony analysis

Colony frequency and area were assessed after 8 days using the bespoke ImageJ script below.

```
// original directory (this is where the file is opened)
	DIRECTORY = File.directory + "plates\\";
	DIRECTORY1 = File.directory
// get an array of all filenames and the number of files in that list
	allfiles = getFileList(DIRECTORY);
	NUMFILES = allfiles.length;
// make a new directory and make it the save point for future images
	File.makeDirectory(DIRECTORY1 + "PROCESSED_PLATES\\");
	DIRECTORY2 = DIRECTORY1 + "PROCESSED_PLATES\\";
// clear any previous results from the ImageJ results window
	run("Clear Results");
// run the edits on the image (this is for a conidiospore count)
	for (filenumber=0; filenumber<NUMFILES; filenumber++){
		open(DIRECTORY+allfiles[filenumber]);
		// this is the important bit – count the conidiospores
		// 16 bit, watershed, select conidiospores, measure
			run("Threshold...");
			setThreshold(150, 255); // chooses just the lighter colours, removes background
			run("Convert to Mask");
			run("Watershed"); // this splits-up overlapping colonies into single colonies
			run("Set Measurements...", "area display redirect=None decimal=3");
			run("Analyze Particles...", "sise=10-1000 circularity=0.70-1.00 display");
		// save and close the new images you made
			run("Save", "save="+DIRECTORY2+allfiles[filenumber]+".tif");
			close();
		// close the original
			close(allfiles[filenumber]);
	}  // end the for statement
// save the details including the file name to an excel file or CSV
	run("Input/Output...", "jpeg=85 gif=-1 file=.csv use_file copy_column copy_row save_column
save_row");
	saveAs("Results", DIRECTORY1+"Results.csv");
```

10.2. ImageJ Script: Disease analysis by pycnidia counting

For all disease assessments, leaves were harvested from plants at 28 dpi. To aid in the data collection process, leaves were then left for 24 hours in a sealed container on top of damp Wypall L20 before being taped, abaxial side up, onto 3 mm white Perspex® sheets and finally weighed down for 24 hours to dry. Leaves were then scanned on a Canon CanoScan LiDE 210 at a setting of 1200 dots per inch. Scanned leaves were measured using a macro designed in Adobe Photoshop®.

Pycnidia were compared to manual counts using the bespoke ImageJ script below. The script was not accurate enough to be used in this thesis.

```
// original directory (this is where the file is opened)
	DIRECTORY = File.directory + "images\\leaves\\";
	DIRECTORY1 = File.directory + "images\\";
// get an array of all filenames and the number of files in that array
	allfiles = getFileList(DIRECTORY);
	NUMFILES = allfiles.length;
// make a new directory and make it the save point
	File.makeDirectory(DIRECTORY1 + "after_ImageJ\\");
	DIRECTORY2 = DIRECTORY1 + "after_ImageJ\\";
// clear any previous results from the window
	run("Clear Results");
// run the edits on the image (this is for a conidiospore count)
	for (filenumber=0; filenumber<NUMFILES; filenumber++){
		open(DIRECTORY+allfiles[filenumber]);
		// this is the important bit – count the conidiospores
		// need to change the brightness and contrast here instead of photoshop
			// run("Brightness/Contrast...");
			setMinAndMax(80, 140);
			run("Apply LUT");
		// 16 bit, watershed, select conidiospores, measure
			run("16-bit");
			setAutoThreshold("Default dark");
			run("Threshold...");
			setThreshold(0, 70);
			run("Close");
			run("Convert to Mask");
			run("Watershed");// this splits up overlapping colonies into single colonies
			run("Set Measurements...", "area display redirect=None decimal=3");
			run("Analyze Particles...", "sise=5-100 circularity=0.70-1.00 display");
		// save and close the new images you made
```

```
                    run("Save", "save="+DIRECTORY2+allfiles[filenumber]+".tif");
                    close();
                // close the original –
                    close(allfiles[filenumber]);
        }  // end the for statment
// save the details including the file name to an excel file or CSV
        run("Input/Output...", "jpeg=85 gif=-1 file=.csv use_file copy_column copy_row save_column
save_row");
saveAs("Results", DIRECTORY1+"Pycnidia_Results.csv");
```

11. Appendix 2: Chitin – A wheat defence elicitor?

11.1. Introduction

Zymoseptoria tritici, the causal agent of *Septoria tritici* blotch (STB), is the number one fungal pathogen of wheat in temperate countries. It is responsible for causing 5-10 % losses even when tolerant cultivars are sown and treated with appropriate fungicides (Fones and Gurr, 2015). Losses are reduced through the foliar application of chemistries such as azoles and QoIs (Quinone outside Inhibitors), yet resistance is becoming widespread. For example, a 2014 study has shown that alleles which confer resistance to current fungicides have evolved independently in several lineages of *Z. tritici* and are now common amongst pathogenic lines in North America (Estep *et al.*, 2015).

Examples of pathogen resistance such as this highlight the short-term efficacy of fungicide use. Consequently, attention is now being paid to other methods of disease control such as the induction of a plant's own defence mechanisms. A plant defends itself in two distinguishable forms: The first is the 'passive' defence system which include preformed, often morphological adaptations such as primary and secondary cell walls, a thick waxy cuticle, variable leaf openings for gas exchange (stomata), and preformed defensive chemicals named phytoanticipins (VanEtten *et al.*, 1994). The second is the 'active' defence system which relies on the recognition of pathogens, specifically, through the perception of the foreign molecules associated with them. These molecules, termed elicitors or PAMPs (Pathogen associated molecular patterns), are highly conserved and found either attached to, or secreted by, microbial cells. They include chitin, glucan, xylanase and ergosterol for fungi; and elongation factor and flagellin for bacteria (Thonart *et al.*, 2012).

PAMP recognition leads to a form of defence titled pattern triggered immunity (PTI). Biochemical and morphological changes resulting from PTI are well documented and include reactive oxygen species production (Apel and Hirt, 2004), localised cell wall reinforcements (Chowdhury *et al.*, 2014), the release of systemic antimicrobial chemicals (Ahuja *et al.*, 2012) and the secretion of cell wall degrading enzymes specific to fungal invaders (Collinge *et al.*, 1993). Although exact mechanisms are unclear, PTI can also lead to long-term systemic

protection via a form of defence named systemic acquired resistance (SAR), as well as increased sensitivity to future pathogen attacks via a form of defence named priming (Gozzo and Faoro, 2013).

While PTI is generally a successful method of pathogen control for plants (Nuernberger and Lipka, 2005), current dogma, based around the Zig-Zag model of plant defence (Jones and Dangl, 2006), states that successful pathogens can evade triggering these defences through the secretion of effectors (Thomma *et al.*, 2011), the outcome being increased virulence and more severe crop losses. However, application of elicitors directly to plants either before or during early pathogen infection may be enough to induce the necessary defences to confer reduced losses – even when challenged with highly virulent, effector producing pathogens. Successful examples which support this hypothesises include β-1-3-glucan application during the effector induced 'latent' stage of the hemibiotrophic pathogen *Z. tritici* (Shetty *et al.*, 2009) and yeast cell wall extracts applied to control the Barley pathogen *Blumeria graminis* (Reglinski *et al.*, 1994).

One well-studied elicitor is the fungal cell wall component chitin – an insoluble linear β-1-4-linked polymer of N-acetyl-D-glucosamine (GlcNAc) (Figure 50). Chitin is thought to be the second most ubiquitous natural polysaccharide on Earth (Dutta *et al.*, 2004) and due to its presence in crustacean exoskeletons, its commercial availability is high via waste from the shrimp fishing industry. The benefits of chitin application (or its de-acetylated form chitosan) in plant based agricultural systems are well documented with yield increases coming via improved germination rates, the stimulation of early growth and perhaps most importantly, the control of plant pathogenic microbes (El Hadrami *et al.*, 2010, Kumar, 2000, Rinaudo, 2006). The vast majority of published studies, however, do not specify a precise mechanism underpinning the recorded benefits, thus any perceived agricultural advantages may be due to indirect effects (such as chitin induced changes to the local soil microbiome) rather than via direct perception by plants.

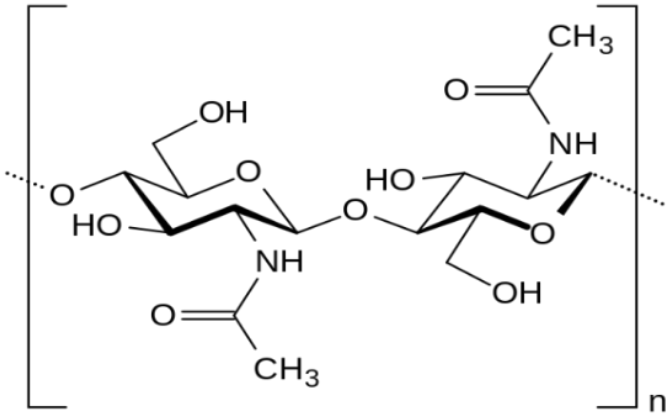

Figure 50: Structure of the chitin molecule, showing two of the N-acetyl-glucosamine units that repeat to form long chains in β-1-4 linkage.
Chitin is a derivative of the 6-carbon sugar glucose, but containing nitrogen in the form of an acetyl group bound to the second carbon atom.

Since the 2006 discovery of CEBiP, a chitin pattern recognition receptor (PRR) in rice (Kaku *et al.*, 2006) and subsequently in Arabidopsis (Miya *et al.*, 2007, Wan *et al.*, 2008), wheat (Sánchez-Vallet *et al.*, 2013) and barley (Tanaka *et al.*, 2010), studies have confirmed a mechanism for direct chitin perception in plants. It has now been shown that downstream signalling events are reliant on a chitin oligomer of more than 6 degrees of polymerisation; as well as the presence of the acetyl groups on each GlcNAc monomer.

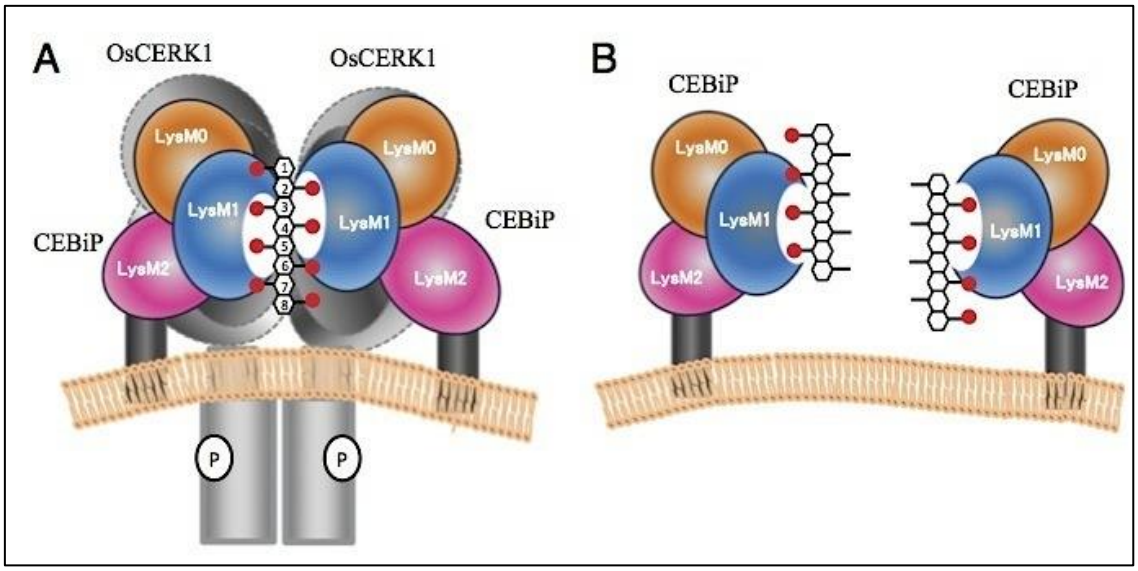

Figure 51: "Sandwich-type" dimerisation in CEBiP receptor
Two CEBiP molecules simultaneously bind to the *N*-acetyl motifs on a single chitooligosaccharide of at least 6 degrees of polymerisation (Hayafune *et al.*, 2014b).

Many studies have also now highlighted the relevance of chitin recognition for pathogen control by showing upregulation of well-known biomarkers of the active defence system such as reactive oxygen species (ROS), pathogenesis-related (PR) proteins and hormones such as salicylic acid (Wan *et al.*, 2008, Vander *et al.*, 1998, Nicaise *et al.*, 2009). However, due to the membrane bound nature of PRRs (Wan *et al.*, 2008, Vander *et al.*, 1998, Nicaise *et al.*, 2009). However, due to the membrane bound nature of PRRs (Macho and Zipfel, 2014), the leaf cuticle, for example, may restrict topical chitin applications from reaching these points of perception. Consequently, almost all evidence for direct chitin induced defences are based on methods that would not be useful in an agricultural context, such as leaf cell suspensions, injections, submersions and wounding (Cabrera *et al.*, 2006, Wan *et al.*, 2008, Paulert *et al.*, 2010, Vander *et al.*, 1998, Barber and Ride, 1988, Barber *et al.*, 1989).

Using agar-based, hydroponic and soil-based systems, this project will assess the ability of wheat plants to perceive chitin when applied using agriculturally appropriate methods, such as by root drenches and foliar sprays. Subsequently, disease assessments will be made in wheat using the foliar pathogen *Z. tritici*, studying variations in wheat disease tolerance due to factors such as method and timing of chitin applications to plants. Finally, negative effects of chitin use such as variations in germination, growth and development or damage due to direct phytotoxicity or chitin-induced transcriptome alterations will be assessed.

11.2. Methods

11.2.1. Crude chitin preparation (from shrimp shells)

For all crude chitin experiments, 10 g batches of chitin flakes from shrimp shells (C9213 Sigma, UK) were ground in a Krupp's Twin Blade Coffee Mill (Amazon.co.uk, Item model number: 203 – 425) for a period of 90 seconds. The resulting powder was sieved, with only particles sized below 500 μM used for subsequent experiments. Chitin was then autoclaved for 15 minutes at 121 °C.

11.2.2. Chitooligosaccharide preparations

Two chitooligosaccharide sources were used in these experiments. These were either purchased from Elicityl Oligotech (France) or received as a gift from Professor Heng Yin, Dalian Institute of Chemical Physics and henceforth subsequently annotated with 'Elicityl' or 'Heng'. For Elicityl solutions labelled 'dp7', only 1 μM of the chitooligosaccharide with 7 degrees of polymerisation was used. For Elicityl solutions labelled 'dpMix' a solution containing 1 μM each of 2, 3, 4, 5, 6 and 7 degrees of polymerisation was used. For 'Heng' mix, the relative chitooligosaccharide concentrations can be seen in the chromatogram in can also be seen labelled as 'COMix' or '1mMCO'. Heng chitooligosaccharides were used at 1 mM for all experiments, unless otherwise stated.

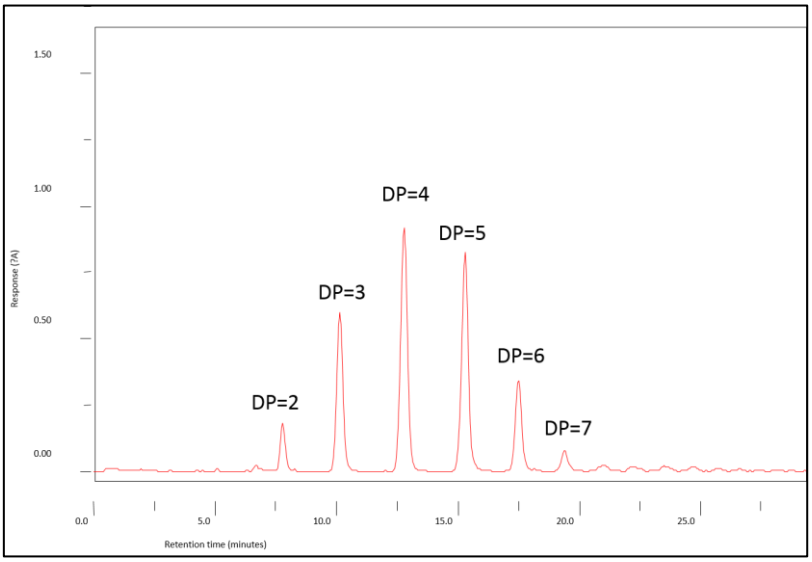

Figure 52: Chromatogram showing concentrations of chitooligosaccharides in CO mix.

11.2.3. Standard Growth Cabinet Conditions

All growth cabinet experiments were run in a Panasonic MLR-352-PE growth cabinet using a long day light cycle (16 hours light and 8 hours of darkness) at 85 % RH at 20 °C using a 4 (out of 5) light setting (~50 μmol light at soil level based on 4 levels in a growth cabinet).

11.2.4. Experimental repeats and statistical analysis

All experiments were carried out in triplicate unless otherwise stated. Residuals from all datasets were analysed for normality using a Shapiro-Wilk test. To achieve normality, non-normal datasets were transformed using either a square root, $(N + 1)^{\wedge 2}$ or natural log transformation. Parametric or nonparametric tests were then carried out accordingly dependent on achievement of normality. Data from repeated experiments were pooled only after running a two-way ANOVA to show that the individual experiments/trials did not significantly differ in their effects on the response variable.

11.2.5. Photography and Scanning

All photography was carried out using a Nikon D7000 with an 18 – 105 vr lens kit. Overhead images were taken using a Kaiser RS1 copy stand. Aperture and shutter settings differed depending on light conditions. Scanning of leaves for staining or disease assessments was carried out on a Canon CanoScan LiDE 210 at a setting of 600 dpi.

11.2.6. Wheat seeds

The seeds used in all experiments were *Triticum aestivum* cv. Consort winter wheat (RAGT). Seeds were surface sterilised before use by completely submerging for 20 minutes in 5 % (w/v) sodium hypochlorite. Seeds were then washed five times (60 seconds each wash while gently stirring) in sterile-distilled water before draining and drying completely by leaving uncovered inside a laminar flow cabinet for at least 60 minutes.

11.2.7. Germination

Germination assays were carried out using 2 pre-cut areas of autoclaved Wypall L20 (KIMBERLY-CLARK PROFESSIONAL, UK) placed inside a Sterilin™ 100 mm Square Petri Dish (Thermo Scientific™, UK). 25 seeds were sown, crease side down, within the petri dishes in a grid at equal distances so to remove contact effects between germinating seeds. Petri dishes were then watered with 7 ml of respective solutions, sealed using para film and incubated using the standard growth cabinet conditions (Methods 2.2). Germination was

assessed every 24 hours with seeds being scored as germinated when the radicle was fully emerged and the hypocotyl was > 5 mm in length.

11.2.8. Soil as a growth medium

In all soil-based experiments seived (10 mm) John Innes No. 2 soil was used. Two seeds were placed in each cell of a 24 cell insert and loosely covered. Trays were watered with 750 ml distilled water and left uncovered for 5 days to germinate under standard growth cabinet conditions (Methods 2.3).

11.2.9. Hydroponic growth medium

All plants grown hydroponically were first grown in soil and then removed after 10 days. Roots were subsequently completely submerged into 0.5 * Hoagland's Solution (H2395, Sigma, UK) within 50 ml Falcon tubes or 250 ml glass duran bottles. Plants were left in solution for 72 hours prior to any subsequent experiments.

11.2.10. Agar as a growth medium

All agar media based experiments were carried out using 7.5 g/l agar and 2.15 g/l Murashige and Skoog Basal Salt Mixture (M5524, Sigma, UK). Solutions were autoclaved for 15 minutes at 121 °C and cooled to 55 °C in a water bath before being mixed with chitin preparations and poured into individual 15 ml centrifuge tubes to set for a period of 2 hours. Seeds were sown, crease side down and tubes were sealed with their lids and incubated for 14 days using standard growth cabinet conditions (Methods 2.3). Lids were removed after 5 days.

11.2.11. Crude Chitin Suspension Agar

Falcon tubes were filled with desired quantity of crude chitin powder preparation (Methods 11.2.1) and topped up to 50 ml with a 55 °C solution containing 7.5 g/l agar and 2.15 g/l MS Basal Salt Mixture (M5524, Sigma, UK). Tubes were placed in racks on their ends at

room temperature and left for 20 minutes until agar had just started to set. Falcon tubes were then shaken vigorously to distribute the chitin homogeneously in the agar. Tubes were tapped from the base to remove excess air bubbles and left to solidify at room temperature until needed (at least 2 hours). Seeds were then sown, crease-side down, onto the agar and incubated for 14 days using the standard growth cabinet conditions (Methods 2.3).

11.2.12. Chitooligosaccharide applications for superoxide staining

Chitooligosaccharides were applied to 14-day old plants that had been grown for 11 days in soil and a further 72 hours in 0.5 * Hoagland's solution. Applications were completed in 3 ways: (i) Leaf sprays, carried out using an Iwata Revolution SAR airbrush using elicitor solutions mixed with 0.01 % Silwet L-77 (Momentive Specialty Chemicals, UK). (ii) Submergence assays, carried out by removing leaves from plants with scissors and submerging in 50 ml Falcon tubes containing elicitor solutions. (iii) Root drenches, carried out by submerging the root systems of plants in 50 ml Falcon tubes containing elicitor solutions. All assays were carried out for 1 hour unless otherwise stated.

11.2.13. NBT staining for superoxide

Leaves were harvested and transferred to 15 ml centrifuge tubes containing 0.1 % (w/v) 3, 30- nitroblue tetrazolium (N5514 Sigma, UK) in demineralised water. Leaves were left to stain for 2 hours, in the dark, and immediately removed into 100 % methanol to clear. Leaves that retained colouration for more than 24 hours were cleared completely by boiling in methanol at 68 °C. Percentage of leaf stained and stain colour intensity was assessed using Adobe Photoshop®.

11.2.14. *Z. tritici* leaf inoculations

Z. tritici IPO323 was used for all inoculations. The fungus as maintained in glycerol at -80 °C. For use, *Z. tritici* was streaked onto YPD agar (Y1500, Sigma, UK) and cultured at 20 °C for five days. Cells were scraped from the agar and suspended in 0.1 % (v/v) tween. Cell

count per ml was estimated using a haemocytometer and adjusted to 5 x 10^6 cfu / ml. *Z. tritici* cell suspension was applied to plants by leaf application with a paintbrush until leaves showed complete coverage with liquid. Inoculated plants were placed in clear autoclave bags for the first 5 days and maintained in growth chambers with standard growth cabinet conditions (Methods 2.3).

11.2.15. Chitooligosaccharide applications for disease trials

Chitooligosaccharides were applied to plants either as a root drench (10 ml of chitooligosaccharide was watered into the soil of plants); or as a foliar application (paint brushed onto leaves in 0.1 % tween). Solutions were applied 24 hours before *Z. tritici* inoculation, as well as 6 days and 10 dpi.

11.3. Results

11.3.1. Superoxide production after chitin foliar spray

To assess whether a foliar spray of chitin is adequate to induce recognition and response, an experiment was conducted which looks for changes in reactive oxygen species (superoxide), molecules which are known to be upregulated after elicitor perception. Foliar applications are the primary method of chemical application in crop based systems and thus this experiment was conducted to assess if it is viable for chitin to be applied this way. 14-day old plants were sprayed with an elicitor solution at a rate of 1 ml per plant until leaf surfaces were saturated and plants were left for either 1 hour, or 2.5 hours.

Both significant upregulation and reduction of superoxide were witnessed in plants sprayed with chitooligosaccharide solutions. Results for this experiment were not be replicated consistently (Figure 53).

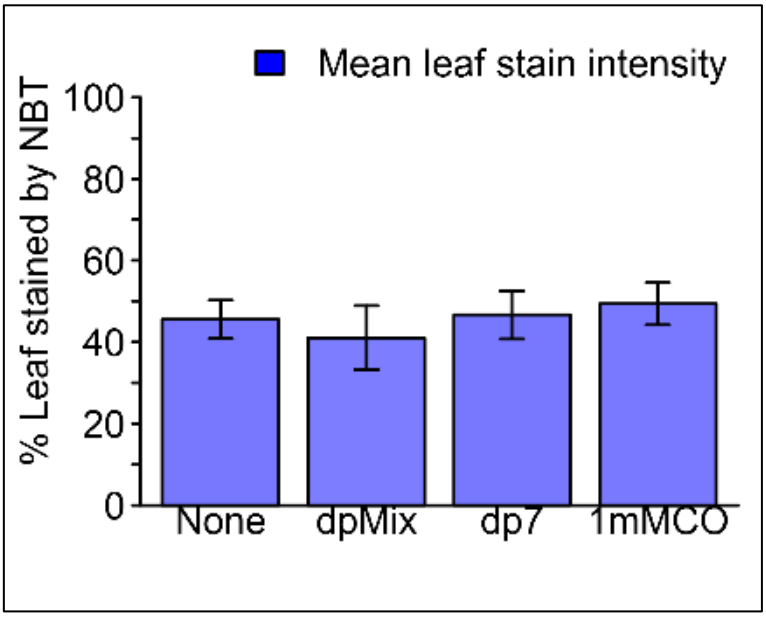

Figure 53: The effect of CO foliar sprays on leaf superoxide production 1-hour post-spray. Data are from 1 representative experiment from 3 independent experiments. Values are means (n = 8). Error bars represent SE. Significant results are indicated by *, ** or *** (α = 0.05, 0.01 or 0.001, respectively). Darker blue shades indicate more intense staining.

11.3.2. Superoxide production in leaves after root drench

The insoluble nature of long chain chitin indicates that a root drench may be an optimal method of delivery in agricultural systems. It is also likely that foliar sprays will lead to run off into soils, therefore assessing the likelihood of chitin perception by roots is important if chitin is to be successful agriculturally. This experiment was conducted using hydroponic media to remove any possible indirect effects from soil microbiota. After an initial 11 days growth in soil, plants were grown for 72 hours in 0.5 * Hoagland's solution before being carefully moved to solutions containing elicitors.

Results for this experiment were mixed. Significant upregulation and reduction of superoxide were both witnessed in plants grown in all chitooligosaccharide solutions (One-way ANOVA, $F = 35.7$, $MSQ = 11998.34$, p-value < 0.001, and One-way ANOVA, $F = 4.91$, $MSQ = 2328.65$, p-value $= 0.008$). Both results suggest that chitin can be perceived by wheat roots during early growth and trigger changes in ROS production but the reasons for different results are currently uncertain. Subsequent experiments showed no significant differences in superoxide production after 1 hour (Figure 54).

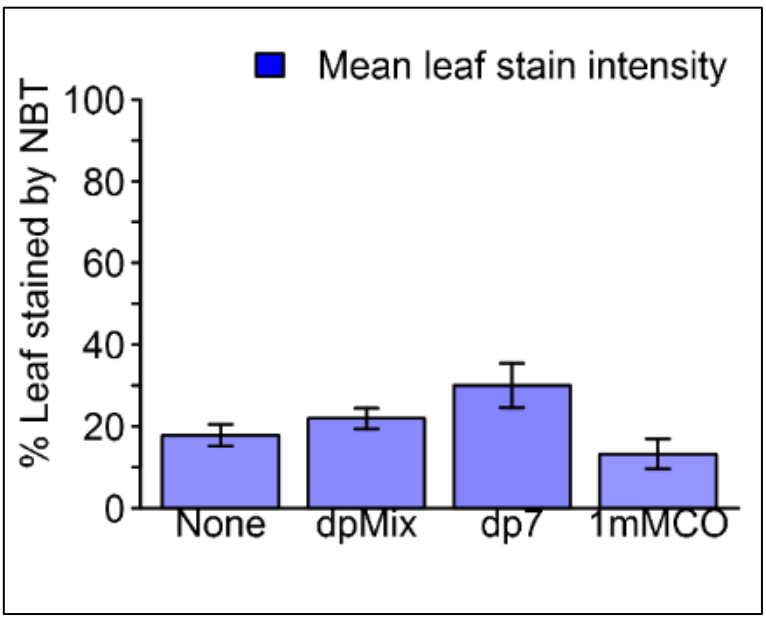

Figure 54: The effect of root applied COs on leaf superoxide production after 1 hour
Data are from 1 representative experiment from 3 independent experiments. Values are means (n = 7). Error bars represent SE. No significant differences were observed between trials (Students *t*-tests, p-value > 0.05).

11.3.3. Superoxide production after detached leaf submergence

To evaluate chitin recognition in leaves, a submerged leaf assay was carried out on detached leaves for 1 hour and superoxide production was assessed.

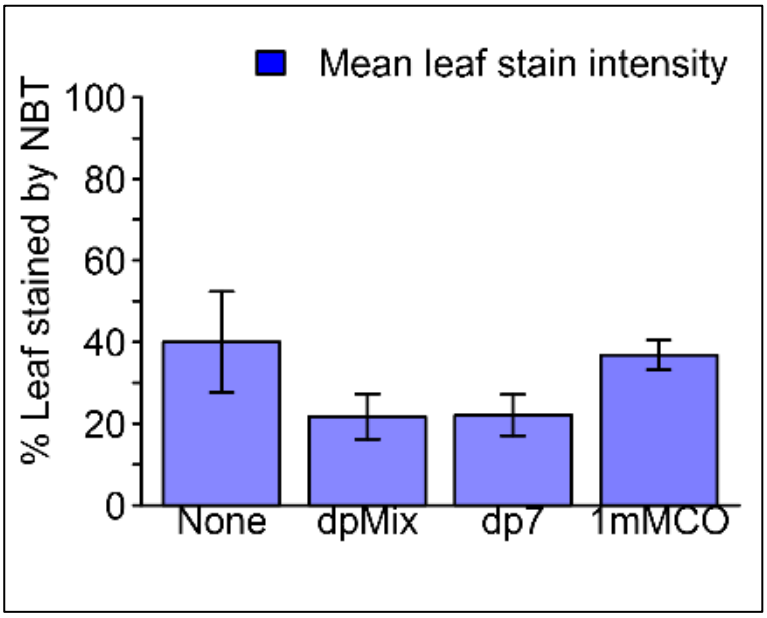

Figure 55: Leaf Superoxide production after detached leaf submergence shown by NBT stain 1 hour after submergence – Trial 3. Values are means (n = 6). Error bars represent SE. Results show no significant differences in superoxide production between treatments.

Results indicate a reduced production of the superoxide ion in plants submerged in Elicityl dpMix solution at 1 μM concentration. This result however, was only witnessed in two out of three trials (p-value = 0.041 and p-value = 0.034 respectively, *t*-tests). A numerical difference was witnessed in the third trial, but this was not significant (p-value = 0.419).

11.3.4. Disease control: Paintbrush Foliar Application

Following the result that chitin affects superoxide production when applied to leaves (Results 11.3.1), an experiment was conducted to determine whether this would impact the development of *Z. tritici* disease in wheat. Although the results show significant differences between pycnidia per leaf in the untreated and chitooligosaccharide treated plants, there are also large numeric differences in the tween only control (Figure 56).

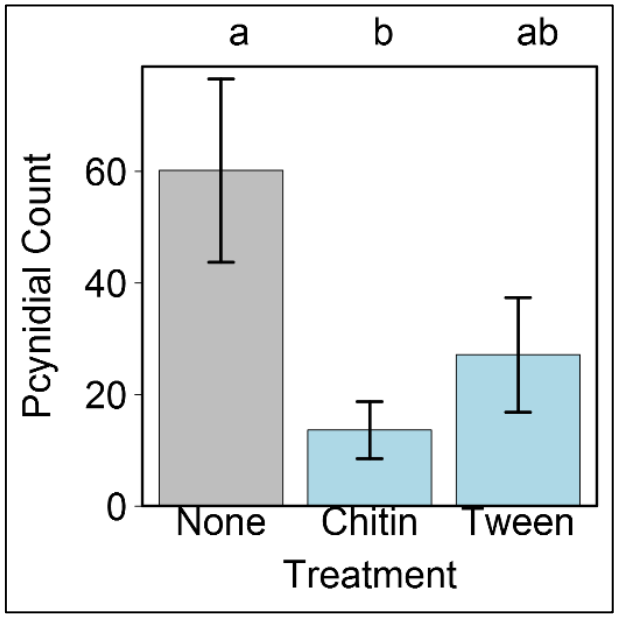

Figure 56: Pycnidia per leaf after treatment with chitooligoacharide solutions
Pycnidia number per leaf infected with *Z. tritici* at 21 days post-infection after treatment with chitooligoacharide solutions applied using a paintbrush on 1 day pre inoculation and days 6 and 10 post-inoculation. Values are means (n = 19). Error bars represent SE. The effect of treatment on pycnidial count was significant: Kruskal-Wallis rank sum test: chi-squared=6.89, df = 2, P = 0.032. Treatment data were not from a normal distribution. Letters indicate significant differences between treatment groups as determined by post-hoc Tukey test; p-value < 0.05.

11.3.5. Disease control: Root Drench

Following the result that chitin seems to be successfully perceived when applied as a root drench, an experiment was conducted to determine whether this would have an effect on the development of *Z. tritici* disease symptoms in wheat. The expensive nature of the Elicityl chitooligosaccharide product led us to only run this experiment using the 1 mM Heng chitin along with a second solution of 1 mM Heng chitosan oligosaccharides. Results have been inconsistent, with chitosan showing reduced disease in experiment number 1 but no significant differences were seen in the first experimental repeat (Figure 57).

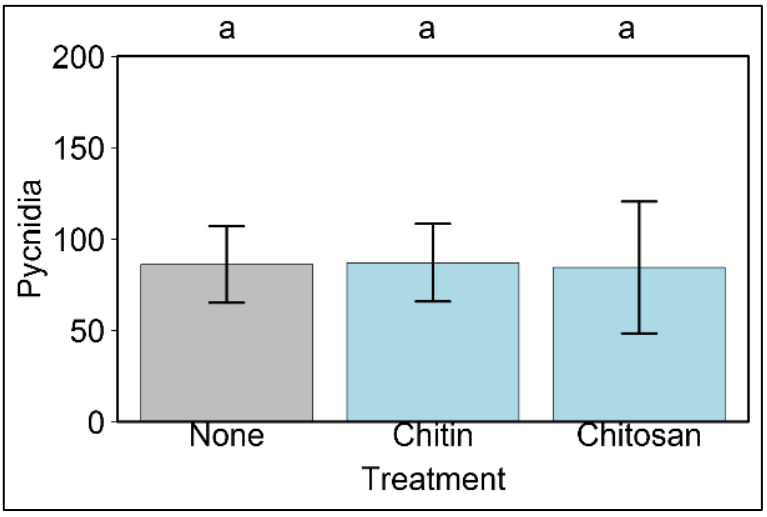

Figure 57: Pycnidia per leaf after treatment with an elector root drench
Pycnidia number per leaf infected with *Z. tritici* at 21 days post-infection after treatment with an elector root drench on 1 day pre inoculation and days 6 and 10 post-inoculation – experiment 2. Values are means (n = 29). Error bars represent SE. The effect of 3-Chitosan on Pycnidia was not significant: Kruskal-Wallis rank sum test: chi-squared=3.27, df=2, P=0.195. Treatment data were not from a normal distribution.

11.3.6. Assessing wheat development: Germination

The possibility of increased disease protection via plant defence priming may, due to the increased energetic costs of defence gene transcription, lead to negative effects on plant growth and development such as germination. This led us to examine the possibility of changes to wheat seed germination rates when grown in either water only or chitooligosaccharide solutions.

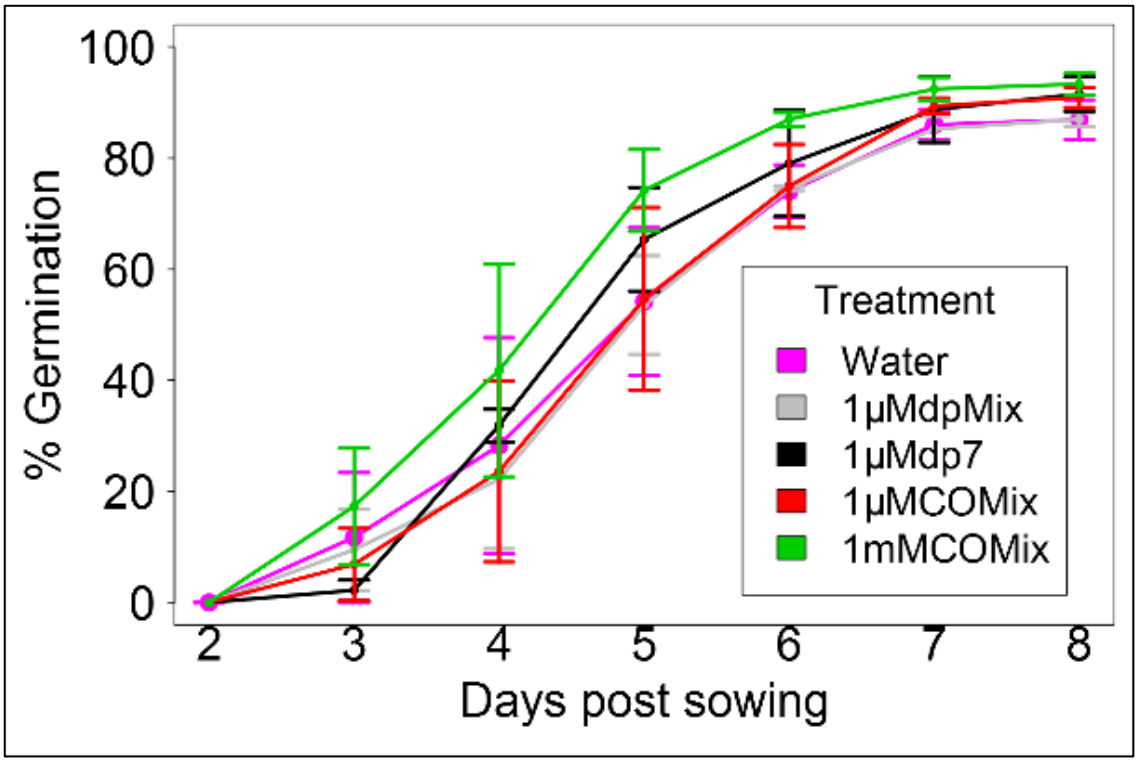

Figure 58: Germination Assay using both Elicityl (dpMix ad dp7) and Heng (COMix) chitooligosaccharide solutions.
Values are means (n = 3). Error bars represent SE. The effect of chitooligosaccharides on Total Germination was not significant: One-way ANOVA, F=1.34, MSQ=24.75, P=0.32. There were also no significant differences in total germination on any of the days assessed.

Results of germination assays showed no significant effects of any chitooligosaccharide mix tested with regards to total seed germination (Figure 58, One-way ANOVA, F=1.34, MSQ=24.75, P=0.3). There was also no effect of increase rate of germination as was tested on each day post-sowing. These results indicate that the use of chitooligosaccharides in growth media have no significant direct effects on wheat seed germination. Further studies will also need to assess effects on post-germination growth.

11.3.7. Assessing Wheat Development in Crude Chitin Agar

The possible energetic costs of chitin recognition and subsequent plant defence induction led us to assess changes to seedling development when plants are grown within a crude chitin suspension agar. To remove any indirect effects from soil-based microbiota, agar was used as a medium for plant growth with insoluble chitin powder suspended within it.

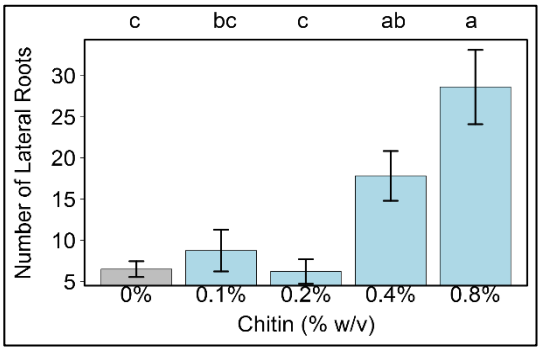

Figure 59: Lateral root count of wheat plants grown in different concentrations of crude chitin suspension agar after 14 days.
Values are means (n = 15). Error bars represent SE. The effect of chitin on Number of Lateral Roots was significant: One-way ANOVA, F=10.29, MSQ=1950.93, $p < 0.001$. Data were transformed using a square root transformation. Letters indicate significant differences determined by Tukey test; $p < 0.05$.

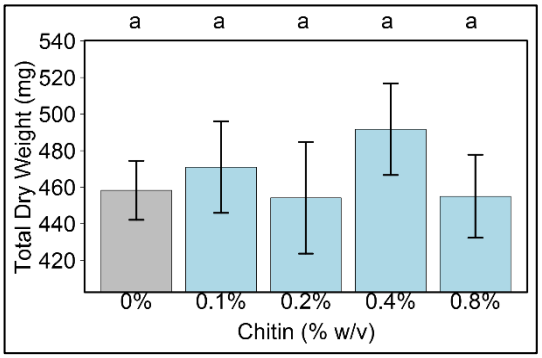

Figure 60: Total plant dry weight of wheat plants grown in different concentrations of crude chitin suspension agar after 14 days.
Values are means (n = 15). Error bars represent SE. The effect of chitin on Total Dry Weight (mg) was not significant: One-way ANOVA, F=0.45, MSQ=5548.91, P=0.771.

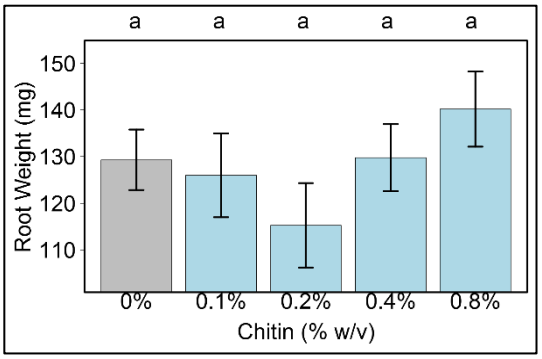

Figure 61: Root dry weight of wheat plants grown in different concentrations of crude chitin suspension agar after 14 days.
Values are means (n = 15). Error bars represent SE. The effect of chitin on Root Weight (mg) was not significant: One-way ANOVA, F=1.1, MSQ=1537.62, P=0.360.

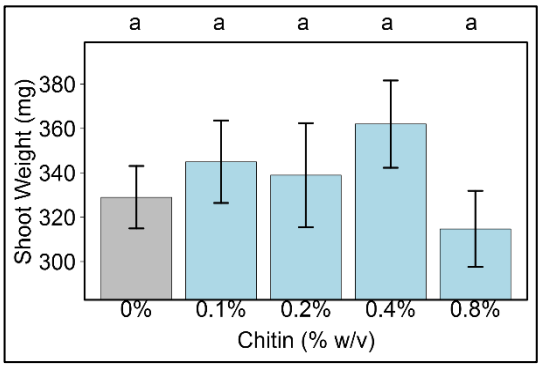

Figure 62: Shoot dry weight of wheat plants grown in different concentrations of crude chitin suspension agar after 14 days.
Values are means (n = 15). Error bars represent SE. The effect of chitin on Shoot Weight (mg) was not significant: One-way ANOVA, F=0.97, MSQ=7182.81, P=0.425.

Results display a significant increase in lateral root production with increasing concentration of chitin (Figure 59, One-way ANOVA, F=10.29, MSQ=1950.93, p-value < 0.001). However, total dry weight (Figure 60), root and shoot dry weight (Figure 61, Figure 62 respectively) were unaffected by the concentration of chitin suspended in the agar. These results suggest that the presence of chitin may indeed affect the development of the root structure during early growth by inducing lateral root growth development. However, it is documented that fragments in soil can lead to auxin induced lateral root induction (Reed *et al.*, 1998): a feature of successful nutrient scavenging, therefore insolubility of the chitin powder may be the reason for the witnessed differences therefore an experiment will be run to assess this.

11.3.8. Assessing wheat growth in chitooligosaccharide agar

Based on the previous results of morphological differences in root growth with wheat grown in an insoluble chitin suspension agar (Figure 59), a subsequent experiment was conducted using soluble chitooligosaccharides. This was achieved diluting both Elicityl and Heng chitooligosaccharide mixes in agar. In these experiments, no lateral roots were recorded over a 14-day growth period regardless of chitooligosaccharide (both Elicityl and Heng) concentration (data not shown). However, at high concentrations of Heng chitin, significant reductions in root growth (length) were witnessed (Figure 63). Interestingly, shoot growth was not significantly affected by the reduced root growth over a 14-day period (Figure 64).

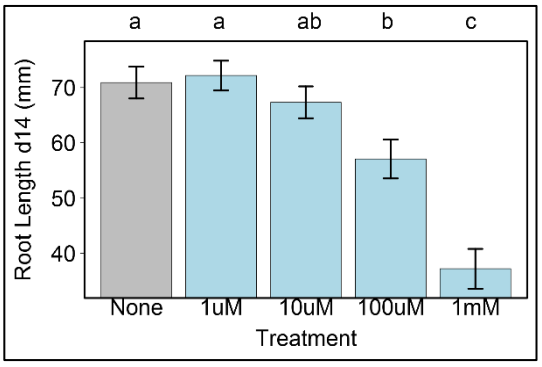

Figure 63: Root length of wheat plants grown in different concentrations of Heng chitin after 14 days.
Values are means (n = 13). Error bars represent SE. The effect of Heng chitooligosaccharide on Root Length (mm) was significant: One-way ANOVA, F=20.74, MSQ=2788.45, p-value < 0.001. Letters indicate significant differences between treatment groups as determined by post-hoc Tukey test; p-value < 0.05.

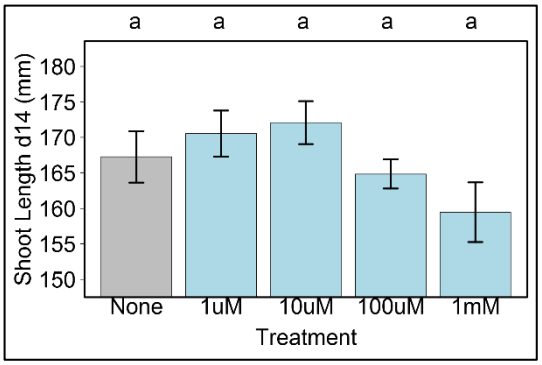

Figure 64: Shoot length of wheat plants grown in different concentrations of Heng chitin after 14 days.
Values are means (n = 13). Error bars represent SE. The effect of Heng chitooligosaccharide on Shoot Length (mm) was not significant: One-way ANOVA, F=2.19, MSQ=324.96, P=0.08.

11.4. Discussion

The recognition of chitin by plants has been shown to induce a form of defence named PAMP triggered immunity (PTI) – a collection of defensive reactions which, via the upregulation of reactive oxygen species (ROS), calcium influx and MAPK phosphorylation cascades, leads to the upregulation of genes which collectively protect the plant from foreign invaders (Zipfel, 2009, Schwessinger *et al.*, 2011, Chisholm *et al.*, 2006). The legitimacy of chitin as an elicitor of PTI in monocots is not in question (Shimizu *et al.*, 2010, Hayafune *et al.*, 2014b, Cao *et al.*, 2014, Liu *et al.*, 2012, Wan *et al.*, 2008, Miya *et al.*, 2007), yet there are few studies which show that these induced changes can affect the development of a virulent pathogen.

One particularly relevant paper by Shetty *et al.* (2009) extracted and purified β-1-3-glucan fragments from the cell walls of *Z. tritici* and used them to elicit a PTI reaction in wheat strong enough to diminish disease symptoms at 14 dpi. The conclusion of this paper is to confirm that, although *Z. tritici* can mask its presence through the secretion of effector proteins, the addition of this elicitor of PTI 1 day before pathogen inoculation was enough

to redress the balance in the plant's favour, thus eliminating disease. The hypothesis follows that if β-1-3-glucan can achieve this result then the same is likely to be true for other *Z. tritici* PAMPs such as chitin, and if this is true, an economically and financially sustainable alternative to chemical pesticides may be found.

11.4.1. Mechanism of recognition

This study has aimed firstly to show that chitin fragments (chitooligosaccharides) are able to elicit a defence response in wheat. This was assessed via three separate methods of application: (i) a leaf submergence assay; (ii) a foliar spray; the most relevant method based on current agricultural practices; and (iii) a root drench; a method which may be used for smaller crops such as those grown under greenhouse conditions. In all three assays the production of the superoxide ion, a known early response of the PTI reaction (Torres, 2010), was used as the assessment for positive recognition.

Results in each of these assays were inconclusive – no experiments showed the same results when repeated in triplicate but each experiment did show significant differences between groups (Results 11.3.1). These results collectively suggest that chitin is in fact perceived and that it is having a significant effect on wheat plants, however, it is not clear if the response is triggering ROS or inhibiting ROS. Within plants there is a fine balance between the upregulation of genes controlling the triggering of different forms of the immune reaction and the balance of a healthy metabolism (Thomma *et al.*, 2011), therefore it may be the case that aspects of the application process differ between experiments and thus present different outcomes. It will be important to repeat these experiments, particularly looking at different time-points post-application.

Although there is strong evidence elsewhere for an ROS burst to occur within minutes of elicitor recognition (Torres, 2010), it is likely that this may differ dependent on the plants pre-elicited state (Apel and Hirt, 2004). Indeed, in the one experiment carried out at a later time course (2.5 hours post-leaf spray application) we see a significant upregulation of superoxide in the dp7 treatment only (p-value < 0.001, Students t-test) – a result that would perhaps be expected based on the knowledge that the chitin receptor needs a chitooligosaccharide greater than 6 monomers long in order to induce the receptor

dimerisation necessary for a downstream response (Hayafune *et al.*, 2014a, Liu *et al.*, 2012, Lee *et al.*, 2013, Vander *et al.*, 1998, Cabrera *et al.*, 2006). It may be the case that in the other elicitor solutions used, the concentration of dp7 is too low (degrees of polymerisation for Heng chitin can be seen in Figure 52) or that the smaller chitooligosaccharide fragments are binding to the chitin receptors and acting as allosteric inhibitors for the larger oligosaccharides (Petutschnig *et al.*, 2010). If successful chitin binding is taking place, it will also be important to look at changes to gene upregulation using methodologies such as RT-PCR.

11.4.2. Disease control

Two experiments have been carried out to assess PTI induced disease control via chitin. The first experiment was a foliar application using a paintbrush to cover leaves with elicitor solutions contained in 0.1 % tween 1 day before inoculation and at 6 and 10 days after. This experiment produced significant differences between the pycnidial counts on untreated leaves than those leaves treated with chitooligosaccharides with tween (Kruskal-Wallis rank sum test: chi-squared=6.89, df=2, P=0.032, Results 0). However, results also showed that there were reductions in leaves treated with a tween only negative control, implying that the method of painting a solution onto wheat leaves is enough to physically remove the pathogen from the leaf surface thus reducing disease incidence. This would follow recent evidence that *Z. tritici* lives mainly epiphytically during its latent stage (~ 10-14 days) before becoming endophytic and finally switching to its necrotrophic stage of nutrition (Fones *et al.*, 2017b).

The second method used was a root drench where chitooligosaccharides were applied to soil in liquid form 1 day before inoculation and at 6 and 10 days after. The results of two experimental repeats showed no difference between pycnidial counts of the water only and the Heng chitooligosaccharide treatment (Figure 57, post-hoc Tukey tests; p-value < 0.05). However, in one of the two trials chitosan application was found to significantly reduce pycnidia. In the case of chitosan, there are many papers which highlight the antimicrobial effects of chitosan application, thought to be via its interaction with negatively-charged phospholipids (El Hadrami *et al.*, 2010, El Hassni *et al.*, 2004, Rabea *et al.*, 2005). However, it is also thought to act as a plant defence elicitor depending on its degree of acetylation (Iriti

et al., 2011, Day *et al.*, 2004). It should be noted that if the chitosan is completely de-acetylated, as is the case for the Heng chitosan used in these experiments, the method of disease reduction must be different from that of chitin as the presence of the acetyl group on chitin is known to be essential for chitin receptor dimerisation: as shown in Figure 51 (Hayafune *et al.*, 2014b).

11.4.3. Chitin: Effects on wheat growth and development

PTI brings about wide scale elicitor dependent changes in hormones involved in the defence reaction such as salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) (Robert-Seilaniantz *et al.*, 2011). Subsequent physiological changes include ROS production (Nars *et al.*, 2013, Ning *et al.*, 2004, Cabrera *et al.*, 2006), cell wall lignification (Barber *et al.*, 1989, Hermans *et al.*, 2010), callose deposition (Kauss *et al.*, 1989) and periderm formation (Beckman, 2000); adaptations designed to halt pathogen movement without the use of the hypersensitive response. Upregulated defence genes code for an array of pathogenesis related (PR) proteins; including antimicrobial chemicals (Hammerschmidt, 1999, Ren and West, 1992) and degradative enzymes such as peroxidases, proteases and chitinases (Linthorst and Van Loon, 1991, Lin *et al.*, 2005).

The elements of this defensive reaction may be strong enough to halt infection before the invader microbe becomes established (Henry *et al.*, 2012). However, it is thought to carry high fitness costs (Katagiri and Tsuda, 2010). Therefore, it should be noted that inducing PTI through the addition of chitin may not prove agriculturally positive in a period of the growing season when pathogen challenge is low.

Initially, positive effects on lateral root growth were witnessed when plants were grown in the crude chitin suspension agar (Figure 59), however, these results were not repeated when high concentrations of soluble chitin were used in agar preparations. This indicates that lateral root growth increases were likely not due to the chitin directly but rather due to physical differences imparted by the relatively large insoluble chitin fragments – this may be a knock on effect of auxin controlled obstacle avoidance in roots (Reed *et al.*, 1998, Grieneisen *et al.*, 2007). Conversely, results in soluble chitooligosaccharide agar show reduced root growth (length) at high concentrations (One-way ANOVA, F=20.74, MSQ=2788.45,

p-value < 0.001, Figure 63), indicating that if Heng chitin was to be used as an agricultural additive, the concentration used would need to be closely monitored to avoid stunting seedling development. Further tests will be run to assess more long-term growth under these conditions, particularly to assess the knock-on effects on above ground development. Germination was not found to be negatively affected, (One-way ANOVA, F=1.34, MSQ=24.75, P=0.32, Figure 58).

These results suggest that if these root growth reductions are due to PTI rather than a directly phytotoxic effect of the Heng chitin preparation used, finding both optimal elicitor concentrations, and a temporal range in which defences should be induced, is extremely important if PTI is to be used successfully in agriculture. More work is needed to determine if these effects are due to chitooligosaccharides being directly toxic to roots.

12. Appendix 3: *Trichoderma* plant growth promotion

12.1. Abstract

Following positive results in an MSc Food Security project, a series of experiments were carried out to assess wheat growth promotion using four *Trichoderma* species isolated from wheat fields. Results showed no significant differences in total, root or shoot growth. ITS sequencing was also carried out on these isolates. *Trichoderma hamatum* GD12, a strain known to induce beneficial growth effects in dicotyledonous plants such as lettuce (Studholme *et al.*, 2013), also showed no significant effects when applied to the soils of wheat.

12.2. Results

12.2.1. Total plant dry weight

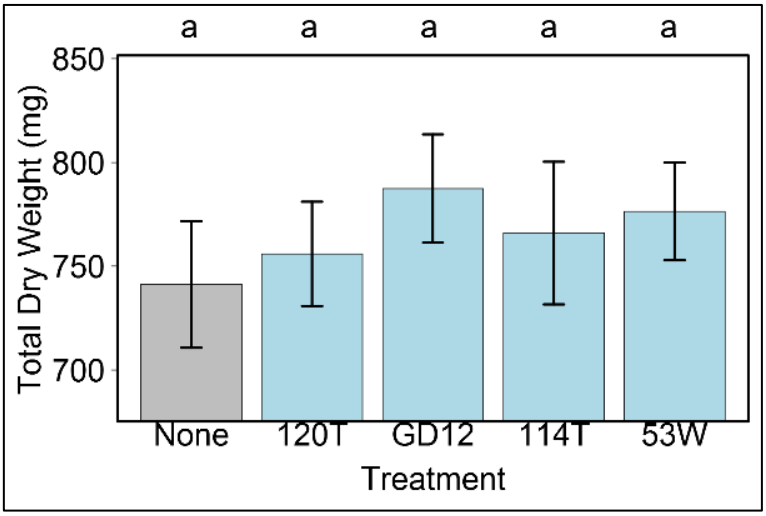

Figure 65: Total plant dry weight after 21 days after addition of *Trichoderma* to soil
Differences in Total Dry Weight (mg) were not significant: One-way ANOVA, F=0.4, MSQ=4797.67, P=0.806.

12.2.2. Total root dry weight

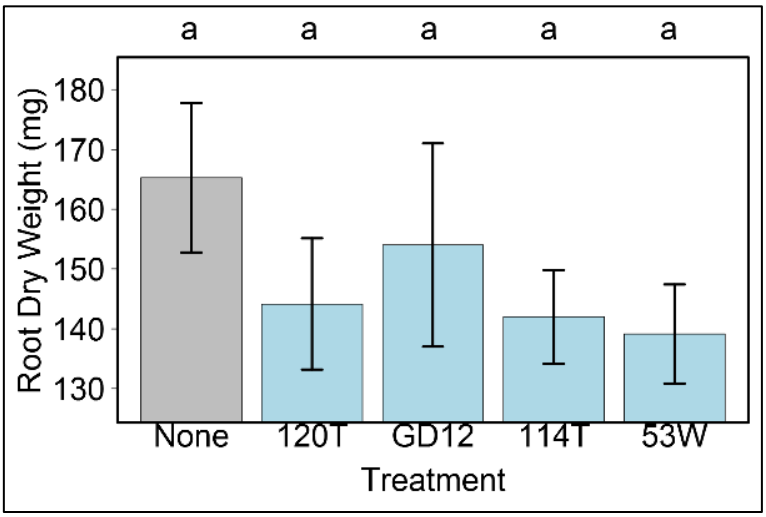

Figure 66: Total root dry weight after 21 days after addition of *Trichoderma* to soil
The effect of treatment on Root Dry Weight (mg) was not significant: One-way ANOVA, F=0.82, MSQ=1722.58, P=0.514To achieve normality, Treatment data were transformed using a square root transformation.

12.2.3. Total shoot dry weight

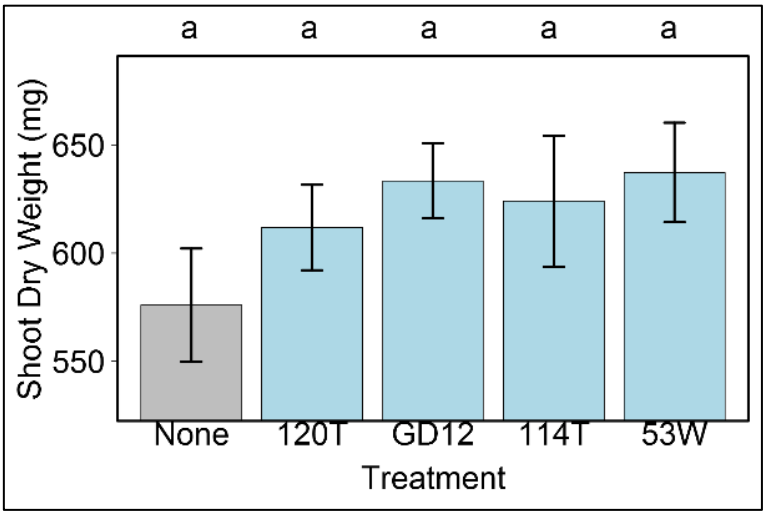

Figure 67: Total shoot dry weight at 21 days after addition of *Trichoderma* to soil
The effect of treatment on Shoot Dry Weight (mg) was not significant: One-way ANOVA, F=1.08, MSQ=9139.86, P=0.373.

12.2.4. *Trichoderma* Isolates (ITS Sequencing)

- WK71W: *Trichoderma harzianum* strain VSL 291
- WK114W: *Trichoderma velutinum* strain ZQ3206
- WK97W: *Trichoderma hamatum* isolate SZMC 20785
- WK53W: *Trichoderma paraviridescens* strain 6804-R7
- WK34W: *Trichoderma harzianum* strain TR274
- WK120T: *Trichoderma velutinum* isolate IIc2a

13. References

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