# EFFICACY OF FUNGICIDES ON COEXISTING *LEPTOSPHAERIA* SPP. CAUSING PHOMA STEM CANKER ON WINTER OILSEED RAPE

Thomas Richard Sewell, B.Sc., M.Sc.

Submitted to the University of Hertfordshire in partial fulfilment of the requirements of the degree of Doctor of Philosophy

School of Life and Medical Sciences, University of Hertfordshire, Hatfield, Herts, AL10 9AB

November 2016

## Abstract

Phoma stem canker is a disease of oilseed rape (*Brassica napus*) caused by closely related plant pathogens *Leptosphaeria maculans* and *L. biglobosa*. It is an economically important disease, causing annual yield losses of approximately £770 million worldwide. When colonising oilseed rape, *L. maculans* and *L. biglobosa* exist in close proximity on the leaf, competing for resources as they move through the main leaf vein and into the stem. Fungicides are commonly used to decrease severity of phoma stem canker on oilseed rape. However, the efficacy and longevity of active chemicals is under threat from evolution of resistance in pathogen populations and government legalisation. Moreover, it has been suggested that both *L. maculans* and *L. biglobosa* differ in their sensitivity to azoles, and important class of fungicides that are used to control the disease through the inhibition of lanosterol 14-α demethylase (*erg11*, CYP51). This project aims to further understand the role that fungicides have in controlling phoma stem canker by investigating their efficacy *against L. maculans* and *L. biglobosa* in crops, *in vitro* and *in planta*. In field experiments, established in Cambridgeshire across four cropping seasons, the fungicide mixture penthiopyrad (SDHI) plus picoxystrobin (QoI) was as effective at controlling phoma leaf spotting and phoma stem canker in winter oilseed rape as prothioconazole (DMI), suggesting that both fungicides could be used to reduce phoma stem canker symptoms. The two pathogens differed in their growth rates *in vitro*, with *L. biglobosa* growing faster than *L. maculans* when untreated or treated with lower fungicide concentrations. Fungicide sensitivity assays suggest that L. *maculans* and *L. biglobosa* are both sensitive to DMI, SDHI and QoI fungicides and that differences between the species are minor. Prothioconazole and penthiopyrad + picoxystrobin had a similar efficacy on oilseed rape cotyledons colonised with either *L. maculans* or *L. biglobosa*. There was no difference between species on prothioconazole treated plants, although there was a difference between *L. maculans* and *L. biglobosa* when treated with 20 µg/ml penthiopyrad + picoxystrobin. Heterologous yeast expression of LmCYP51B and LbCYP51B with fungicide sensitivity testing of the yeast transformants suggests that LmCYP51B and LbCYP51B are similarly sensitive to azole fungicides flusilazole, prothioconazole-desthio and tebuconazole. These findings are supported by homology protein modelling, which predicts that LmCYP51B and LbCYP51B are structurally very similar, specifically at the azole-binding site. In conclusion, fungicides are still an effective control method for reducing phoma stem canker symptoms caused by *Leptosphaeria* species in the UK, and a useful tool to in the sustainable production of oilseed rape.

## Acknowledgements

Firstly, I would like to acknowledge the financial contributions made by DuPont Ltd UK, The Perry Foundation, The Felix Thornley Cobbold Agricultural Trust and The University of Hertfordshire, together with the 'in kind' contribution made by ADAS Ltd. Without your support, this work would not have been possible.

I am grateful to all members of the Crop and Environmental Protection Group at the University of Hertfordshire; it has been a pleasure to work with you over the past three years. More specifically, I would like to thank my supervisory team: Professor Bruce Fitt, Dr Henrik Stotz and Dr YongJu Huang. As mentors you have taught me a great deal about being an independent and mature scientist. I would also like to thank Coretta Kloppel and Dr Georgia Mitrousia for dragging me through field experiments, rain and shine, and Dr Pryank Patel, Gala Ramón Zamorano, Arohi Srivastava, Chinthani Karandeni Dewage and the technical team at the University for their support, discussions and ideas.

This project would not have been accomplished without the contributions made by Dr Bart Fraaije and Dr Nichola Hawkins of the fungicide resistance group at Rothamsted Research. I am indebted to them for allowing me to use their facilities and appreciate their time.

Support from all my friends and family has been incredibly important during my time as a PhD student. Particularly, I wish to thank the Cleobury Massive and Aberystwyth pals for their continuous friendship. I would like to thank Sticko, Carrie, Rosie and Zoe for their support throughout my studies. I would like to thank my oldest sister Andrea, who has always been there for me. I would like to thank Ronald and Denise Bigg for adopting me every weekend. Most importantly, I wish to thank Amélie Brackin for her unwavering support throughout my studies. I could not have done this without you.

Finally, I would like to dedicate this thesis to my mother, Susan Sewell, who sadly passed away during my childhood. My mother ignited my fascination of the natural world and unquestionably guided me along this biological journey.

# List of publications

Research publications

Sewell, T.R., Moloney, S., Ashworth, M., Ritchie, F., Mashanova, A., Huang, Y.J., Stotz, H.U. and Fitt, B.D.L., 2016. Effects of a penthiopyrad and picoxystrobin fungicide mixture on phoma stem canker (*Leptosphaeria* spp.) on UK winter oilseed rape. *European Journal of Plant Pathology*, 145, pp.675-685.

Conference proceedings

Sewell, T.R., Huang, Y., Stotz, H. and Fitt, B.D.L., 2016. Azole sensitivity in *Leptosphaeria* species: The role of lanosterol 14-α demethylase. *Brassica 2016, Melbourne, Australia.* Poster presentation. Abstract.

Sewell, T.R., Huang, Y., Stotz, H., Ashworth, M., Ritchie, F. and Fitt, B.D.L., 2015. The effect of fungicides on *Leptosphaeria biglobosa* and *L. maculans*, phoma stem canker severity and oilseed rape yield. *14th International Rapeseed Congress, Saskatoon 2015, Canada.* Oral presentation theme B. Abstract, p111.

Sewell, T.R., Huang, Y., Stotz, H., Ashworth, M., Walker, P., Ritchie, F. and Fitt, B.D.L, 2014. Effects of different fungicides on the severity of phoma stem canker. *BSPP Presidential Meeting 2014 , St Andrews*, *UK*. Poster presentation. Abstract, p8.

Sewell, T.R., Huang, Y., Stotz, H., Ashworth, M., Ritchie, F. and Fitt, B.D.L., 2014. Effects of different fungicides on development of phoma stem canker and oilseed rape yield. *11th Conference of the European Foundation for Plant Pathology, 2014, Cracow, Poland*. Poster presentation. Abstract, S10, P3, p350.

# Table of Contents

# List of Figures

# List of Tables

# List of Equations

## List of Acronyms

TE – Transposable elements

RIP – Repeat induced polymorphism

MBC – Methyl benzimidazole carbamate

DMI – Demethylation inhibitor

*Erg11* – Gene that codes for Lanosterol 14-α demethylase

CYP51 (Paralogs: CYP51A, CYP51B and CYP51C) – Lanosterol 14-α demethylase

SDH – Succinate dehydorgenase

UQ binding site – Ubiquinone-binding site

SDHI – Succinate dehydrogenase inhibtor

QoI – Quinone outside inhibitor

SHAM – Salicylhydroxamic acid

ABC transporter – ATP-binding cassette transporters

PCR – Polymerase chain reaction

QPCR – Quantative polymerase chain reaction

BLAST – Basic local alignment search tool

PDB – Protein data bank

SD – Selective media

GAL – Galactose

RAF – Raffinose

YUG37 – *Saccharomyces cerevisiae erg11* yeast expression strain

# Chapter 1 General introduction

## 1.1 Fungal plant pathogens

Fungi, from the domain *Eukaryota*, are an extremely diverse kingdom of organisms with an estimated 1.5 million species in existence worldwide (Hawksworth 1991, 2001). They occupy a wide range of habitats and succeed through the adoption of variable life-styles. One of these habitats is the tissue of another kingdom of organisms, *Plantae* (Figure 1.1). Fungal plant pathogens are so called because they cause disease in plants. Some common, well-documented fungal plant pathogens are listed in Table 1.1.

Fungi that utilise nutrients from plants do so using a variety of approaches, most notably through biotrophic, necrotrophic or hemibiotrophic nutrient uptake strategies. Obligate biotrophs, such as *Blumeria graminis* (powdery mildew) produce hyphae that penetrate into epidermal cells of plants where they develop nutrient-uptake structures such as haustoria. These are used to extract nutrients out of the plant cells at a slow rate that only weakens, and does not kill, the host plant (Oliver and Ipcho 2004; Stotz et al. 2014). Conversely, necrotrophs, such as *Botrytis cinerea*, cause much more destructive symptoms by producing macerating enzymes that quickly break down plant material, thus releasing nutrients for uptake. This strategy is much more damaging to the plant's health and over time results in the death of the plant (Oliver and Ipcho 2004; Prins et al. 2000). Table 1.2 compares some general characteristic differences between necrotrophic and biotrophic fungal pathogens. Hemibiotrophs, pathogens that adopt both endophytic and necrotrophic strategies during some stages of their life cycles, are also of importance, with *Leptosphaeria maculans* being a relevant example (Stotz et al. 2014).

Plant pathogens, and in general most fungi, have a complex reproductive strategy, with many species undergoing both sexual and asexual reproduction (Taylor et al. 1999). This combination of reproductive approaches results in a high frequency of genetic recombination. Combining this with the fact that fungi produce large numbers of spores, it is evident that intraspecific variation is substantial (Giraud et al. 2010). The adaptability factor of intraspecific variation is one of the most important difficulties affecting the control of agricultural plant pathogens. Just as many human pathogens evolve new strategies for infection (Dethlefsen et al. 2007), so do plant pathogens (Hammond-Kosack and Jones 1997).

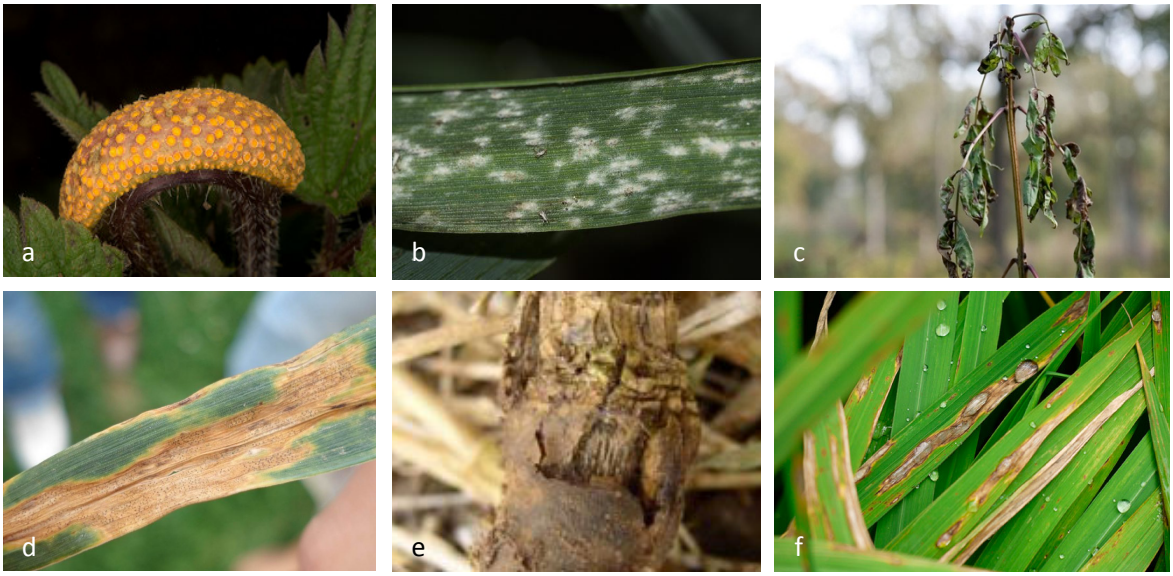



**Figure 1.1.** Six important plant pathogens affecting their relevant host plant: a) *Puccinia urticata* (nettle clustercup rust*)* affecting nettle (*Urtica dioica*); b) *Blumeria graminis* f. sp. *tritici* (powdery mildew*)* affecting wheat (*Triticum aestivum*); c) *Hymenoscyphus fraxineus* (chalara dieback of ash) affecting ash (*Fraxinus excelsior*); d) *Zymoseptoria tritici* (septoria) affecting wheat (*Triticum aestivum*); e) *Leptosphaeria maculans* (phoma stem canker*)* affecting oilseed rape (*Brassica napus)*; f) *Magnaporthe oryzae* (rice blast) affecting rice (*Oryza sativa*). *Images courtesy of Brain Eversham,* www.biopix.com*, telegraph.com, Chambre d'Agriculture,* Bayer Crop Science and 6th International Rice Blast Conference, *respectively.*

**Table 1.1.** Three economically important fungal plant pathogens. Importance decided by 495 authors, reviewers, editorial board members or senior editors of the journal Molecular Plant Pathology. Scientific and economic importance of fungal plant pathogens was decided by a vote, with *M. oryzae*, *B. cinerea* and *Puccinia* spp. at the top of a final list of 10 pathogens (Dean et al. 2012).

| Pathogen | Host | Importance | Reference |
|---|---|---|---|
| *Magnaporthe oryzae* | Rice | One-half of the world's population relies on rice as a staple crop | (Couch et al. 2005) |
| *Botrytis cinerea* | Various (> 200 plants) | Global control costs £1 billion | (Williamson et al. 2007) |
| *Puccinia* spp. | Wheat and barley | Novel epidemics ascribed to new aggressive lineages adapted to warmer environments | (Chen 2005) |

**Table 1.2.** General comparison between two types of plant pathogenic fungi with different nutrient uptake strategies. Modified from Oliver et al. (2004).

| Biotrophic | Necrotrophic |
|---|---|
| Derives energy from living cells | Derives energy from killed cells |
| Obligate | Non-obligate |
| Narrow host range | Wide host range |
| Secretes few cell-wall degrading enzymes | Secretes limited levels of lytic enzymes |
| Causes little damage to host | Produces toxins causing damage to host |
| Possesses haustoria | Generally lacks haustoria |
| Controlled by salicylate-dependent defence pathways | Controlled by jasmonate- and ethylene-dependent defence pathways |
| Controlled by host-specific gene-for-gene responses | Controlled by quantitative resistance genes |

Fungi that infect plants are of particular economic importance because of their threat to agricultural crops. Particularly devastating crop pathogens, such as *Magnaporthe oryzae,* can cause typical losses of 10-30% of potential yield (Couch et al. 2005; Dean et al. 2012; Kang et al. 2001). Using data from 2009 and 2010, it was estimated that pathogens affecting the five globally most important agricultural crops cause a deficit in yield that could feed 595 million people if disease severity was low (Fisher et al. 2012). With this in mind, combating crop pathogens with cultural, chemical and genetic innovations has become an important tool exploited by the agricultural industry. Projected world population increases to 9 billion by 2050 will add further strain on food production globally (Godfray et al. 2010).This in turn will affect the potential losses from both current and emerging diseases.

### 1.1.1 Plant-pathogen interactions

A notable feature of fungal plant pathogens is their interaction with the host environment they exist within. As this host is other living organisms (plants), it is inevitable that there are complex interactions between the two that have evolved over millions of years (Agrios 2005; Chisholm et al. 2006), just as there are complex interactions between humans and their resident microbes (Dethlefsen et al. 2007; Kinross et al. 2011). To defend themselves against invading pathogens, plants elicit defence responses that stimulate an innate immune system, which resists pathogen invasion (Hammond-Kosack and Jones 1997; Stotz et al. 2014). There are two types of resistance, namely qualitative resistance and quantitative resistance.

### 1.1.2 Qualitative resistance

Qualitative resistance, also known as race-specific resistance, was first described by Flor (1956) in *Linum usitatissimum* (flax). Using *L. usitatissimum* and *Melampsora lini* (flax rust), Flor identified a pathogen-host interaction that involves just a single pair of genes (Flor 1956). This single-gene interaction is dependent on gene products from both the pathogen and the host; the pathogen produces an effector whereas the host *R*-gene produces a protein receptor that interacts with the effector, sometimes as a receptor. When resistance occurs in the plant, the receptor, produced from the resistance gene (*R* gene) of the plant, recognises an effector produced from the corresponding *Avr* gene (avirulence) of the pathogen (Table 1.3). This recognition stimulates a defence response

**Table 1.3.** Table of gene-for-gene interactions between plant and pathogen. Only when a dominant allele receptor product interacts with a dominant allele effector product does resistance occur. All other scenarios result in disease. Modified from Agrios 2005.

| | | **Plant (receptor)** | |
|---|---|---|---|
| | | *R* (resistant) dominant | *r* (susceptible) recessive |
| **Pathogen (effector)** | *A (avirulent) dominant* | *AR* - **Resistance** | *Ar* - **Disease** |
| | *a (virulent) recessive* | *aR* - **Disease** | *ar* - **Disease** |

in the plant that limits the spread of the pathogen. Conversely, if the pathogen effector and the plant receptor do not interact then disease will occur (Flor 1971).

Plant breeding programs take advantage of major gene resistance by identifying and inserting novel resistance genes into the genomes of host plants. This practice is now widespread and a vital tool used in agriculture across the world, where it has shown much success in controlling crop pathogens without reliance on chemicals (Niks et al. 2011).

Nonetheless, the gene-for-gene interaction is founded on the coevolution of both pathogen and host, and as evolution is continuous, new pathogen lineages lacking the corresponding *Avr* genes will inevitably arise. When this happens, a breakdown in qualitative resistance occurs, resulting in disease epidemics (Li et al. 2003). Agricultural ecosystems are an ideal environment for the emergence of new plant pathogens or fitter lineages of established plant pathogens (Stukenbrock and McDonald 2008).

The fragility of the gene-for-gene interaction has been highlighted in the hemibiotrophic tomato (*Lycopersicon esculentum*) pathogen, *Cladosporium fulvum.* A single base-pair change in the *Avr4* effector gene, that was initially recognised by the host resistance gene *Cf4*, altered pathogen virulence, resulted in colonisation and disease (Joosten et al. 1994). This shows how malleable the gene-for-gene interaction is to mutational change and the influence this can have on host-plant interactions.

### 1.1.3 Quantitative resistance

Quantitative resistance is a complex non-host specific mechanism involving a polygenic response to the invading pathogen. Quantitative resistance does not provide complete disease control as qualitative resistance does and instead confers a partial resistance response. This usually means that disease still occurs in the plant but the epidemic is of a reduced severity compared to that on a host not expressing a similar polygenic response (Delourme et al. 2006; Huang et al. 2009; Huang et al. 2014b).

Little is known about quantitative resistance in comparison to qualitative resistance. Mapping of minor genes in regions designated quantitative trait loci (QTLs) has been used to further understand the resistance mechanism (Young 1996). Recent research comparing the combined effects of both qualitative and quantitative resistance over a five-year period has concluded that both strategies should be used in breeding programs (Brun et al. 2010). When the disease response using near-isogenic *Brassica napus* lines with several QTLs as well as various major resistance genes was compared to that of

other more susceptible cultivars, the combined minor and major resistance genes complemented each other, resulting in an increased durability of the major resistance gene.

### 1.1.4 Evolutionary potential of plant pathogens

Fungal pathogens evolve reduced sensitivities to fungicides or new mechanisms to overcome host plant resistance. The evolutionary potential of a population can determine the risk that that population will overcome a particular control method (Table 1.4). Pathogens that have large populations, mixed reproductive life cycles, high gene flow and high mutation potential are classed as at high risk of overcoming host-resistance or fungicide control (McDonald and Linde 2002). In contrast, pathogens that have small populations, are stringently asexual, have a low gene flow and low mutation rate are much less of a risk.

The evolutionary potential of plant pathogens is of particular importance when considering coexisting sibling species. If one species has a difference in sensitivity to a certain control method, but that control method is used to target both species, then the population structure of the pathogens may be altered. This phenomenon has been experimentally investigated using *Oculimacula yallundae* and *O. acuformis* and their differences in sensitivity to prochloraz. Mycelial growth tests on agar with different prochloraz concentrations showed that the fungicide affected the two pathogens differently. In theory, if the same response to mycelial growth takes place *in planta* then a population change may occur (Bierman et al. 2002; Fitt et al. 2006b).

## 1.2 Coexisting plant pathogens

### 1.2.1 Speciation

Speciation can be described as the division of one biological species from another (Mayr 1963, 1970, 1996). It is a mechanism of divergence that is recurrent in nature owing to evolution (Dobzhansky 1973). There are several known types of speciation. Figure 1.2 describes three commonly applied mechanisms of speciation, which are adopted by a wide range of organisms in all kingdoms of life. The shared characteristic between all three of the mechanisms described is the formation of a reproductive barrier. This barrier can be termed reproductive isolation. It is this isolation that nullifies the potential flow of

**Table 1.4.** Factors affecting potential evolutionary risk raised by plant pathogens. Many pathogens adopt a mixture of high and low risk potentials. Modified from McDonald and Linde (2002).

| **High risk of evolution** | **Lowest risk of evolution** |
|---|---|
| High mutation rate | Low mutation rate |
| Large population size | Small population size |
| High gene/genotype flow* | Low gene/genotype flow* |
| Mixed reproductive system | Asexual reproductive system |
| Efficient directional selection^ | Disruptive selection[+] |

* exchange of genes or genotypes among geographically separated populations

^ Uniform deployment of *R*-genes over a large area

+ Multiple deployment of *R*-genes rotated in time and space

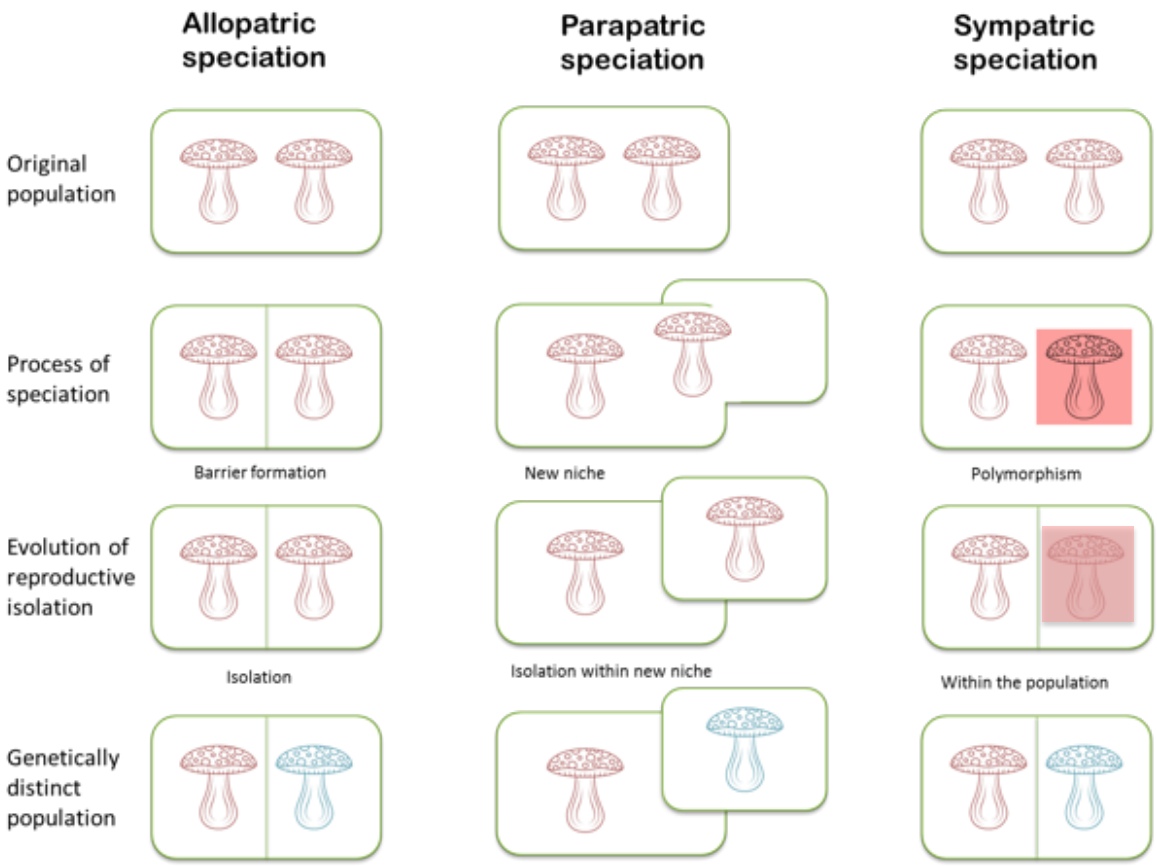



**Figure 1.2.** Three common mechanisms of speciation. Reproductive isolation has been represented using a green line separating the two populations. The genetic amalgamation event in sympatric speciation has been represented using a red square and labelled polymorphism. Modified from University of Miami. Accessed: 17 May 2014.

alleles between two populations, which in turn causes genetic divergence and differentiation between the genotypes within each population (Giraud et al. 2008; Mayr 1996; Rundle and Nosil 2005).

Dodd (1989) provided an experimental example of allopatric speciation *in vitro* using *Drosophila pseudoobscura*. Feeding a population of *D. pseudoobscura* on either starch-based or maltose-based medium, it was shown that after many generations, when the two food-restricted populations were introduced to one another, interbreeding between the two populations was non-existent and the food-adapted populations would breed only amongst themselves (Dodd 1989).

Reproductive isolation evolves from selection, genetic drift, hybridisation and alterations in chromosome number; its development is different depending on the mechanism (Coyne and Orr 2004; Giraud et al. 2008; Kohn 2005). For example, species undergoing allopatric speciation have no opportunity to reproduce, so reproductive isolation is slow and develops over time through mutations, genetic drift and localised adaptation. Species undergoing sympatric speciation, in comparison, can reproduce; therefore a strong and rapid selection that blocks interspecific crosses is required for speciation to occur.

These differing approaches can be used to investigate the mechanism of speciation adopted by different organisms. Giraud et al. (2006) exploited these using fungi in a comparative study of *Homobasidomycota* and *Ascomycota* species complexes amounting to 431 species pairs. *Homobasidomycota* species pairs in sympatry had a strong premating isolation, whereas allopatric species pairs displayed a greater affinity. The *Ascomycota* species complexes, conversely, showed a low level of reproductive isolation in both allopatric and sympatric species pairs. These results suggested that the group of *Ascomycota* species pairs selected had high mate recognition in sympatry but had possibly diverged due to a strong selection pressure, with host specialisation acting as the premating reproductive barrier (Giraud et al. 2006).

### 1.2.2 Speciation in ascomycete plant pathogens

Many fungal species have complex life-cycles that involve interactions with other host organisms. Therefore, it is understandable that speciation of host-dependent organisms or parasites is also rather complex. In ascomycete plant pathogens, the plant host plays an important role in a mechanism called ecological speciation. Ecological speciation can be described as the development of reproductive isolation through ecologically based divergent selection (Giraud et al. 2010; Rundle and Nosil 2005).

An example of potential ecological speciation has been identified among populations of *Venturia inaequalis*, the causal agent of apple scab disease. *V. inaequalis* is a hemibiotrophic filamentous ascomycete fungus that causes significant damage to trees of the genus *Malus* (Bowen et al. 2011; Guérin and Le Cam 2004). It is of particular economic importance to apple producers due to its effect on yield and visual appearance of the apples.

In this example, host-specificity has acted as an efficient barrier to genetically isolate two *V. inaequalis* populations within an orchard, where different apple tree cultivars with different resistance genes (*Vf* cultivar and non-*Vf* cultivar) are grown in sympatry. This differentiated population has developed because of the gene-for-gene interaction between pathogen and host.

Interestingly, Gladieux et al. (2011) found that, although the pathogen was now able to cause disease on *Vf* apple cultivars, it was unable to infect the non-*Vf* apple tree cultivars also present in the orchard. Therefore, host-specificity could be hypothesised as the restriction to gene flow. An investigation into this, compared with pre- or post-zygotic speciation mechanisms, showed that it was in fact host-specificity that restricted gene flow through the inability of *Vf* and *non-Vf* fungal populations to obtain nutrients, survive and thus breed on their non-host cultivar (Gladieux et al. 2011).

In this example, reproductive isolation has resulted from a strong host-specific selection, due to immigrant in-viability, which in turn has resulted in an allelic differentiation within a sympatric population (Giraud et al. 2010; Gladieux et al. 2011). This can be described as an ‘automatic magic trait’, which assumes that there is a pleiotropic relationship between divergent selection and non-random mating (Servedio et al. 2011).

Further work will shed light on whether host adaptation instigating pleiotropic reproductive isolation in line with the ‘magic trait’ hypothesis can be applied to other ascomycete plant pathogens that reproduce sexually within a host. It is likely that this mechanism of reproductive isolation, the precursor to ecological speciation, has been involved in the continuous emergence of closely related sibling pathogen species or linages that have an advantageous virulence over their target host (Gladieux et al. 2011).

### 1.2.3 Strategies in coexistence

One importance of speciation events, in relation to plant pathology, is the emergence of a new disease similar to one already in existence that is caused by a novel, genetically distinct, but closely related, species (Figure 1.5). These diseases may develop into an emergent threat if the novel pathogen is more virulent than its ancestor.

**Table 1.5.** Three examples of closely related, coexisting crop pathogens. Each pair of species cause either the same or very similar disease symptoms on their relative hosts.

| Pathogens | Host | Disease | Reference |
|---|---|---|---|
| *Leptosphaeria maculans* and *L. biglobosa* | Oilseed rape and other brassicas | Phoma stem canker | Fitt *et al*., 2006a, Mendes-Pereira *et al*., 2003 |
| *Mycospherella musicola* and *M. fijiensis* | Banana and plantain | Yellow and black sigatoka leaf spot/streak | Churchill, 2011 |
| *Oculimacula yallundae* and *O. acuformis* | Cereals | Eyespot of cereals | Bierman *et al*., 2002 |

In nature, there are many examples of closely related pathogens that exist in sympatry yet maintain their genetic identity. This differentiation may be maintained by epidemiological and ecological mechanisms that include host-pathogen, sibling, abiotic and biotic interactions. A slight difference in life cycle in either space or time could allow coexistence of sibling pathogens on the same host (Fitt et al. 2006b).

Eyespot disease of winter wheat, caused by sibling pathogens *O. yallundae* and *O. acuformis,* is an example of a disease caused by two closely related pathogens that coexist on the same host. Niche differences probably developed from biological dissimilarities between the two pathogens. In the UK, it is thought that *O. yallundae* progresses more rapidly than *O. acuformis* through the leaf sheaths (Bierman et al. 2002). Both *in vitro* and *in planta* investigations support this differentiation of growth. On artificial medium, *O. yallundae* has a greater mycelial growth rate than *O. acuformis,* and when tested on leaf sheaths *O. yallundae* was shown to have a greater penetration success rate than *O. acuformis*. In cereal crops there also appears to be a notable difference that is likely to be temperature-dependent (Fitt et al. 2006b). *O. yallundae* is more noticeable in early spring when temperatures are fairly low. *O. acuformis*, conversely, is more prevalent later in the season when the temperatures have increased. The combination of these three adaptive differences could allow the pathogens to coexist on the same host at different times and in slightly different niches, while both cause significant disease on winter wheat.

## 1.3 Plant pathogen invasion

Recently, numerous economically important agricultural crops have experienced invasive diseases caused by plant pathogen populations exploiting novel virulence. It can be hypothesised here that replacement of one pathogen population by a fitter, more invasive pathogen population can result from an altering of the genome through a mechanism such as mutation, sexual recombination, directional selection or genome expansion. It can therefore be suggested that a genome alteration, or an invasion from transposable elements, over a period of time, can result in a large-scale, often global, pathogen invasion due to population replacement.

### 1.3.1 Invasion on a small scale

Transposable genetic elements (TEs), sometimes referred to as ‘jumping genes’, are fragments of DNA that can autonomously move from one location in the genome to

another location. Since their identification, more than 50 years ago (McClintock 1951), transposons have been found in almost all prokaryotic and eukaryotic organisms, with fungi being no exception (Daboussi et al. 1992; Daboussi and Capy 2003; Muszewska et al. 2011).

There are two major classes of TEs: class I and class II. Class I TEs, also known as retrotransposons, synthesize cDNA using an RNA intermediate, which is governed by reverse transcriptase. Class II TEs, also known as DNA transposons, have two terminal inverted repeats and use a cut-and-paste mechanism to insert directly into the DNA (Daboussi and Capy 2003; Muszewska et al. 2011).

The genome has evolved some defence strategies against TEs. In fungi, three mechanisms have been identified: repeat induced polymorphism (RIP), methylation induced premeiotically (MIP) and quelling (Daboussi and Capy 2003). RIPs silence genes via extensive CG to TA polymorphic transitions (Selker 1997). MIPs have no polymorphisms and silence genes through methylation of nucleotides (Faugeron 2000). Quelling targets repeated mRNA sequences in the cytoplasm and either inhibits them or marks them for disintegration (De Backer et al. 2002). These mechanisms are used by the host genome to control the effect of TEs. Gene silencing of TEs protects against genome invasion by disrupting the replicative exploitation and autonomous nature of TEs, and by blocking possible gene rearrangement (Daboussi and Capy 2003).

TEs have the potential to modify a host's genome by transposing into or near active genes. This action can result in the partial or complete inactivation of genes and is instigated by both class I and II TE's (Daboussi and Capy 2003; Kidwell and Lisch 1997). Furthermore, the high frequency of LTR retrotransposons within fungal genomes (Muszewska et al. 2011), the knowledge that they contain replicative machinery and the ability to affect gene expression suggests that TEs could be an important factor in fungal evolution (Daboussi and Capy 2003). The importance of this in plant-infecting fungi is that TEs can influence the gene-for-gene interaction (Kang et al. 2001).

A notable example of this, involving fungal-plant interactions, is the development of virulence observed in *Magnaporthe oryzae* after the invasion of the class I TE *Pot3* (Kang et al. 2001). Rice blast caused by *M. oryzae* is a devastating crop disease of rice, causing serious yield losses to countries heavily reliant on the crop (Zeigler et al. 1994). An investigation done by Kang et al. (2001) showed that the insertion of *Pot3* into an essential promotor region of the disease-preventing effector gene, *AVR-Pita*, physically disabled the expression of this gene, which resulted in a disease-causing gene-for-gene interaction with rice cultivar Yashiro-Mochi. The movement of *Pot3* within the genome of

*M. oryzae* has consequently been shown to influence the diversification of the rice blast pathogen population, thus acting as a precursor to potential disease invasion (Kang et al. 2001).

TEs are not restricted to movement solely within a genome. Horizontal transmission, or interspecific gene transfer, of virulence factors is well described in bacterial pathogen populations, so it has always been assumed likely that the same mechanism is applicable to fungi (Rosewich and Kistler 2000).

An early example of interspecific virulence gene transfer was identified using wheat (*T. aestivum*) pathogen *Pyrenophora tritici-repentis*, the causal agent of tan spot on wheat. An investigation comparing the sequence of host-specific toxin *ToxA,* from *P. tritici-repentis* and that for the major wheat pathogen *Stagonospora* (syn. *Septoria*, teleomorph *Phaeosphaeria nodorum*)*,* identified a 99.7% similarity between the two. With such a high likeness both structurally (both having three exons and two introns) and within the sequence itself, it was possible to propose a recent common ancestry (Friesen et al. 2006). A further analysis of the two *ToxA* sequences identified a single haplotype in the *P. tritici-repenti* sequence and 11 haplotypes in the *S. nodorum* sequence. This differing diversity of nucleotides between the two can be used to classify evolutionary distances (Ridley 2004). The lack of variation in the *ToxA* sequence of *P. tritici-repentis,* therefore, suggests that it was a relatively recent introduction into its genome. This combination of sequence similarity, nucleotide diversity and the fact that other closely related species do not possess the gene, can be used to propose the interspecific transfer of the gene coding for *ToxA* from *S. nodorum* into *P. tritici-repentis* (Friesen et al. 2006).

Furthermore, the original description of *P. tritici-repentis* in 1902 stated that the fungus was a pathogen only occasionally and was predominantly a saprophyte. In 1941, however, the emergence of a new disease, probably caused by *P. tritici-repentis* was described in the USA, Australia and Africa (Friesen et al. 2006). The symptoms were those of *ToxA*-induced necrosis; a brown lesion with a yellow border.

In relation to this investigation, another example, this time involving a much larger transposable fragment, has also been shown to affect pathogenicity. Mobile pathogenicity chromosomes, known as lineage-specific genomic regions, are large sections of DNA that can span many chromosomes. They consist of multiple transposons as well as genes related to host-pathogen interactions (Ma et al. 2010).

An extensive collaborative analysis using the genomes of three *Fusarium* species (*Fusarium graminearum, Fusarium verticillioides* and *Fusarium oxysporum* f. sp.

*lycopersici*) concluded with the identification of a large lineage-specific genomic region that spanned four whole chromosomes. Experimentation, using two of the chromosomes, found that by transferring the chromosomes between *F. oxysporum* isolates, the fungus was converted from a non-pathogen into a pathogen, which in turn caused disease on the tomato host.

The consequences of such a large fragment of transportable DNA are far-reaching. In a single event, host-specific virulence genes and pathogenicity genes can be inserted into the genome of another organism, potential creating a novel pathogen. This novel pathogen may already be perfectly adapted to its local environment and would therefore flourish on its new host, potentially causing disease.

### 1.3.2 Invasion on the large scale

Pathogen invasion, causing novel disease on a specific host, can be the consequence of pathogen population replacement. Pathogen population replacement can generally be described as the emergence of a new fitter population that, depending on circumstances, may replace a presently established local or distant population. The new population may consist of either a new lineage of a current species or a whole new species. In plant pathogenic fungi, the improved fitness is likely to be host-specific (Chen et al. 2010). It may also be a difference in epidemiology that results from a change in climate sensitivity or changes to key life cycle timings, such as sporulation (Fitt et al. 2006b). It could also be due to anthropogenic influence, such as an increase in pollution effecting population dynamics (Shaw et al. 2008) or the application of fungicides (Bierman et al. 2002).

There are four interlinked mechanisms of pathogen emergence through population replacement:

1. **Emergence:** Novel pathogen evolves. Its expansion is limited by competition from existing populations and resource obtainability.
2. **Competition:** The novel pathogen competes with the existing pathogen for its niche; if the new pathogen is fitter than the original, it may begin to replace it.
3. **Migration:** The new pathogen is transported from its origin to another location, either by using its own dispersal mechanisms or by influence by another species.
4. **Expansion:** If the new pathogen has been able to adapt to, or cope with, the environmental conditions in the new locations then the pathogen may replace local populations and/or hybridise with existing populations.

Many examples of these mechanisms regulating disease invasion have occurred, with numerous novel pathogens targeting staple agricultural crops, such as rice, wheat, and banana (Chen et al. 2010; Churchill 2011; Couch et al. 2005; Fisher et al. 2012). This is of particular importance currently due to the pressure to maintain global food security with a rapidly increasing population that has a progressively diversifying diet (Godfray et al. 2010). A need for increased knowledge about the mechanisms behind pathogen population replacement is required in order to maintain and control emerging plant diseases. Three examples of diseases influenced by pathogen population replacement are yellow rust (caused by new lineages), banana sigatoka and ash dieback (both caused by a new species).

#### 1.3.2.1 Yellow rust

New fitter lineages are a critical product of evolution and their continuous emergence, particularly concerning host specificity, is inevitable. Yellow rust on wheat (*Triticum* spp.) is a notable example of novel variation resulting in pathogen replacement. The causal agent of yellow rust, *Puccinia striiformis* f. sp. *tritici*, is a biotrophic basidiomycete fungus with a polycyclic, heterothallic life cycle (Chen 2005). The pathogen produces, and then discharges, urediniospores, which when in contact with a susceptible host cause necrotic striping on green leaves; this is actually the host's hypersensitive response to infection. Additionally, the fungus produces uredia to manufacture more urediniospores for dispersal. The fungus obtains nutrients and water from the host, and largely weakens the plant. This is of importance to agriculture as it results in reduced growth, loss of photosynthetic productivity and ultimately a loss of yield.

Genotype variation in *P. striiformis* f. sp. *tritici* is well documented globally and in the USA 109 lineages have been identified (Chen 2005; Line 2002). Using this wealth of data on pathotype variation, it was possible to map changes in population structure over an eight-year period. New lineage populations, replacing established pathogen populations, caused disease epidemics in three major waves. These waves, directly linked to the planting of cultivars with differing host-specific genes, were seen to be associated with novel lineages encompassing a greater number of virulence factors. Pathogens with more virulence alleles were able to infect more wheat cultivars and thus have an advantage over pathogen populations of the same species but with fewer host-specific virulence alleles (Chen et al. 2010).

#### 1.3.2.2 Sigatoka leaf spot/streak

The emergence of new species from ecological speciation has been described in Chapter 1.2.2. The relevance of these speciation events is the emergence of novel species that have the potential to replace an existing pathogen population through pathogen population replacement.

Sigatoka leaf spot and black sigatoka leaf streak are diseases of banana (*Musa* spp.) caused by closely related plant pathogens *Mycosphaerella musicola* and *M. fijiensis,* respectively. *M. musicola* and *M. fijiensis* are both haploid, heterothallic ascomycete fungi that follow a hemibiotrophic nutrient uptake strategy and a polycyclic life-cycle. Both pathogens produce similar leaf lesions that differ slightly in colour (Figure 1.3) but they can be distinguished non-molecularly using their conidiophore structure (Agrios 2005; Arzanlou et al. 2008; Churchill 2011). The host, banana (including plantain), is a crop of social and economic importance to approximately 120 countries globally and is generally grown in areas of high rainfall and humid climate. Many countries rely on the crop as a staple food source or for export trade (Churchill 2011; Friesen 2016).

At first it was thought that banana leaf spot, caused by *M. musicola,* was the only real threat to banana production (Leach 1946). However, in 1969, in Sigatoka, Fiji, a new disease was discovered; black leaf streak (Leach 1964), which was found to be caused by *M. fijiensis* and was shown in Hawaii to be just as severe as banana leaf spotting (Leach 1964; Meredith and Lawrence 1969). Using 19 DNA restriction fragment length polymorphism (RFLP) markers, it has been predicted that the centre of origin for *M. fijiensis* was South East Asia or Australasia (Carlier et al. 1996) and that the disease has spread globally through the distribution of plant material (Hayden et al. 2003). Additionally, another closely related pathogen, *M. eumusae*, has been more recently identified as the causal agent of another similar disease on banana, which is known as septoria leaf spot disease (Carlier et al. 2000).

A study in Nigeria, comparing the genetic structure of *M. musicola*, *M. fijiensis* and *M. eumusae* populations, identified *M. fijiensis* as the most virulent of the three pathogens (Zandjanakou-Tachin *et al*., 2009). Using 95 *Mycosphaerella* spp. isolates and analysing single nucleotide polymorphisms, *M. fijiensis* was identified as the dominant species and *M. eumusae* as present but in small numbers. *M. musicola*, once the foremost pathogen in Nigeria, after being introduced in the 1930's (Hayden et al. 2003; Rhodes 1964), was not identified in the isolate collection (Zandjanakou-Tachin et al. 2009). Interestingly, genetic diversity in *M. eumusae* was shown to be greater than that of *M. fijiensis,* suggesting that the pathogen had been present before the introduction of *M. fijiensis*.

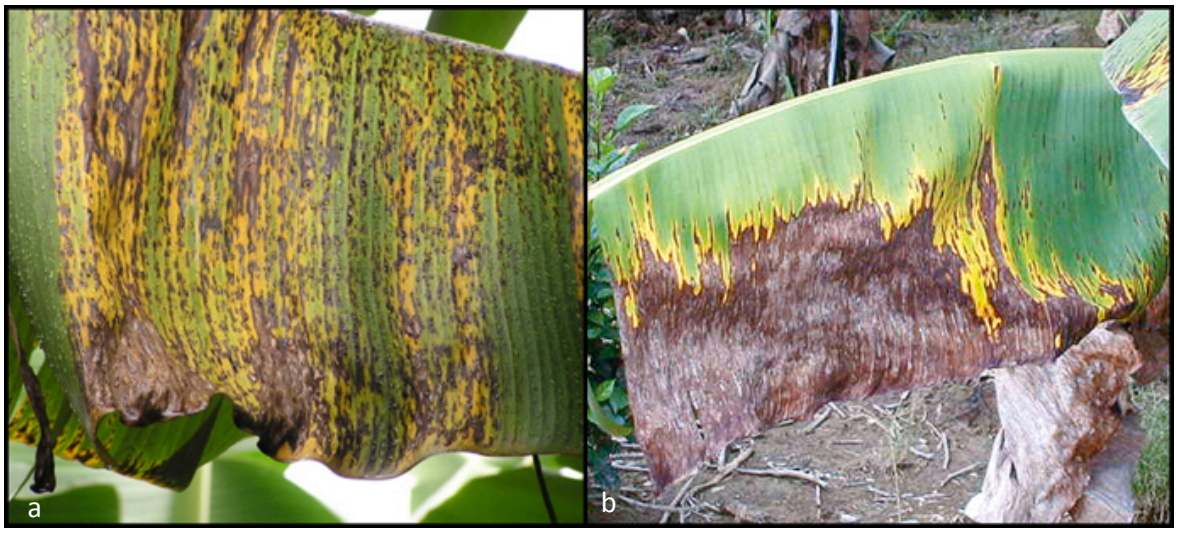



**Figure 1.3.** Banana leaves with necrotic lesion streaks caused by a) *M. musicola* and b) *M. fijiensis*. Images sourced from www.agfax.net. Accessed: 3rd June 2014.

It could be possible that *M. eumusae* was introduced before *M. fijiensis* but did not adapt as successfully to the environmental conditions. It may also be that the *M. fijiensis* and *M. eumusae* have been confused in the past due to similarities in their leaf lesions and that *M. eumusae* is more widespread than was originally suggested.

One preliminary theory to explain the current predominance of *M. fijiensis* over *M. musicola* is differing temperature sensitivity between the two pathogens. Preliminary analysis of germ tube growth at different temperatures showed that growth of *M. fijiensis* was faster than that of *M. musicola* at higher temperatures. However, *M. musicola* grows faster at lower temperatures and the two pathogens have been shown to coexist in environments with fluctuating temperatures (Marín et al. 2003). It is likely that there are other mechanisms underlying the population changes between the pathogens. It could be hypothesised, however, that increasing temperature through global climate change could be affecting the population structure of *Mycosphaerella* spp. infecting banana.

#### 1.3.2.3 Ash dieback

Chalara dieback of ash is a monocylic disease of ash trees (*Fraxinus* spp.) caused by the ascomycete fungus *Chalara fraxinea*. *C. fraxinea* is the anamorphic (asexual) form of *Hymenscyphus pseudoalbidus*. The disease is currently epidemic in Europe where it causes loss of leaves, crown dieback and ultimately death of the tree. The disease produces stem and leaf lesions, bark cankers and causes both leaves and branches to die (Halmschlager and Kirisits 2008; Kowalski and Holdenrieder 2009).

First identified in Poland (Kowalski 2006), *C. fraxinea* was originally believed to be the anamorphic form of the non-pathogenic *H. albidus*. However, analysing the DNA sequences of loci calmodulin, translation elongation factor 1-α and the internal transcribed spacers (ITS) of the rDNA genes, it was shown that *C. fraxinea* was in fact the anamorph of *H. pseudoalbidus; a '*cryptic' species of *H. albidus* (Queloz et al., 2011). Additionally, recent studies have confirmed substantial genetic differences between the two species, suggesting that they have not evolved from a recent common ancestor (Bengtsson et al. 2012). Due to the sudden appearance and rapid emergence of *H. pseudoalbidus,* it is likely that the origin of the pathogen was not Europe and that it was somehow recently introduced (Timmermann et al. 2011).

One possible consequence of an invading species is niche displacement of an existing species (Mooney and Cleland 2001). It has been suggested that invading *H. pseudoalbidus* has occupied the niche of the non-pathogenic and ecologically important

saprophyte *H. albidus*. Genetic analysis of old herbarium samples, originally thought to be *H. pseudoalbidus,* found that the samples taken between 1989 and 1994 were in fact *H. albidus*, whereas the newer samples taken in 2005 were *H. pseudoalbidus.* The most recent collection of samples (2010), taken from the same regions as the original samples (1989-2005), displays an absence of *H. albidus* with only *H. pseudoalbidus* identified. It would appear that *H. pseudoalbidus* has displaced the originally dominant species of fungus (McKinney et al. 2012). It is likely, despite a lack of understanding of the life-cycle of both pathogens, that because both species develop fruiting bodies on the petioles on young, one-year-old ash trees, there could be a competitive interaction between the two species, with *H. pseudoalbidus* out-competing *H. albidus.*

## 1.4 *Leptosphaeria* species complex:

The *Leptosphaeria* species complex, the causal pathogens of phoma stem canker, has the potential to be used as a model example of a genome invasion that eventually leads to invasion at the global scale. Here we compile, describe and relate the events. Firstly however, the host, the disease and the species complex must be introduced, respectively.

### 1.4.1 Oilseed rape

Oilseed rape (canola, *Brassica napus* L. ssp. *oleifera* Metzg (AACC, n = 19)) is a common cultivated crop worldwide. It is a member of the Brassicaceae (or Cruciferare) family, which it shares with other economically important crops, such as cabbage (*Brassica oleracea*) and turnip (*Brassica rapa* subsp. *rapa*). *B. napus* originated from interspecific crosses between *B. oleracea* and *B. rapa* (Kimber and McGregor 1995; Larkan et al. 2013).

It is one of the most important oil-producing crop grown in Europe and is used in a variety of industrial, commercial and agricultural practices; namely biofuels, vegetable oils and livestock feed (Eckert, 2005). Most notable for its yellow flowers visible in spring, the crop has become an integral part of agriculture across the world; made noticeable by the increase in area dedicated to growing the crop (Figure 1.4).

### 1.4.2 Phoma stem canker

Phoma stem canker (blackleg) is a significant disease of oilseed rape that has as an influence on the commercial productivity of crops worldwide. It is of significant importance in Australia, North America and Europe. It is estimated that on a global scale the disease can causes losses of approximately £770 million (Fitt et al. 2006a).

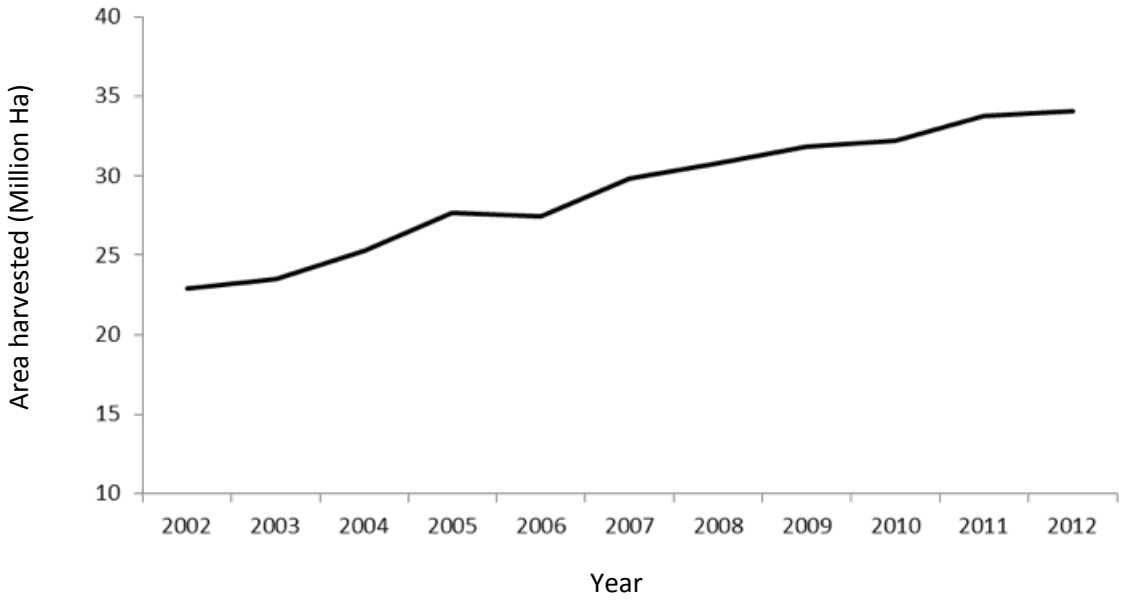



**Figure 1.4.** Total world area (hectares) of oilseed rape harvested annually between 2002 and 2012. Graph produced using data downloaded from Food and Agriculture Organisation of the United Nations, 2014 (http://faostat3.fao.org). Accessed: 12th June 2014.

*L. maculans* and *L. biglobosa*, the two species that make up the *Leptosphaeria* species complex, coexist together on a variety of Brassica hosts (including oilseed rape, turnip and cabbage), causing four frequently noticeable disease symptoms: phoma leaf spotting, phoma stem lesions, basal stem cankers and upper stem lesions (Figure 1.5a-d, respectively). Symptoms can occur on all parts of the plant but most common symptoms are seen on the leaves, upper stem and basal stem.

The pathogens survive over-summer between crops on old plant stubble from previous cropping seasons, and release ascospores after a period of dry then wet weather (Huang et al. 2005). When the ascospores land on the leaves of new plants, they germinate and the pathogens grow through the leaf stomata and into the intercellular spaces (Stotz et al. 2014; Toscano-Underwood et al. 2003; Toscano-Underwood et al. 2001). After a period of incubation, the pathogens begin to cause leaf lesions (phoma leaf spotting). *L. maculans* and *L. biglobosa* cause phenotypically different leaf lesions. *L. maculans* produces large, grey lesions with visible black pycnidia (Figure 1.6), whereas *L. biglobosa* produces much smaller, black lesions with no visible pycnidia (Figure 1.6) (Toscano-Underwood et al. 2001).

In the spring/summer months, following a symptomless stage when the pathogen is progressing through the leaf veins and along the petiole, phoma stem cankers form (Huang et al. 2014b). It is generally accepted that *L. maculans* is the more damaging of the two pathogens and is often associated with basal stem cankers, while *L. biglobosa*, the less damaging pathogen is often associated with upper stem lesions (Huang et al. 2014b; West et al. 2002). Preliminary results, however, suggest that both pathogens have the potential to be equally damaging (Huang et al. 2014a). Nonetheless, the development of the canker is considered the most damaging stage of the disease and the most severe cankers decrease transportation of water and nutrients to the developing seeds. They eventually result in lodging, reduced seed production and premature ripening (Eckert 2005).

### 1.4.3 The *Leptosphaeria* species complex

The *Leptosphaeria* species complex consists of two defined fungal species: *Leptosphaeria maculans* (Desm.) Ces. & De Not., 1863 [anamorph Plenodomus lingam (Tode: Fr.) Desm.] and *L. biglobosa* sp. Nov. [anamorph Plenodomus biglobosus comb. nov.] (De Gruyter et al. 2013; Shoemaker and Brun 2001). In addition to these two species, the complex consists of various lineages that have been identified throughout the world and appear to differ through geographic origin or host-specificity (Dilmaghani et al. 2009; Mendes-Pereira et al. 2003; Vincenot et al. 2008) (Figure 1.7).

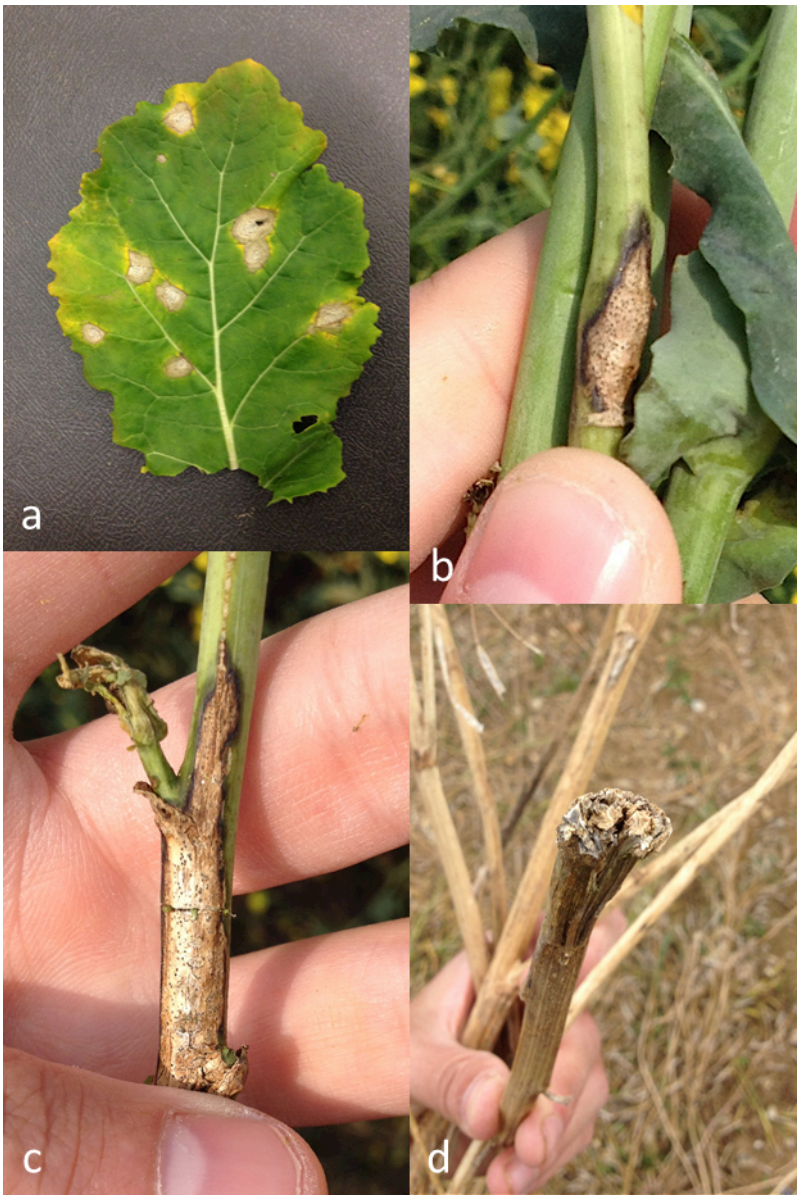



**Figure 1.5.** The various symptoms of phoma stem canker during different stages in the disease cycle. a) phoma leaf spot lesions; b) phoma stem lesion; c) upper stem lesion; d) basal stem canker. Pictures taken by Thomas Sewell.

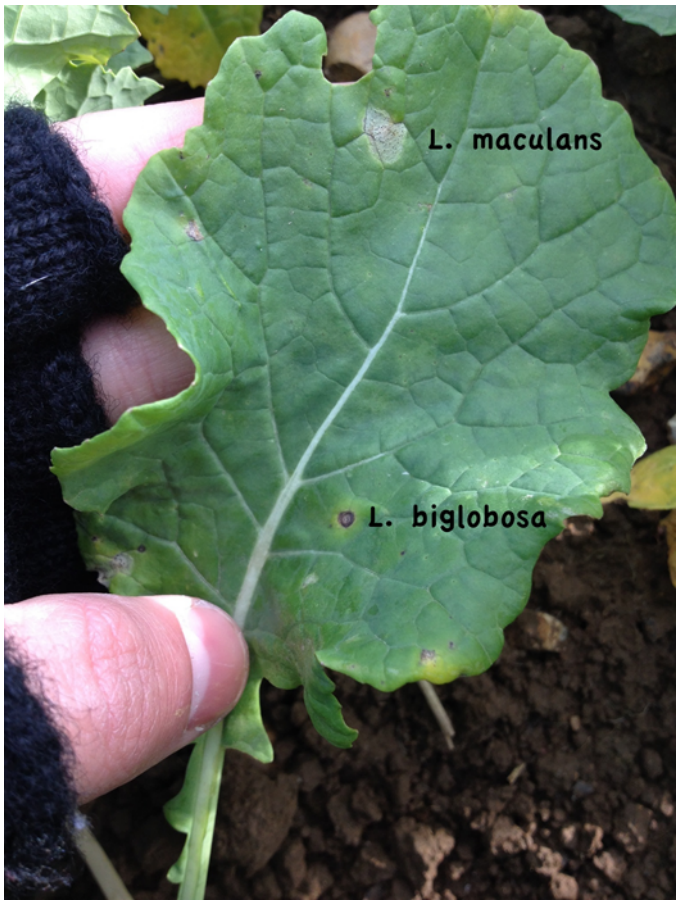



**Figure 1.6.** *Leptosphaeria maculans* and *L. biglobosa* lesions clearly contrasting on an oilseed rape (cv. Catana) leaf. *L. maculans* lesions are large and grey with visible pycnidia. *L. biglobosa* lesions are small, dark and have no clearly visible pycnidia. Picture taken by Thomas Sewell, 10 October 2014.

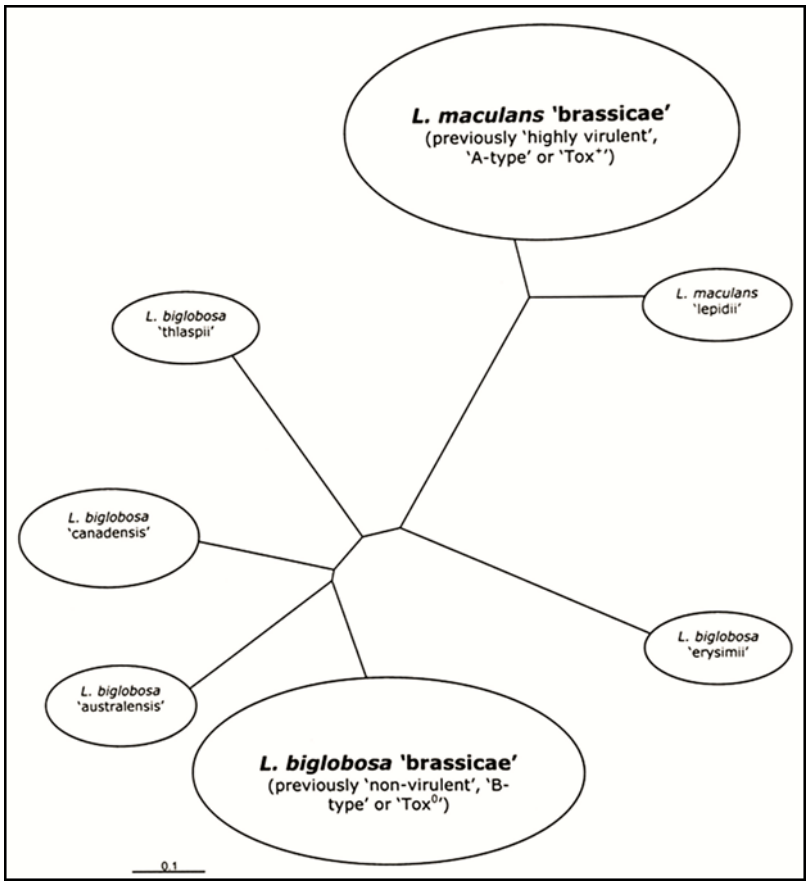



**Figure 1.7.** A phylogenetic tree of the *Leptosphaeria* species complex. Unattached distance tree generated using Neighbour-Joining analysis. Data taken from isozyme, soluble protein profiles and toxin production. Branch lengths measured in the number of substitutions per site. Source: Modified from Mendes-Pereira et al. 2003.

*L. maculans* and *L. biglobosa* are heterothallic ascomycetes, with branched septate hyphae, of the family Dothideomycetes (Loculoascomycetes) and order *Pleosporales*. Other economically important crop pathogens, such as *Venturia* spp. and *Alternaria* spp. are also in this same order (Zhang et al. 2009, 2012). Both species have sexual and asexual stages in their life-cycles. Following sexual reproduction, yellow-brown ascospores (5-8 µm) contained inside bitunicate asci (15-22 µm) are produced within black, globosa pseudothecia. Asexual reproduction produces unicellular conidia (1.5-2µm) inside black pycnidia (Howlett et al. 2001; Shoemaker and Brun 2001).

As they are closely related species, *L. maculans* and *L. biglobosa* are biologically similar. They were once thought to be the same species but of differing subgroups (A and B) and shared the name *L. maculans*; they had different virulence values for identification which related to the fact that *L. maculans* produces the phytotoxic compound sirodesmin ($Tox^+$) whereas *L. biglobosa* does not ($Tox^-$) (Huang et al. 2003; Shoemaker and Brun 2001; Toscano-Underwood et al. 2003; West et al. 1999; West et al. 2002). Nonetheless, further observation of the morphology of the pseudothecia provided the evidence that the differing groups were in fact two species (Shoemaker and Brun 2001). Following this, using ITS sequences for a range of isolates covering the two species, it was shown that there was genetic differentiation between the two pathogen species and their respective lineages (Mendes-Pereira et al. 2003).

### 1.4.4 Pathogen life cycle

*L. maculans* and *L. biglobosa* have similar life cycles, although there are some slight differences, which is to be expected when closely related pathogens exist in the similar ecological niches (Fitt et al. 2006b). Leaf colonisation has been studied in detail for *L. maculans* but less in known about for *L. biglobosa*; *L. maculans* will therefore be used as the example here.

Both *L. maculans* and *L. biglobosa* generally follow monocyclic life cycles (Figure 1.8) with ascospores disseminated by wind over large distances in the autumn in Europe (Huang et al. 2005). The timing of ascospores release varies from region to region and is dependent on the weather conditions at the time (Huang et al. 2005; West et al. 2001). The ascospores, which form within pseudothecia developing on infected oilseed rape stubble, are deposited on new leaves produced by freshly planted oilseed rape. Here the ascospores germinate and the pathogen uses the leaf stomata as an entry point to penetrate into the mesophyll (Huang et al. 2014b).

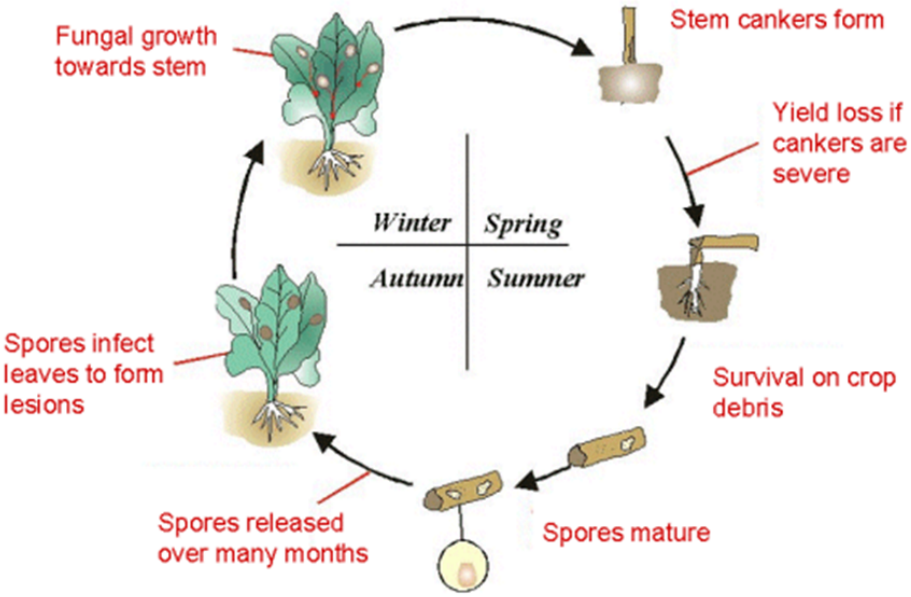



**Figure 1.8.** The life cycle of *L. maculans* causing phoma stem canker on oilseed rape. Less is known about the *L. biglobosa* life cycle. It is currently considered that both species follow a similar life cycle but with slight differences in spore release timing and growth *in planta*. Life-cycle diagram from: http://www.rothamsted.ac.uk/. Accessed: 20th June 2014. Modified from West et al. (1999).

*L. maculans*, now acting as an endophyte, develops within the apoplast, adapting to the antimicrobial compounds within the leaf. After an incubation period of 7 days, the pathogen begins to produce a large necrotic leaf spot lesion in susceptible cultivars. This endophyte stage activates an effective host response in resistant cultivars, stimulating cell death (Stotz et al. 2014).

Dotted within the necrotic lesions are numerous, visible pycnidial fruiting bodies. Inside these are asexual conidia. Although produced in great quantity, conidia are not thought to play an important role in disease epidemics in the UK. In Australia, there is evidence suggesting that rain-splash dispersal of conidia may play a part in the disease cycle (Barbetti 1975); in Europe there is no evidence of this.

Following leaf colonisation and development of leaf spots, a period of symptomless growth takes place. It is during this stage that the pathogen symptomlessly advances along the vascular tissue in the leaf petioles until it reaches the leaf stem. One study, using GFP-transformed isolates of *L. maculans,* has developed our understanding of this symptomless stage (Huang et al. 2009). The same study also showed a correlation between leaf lesion size and speed of movement to the petiole.

During growth along the petiole, the pathogen moves down and into the vascular bundle, in particular the xylem vessels. In stems, further infection of the xylem parenchyma and stem cortex takes place. Following this, the pathogen begins to break down the cortex cells to form stem cankers (Howlett et al. 2001).

Eventually the plant dies and the crop is harvested. The pathogen, now behaving as a saprophyte, colonises dead stem tissues and eventually undergoes sexual outcrossing (Fitt et al. 2006a; West et al. 2001).

### 1.4.5 Genome invasion

The recently sequenced lineages of the *Leptosphaeria* species complex, *L. maculans* 'brassicae', has a genome size of 45,124,619 base pairs, scaffolded into 76 SuperContigs and 17-18 chromosomes (NCBI Assembly No: ASM23037v1). The genome is considered unusual because it has a distinct nucleotide composition of compartmentalised guanine and cytosine (GC)-equilibrated and alternating adenine and thymine (AT)-rich blocks (Rouxel et al. 2011). The genome is of a much larger size than those of other closely related Dothidiomyctes, such as *Mycosphaerella graminicola* and *Phaeosphaeria nodorum*; a trait that is consistent with a large-scale invasion by transposable elements

(TEs). In comparison, the *L. biglobosa* genome is much smaller, with analysis suggesting a size nearer that of other Dothidiomycetes (Grandaubert et al. 2014a, 2014b). The larger genome size can be used to suggest that *L. maculans* has had some degree of genome amalgamation.

A detailed analysis of the *L. maculans* genome has now shown that a large scale, and probably quite recent genome invasion, consisting of both class I and class II TEs (12.30 Mb and 1.19 Mb, respectively), has influenced the genomic structure of *L. maculans* post-divergence. Following the insertion of TEs, a few TE families underwent amplification that overlapped within the genome. In addition to this, the activity of these TEs was then degenerated by multiple rounds of repeat induced polymorphisms (RIP). The reduced GC content, plus the evidence that there are no duplicated copies of effector genes, suggests that over time TEs have become inactivated (Attard et al. 2005; Rouxel et al. 2011).

Additionally, the AT rich regions that make up one-third of the genome have been shown to contain host specific effector genes, which have also been affected by RIP. This novel RIP-based mechanism controls the diversification of effectors within the genome. This, combined with the plasticity of AT-rich regions, allows the pathogen to rapidly respond to selective pressures; thus dramatically increasing the evolutionary potential of the pathogen (Rouxel et al. 2011).

### 1.4.6 Pathogen population replacement

Life cycle similarities are differentiated by two main traits. Firstly, *L. maculans* generally releases its ascospores earlier than *L. biglobosa*, an important factor when considering inoculum density during the emergence of young basal leaves in the autumn (Fitt et al. 2006). Secondly, temperature responses appear to differ between the two pathogens. Studies investigating pseudothecial maturation of the two pathogens showed that *L. biglobosa* germinates earlier at 5-10 $^{O}$C and *L. maculans* later at 5-10 $^{O}$C (Huang et al. 2001; Toscano-Underwood et al. 2003).

Phylogenetic studies suggest that *L. maculans* is, in evolutionary terms, younger than *L. biglobosa* (Dilmaghani et al. 2009; Mendes-Pereira et al. 2003). This is because *L. maculans* is very monomorphic, whereas *L. biglobosa* has many lineages, one of which (*L. biglobosa* 'brassicase') is almost endemic worldwide. Together with the fact that *L. maculans* has slowly been moving into areas where it was originally absent (Figure 1.9), this suggests that the species is an emerging pathogen that is replacing less virulent *L. biglobosa* populations (Fitt et al. 2006).

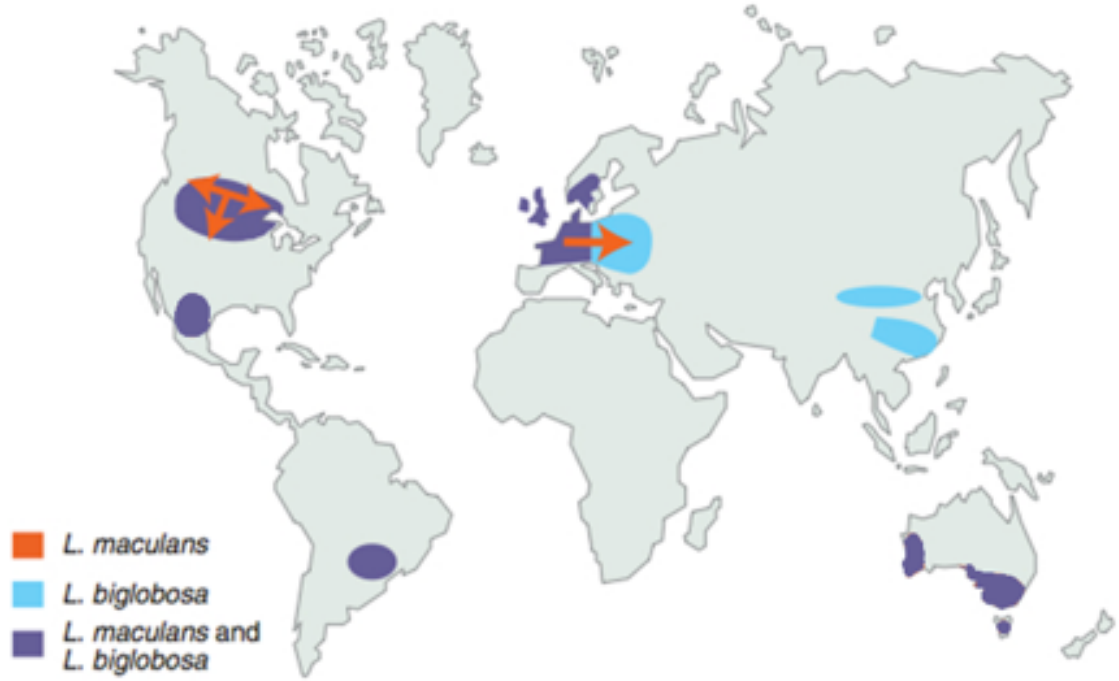



**Figure 1.9.** The global distribution of *Leptosphaeria maculans* and *L. biglobosa. L. maculans*, the younger emergent species, is moving across Europe into Eastern European countries such as Poland. In China, only *L. biglobosa* is endemic. However, concern has been raised that soon crops in China could to be infected by *L. maculans* as they have very little resistance to the pathogen. Modified from Fitt et al. (2006a).

.

An investigation using 465 haploid isolates sampled from 16 locations around the world has suggested that the centre of origin for *L. maculans* is the USA (Dilmaghani et al. 2012). Moreover, a comparison of approximate Bayesian computation outputs of suggested distribution scenarios matches the first citation of the disease on red cabbage (Henderson 1918; Rouxel and Balesdent 2005).

The global spread of the *L. maculans* and *L. biglobosa* is likely to have been aided by the dissemination of wind-dispersed ascospores over long distances. Nevertheless, because of the genetic variability observed in areas of introduction, plus the reasonable differentiation between populations, it can be considered that introduction of the pathogen has happened on several occasions, probably by the transportation of infected debris or seeds (Dilmaghani et al. 2009; Zhang et al. 2014).

Controversially, another hypothesis regarding the origin and subsequent global spread of *L. maculans* has been suggested. The idea is based on the transportation of plant material from Australia to Canada for analysis; it is possible that the pathogen could have been inadvertently released and infected local crops (Zhang et al. 2014). This would suggest that the origin of *L. maculans* was Australia and that the North American spread of phoma was initiated in Canada, later moving into the USA. An extensive genotype analysis on a large set of isolates would need to take place before this can be confirmed.

The full genome sequence of *L. biglobosa*, plus a detailed comparative analysis with the *L. maculans* genome, will provide further information on the difference in virulence between the two pathogens in relation to the invasion of TEs. Additionally, an increased understanding of the speciation event between the two species will provide new knowledge on the interactions between closely related, coexisting pathogens. In combination, these studies will provide further evidence of the impact these events have on pathogen population replacement and global species invasion.

## 1.5 Fungicides and crop protection

### 1.5.1 Fungicides

Fungicides have been relied upon for many years to control fungal crop pathogens (Morton and Staub, 2008). They are economically very important, providing growers with the potential to improve the value of their crop. For example, in Australia, it is estimated that of for every AUS$1 spent on fungicide a gain of AUS$8 is made (Murray and Brennan 2009). However, the situation is complex, and relative benefits vary due to crop

type, colonising pathogen and disease severity (Oliver and Hewitt 2014). Moreover, evolution of fungicide resistance and legalisation-led withdrawal of fungicides both actively reduce the available effective compounds. Consequently, agro-chemical companies are actively seeking new fungicides that control novel and existing plant diseases, which outperform the competition and secure a substantial portion of the fungicide market.

Different fungicides use different active ingredients along with different application strategies (Table 1.6). The variation is dependent on crops, target disease and efficacy of the compound. In the UK, oilseed rape receives around 2-3 sprays per season for disease control. The first spray (T1) is applied when the phoma leaf-spotting incidence is at around 10% plants affected. The second spray (T2) is usually sprayed around 3 to 4 weeks after the first spray. The third spray (T3), which takes place at the flowering stage of the oilseed rape growth cycle, is applied for control of both light leaf spot and sclerotinia (Huang et al. 2011; Sewell et al. 2016; Steed et al. 2007; West et al. 2002).

Currently, three classes of fungicides are commonly used on winter oilseed rape in the UK. These are demethylation inhibitors (DMI), quinone outside inhibitors (QoI) and modern succinate dehydrogenase inhibitors (SDHI). All three classes are currently used to control both *Leptosphaeria* spp. in the UK (Sewell et al. 2016). A previously important class of fungicides, the methyl benzimidazole carbamates (MBC), are no longer extensively applied on winter oilseed rape crops due to widespread resistance reported in Europe and therefore will not be discussed.

### 1.5.2 Demethylation inhibitors

Demethylation inhibitors (DMIs), such as triazoles, triazolinthiones and imidazoles (Figure 1.10) can be further defined as sterol biosynthesis inhibitors (SBIs). They are single site fungicides that target lanosterol 14α−demethylase (*erg11,* CYP51); a membrane bound P450 mono-oxygenase and key component of the ergosterol biosynthesis pathway in fungi. DMI fungicides disrupt the production of ergosterol by inhibiting the removal of the C14-methyl group of 24-methylenedihydrolanosterol (Kelly et al. 1995).

Commonly used agricultural DMIs such as tebuconazole, prothioconazole-desthio and fluquinconazole directly interact with the enzyme. Acting as the sixth ligand of the heme cofactor they bind to the iron atom via N-4 nitrogen on the triazole ring (N-3 nitrogen in imidazoles), inactivating the catalytic capacity of the enzyme and disrupting its native demethylation potential, which in turn disrupts ergosterol biosynthesis.

**Table 1.6.** List of common commercially available fungicides that are used to control phoma stem canker on oilseed rape in the UK. Information taken from a summary of HGCA fungicide project 2010–2013. Accessed 20th June 2014. Information on class taken from www.alanwood.net. Accessed 20th June 2016.

| Fungicide | Active chemical(s) | Class | Company |
|---|---|---|---|
| Orius P | Tebuconazole + prochloraz | DMI + DMI | ADAM |
| Proline 275 | Prothioconazole | DMI | Bayer CropScience (UK) |
| Prosaro | Prothioconazole + tebuconazole | DMI + DMI | Bayer CropScience (UK) |
| Sanction* | Flusilazole | DMI | DuPont (UK) Ltd |
| Refinzar | Penthiopyrad + picoxystrobin | SDHI + QoI | DuPont (UK) Ltd |

.*Sanction, from October 2013, is unavailable for purchase due to EU legislation.

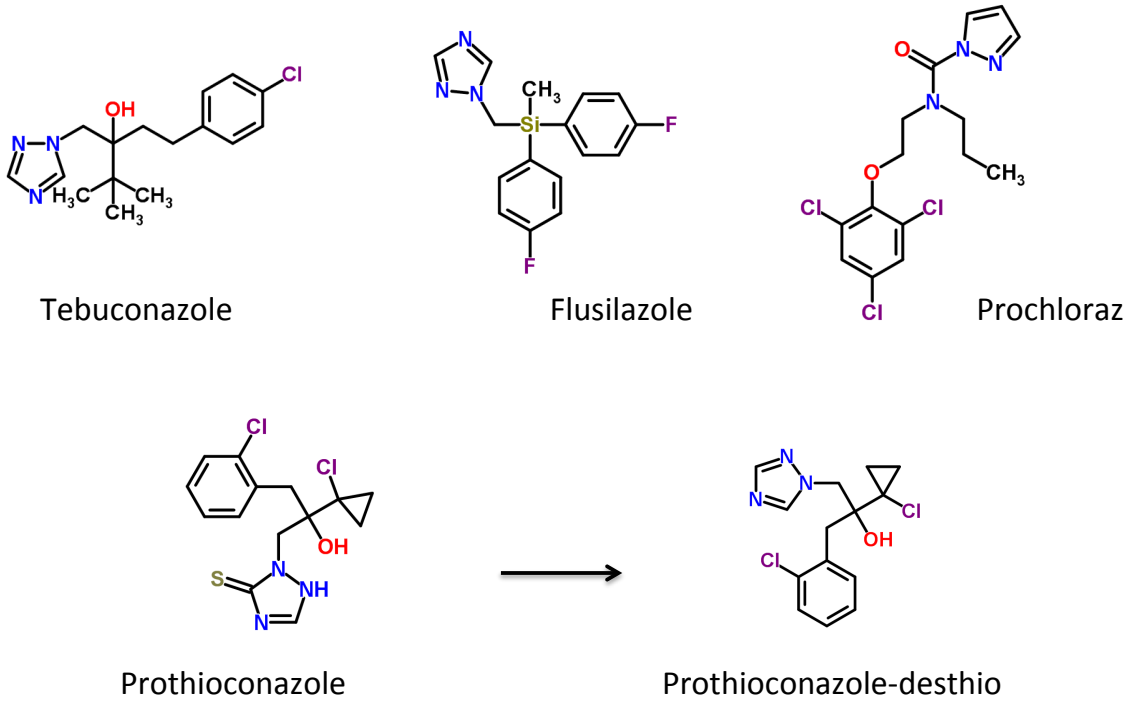



**Figure 1.10.** Chemical structures of five DMI compounds used for the control of plant pathogens in agriculture. The arrow represents the metabolism of prothioconazole into the true antifungal compound prothioconazole-desthio.

The combination of accumulating 14α-demethylated sterol precursors and ergosterol depletion in the cell is the basis of antifungal activity of DMIs (Marichal et al. 1999). When production of ergosterol is inhibited, the permeability and fluidity of the fungal membrane is repressed, causing the fungus to develop incorrectly (Griffiths et al. 2003; Odds et al. 2003).

#### 1.5.2.1 DMI resistance

There are three primary mechanisms of DMI resistance (Figure 1.11): accumulation of mutations in the coding region of CYP51B, increased levels of intracellular CYP51B expression and enhanced fungicide efflux (Cools et al. 2013; Parker et al. 2014; Price et al. 2015). The most common mechanism of resistance is the accumulation of polymorphisms in CYP51B, although if the intensity of azole application endures, overexpression of CYP51B and increased fungicide efflux could become more predominant in insensitive populations due to their characteristics of cross-resistance to different azoles (Cools et al. 2013).

For many years, azole fungicides had been considered resilient to resistance evolution due to a) the necessity of multiple target site polymorphisms to confer notable field resistance and b) the reasonable diversity of agricultural azole fungicides (Cools et al. 2013; Lucas et al. 2015). Moreover, many of the polymorphisms are compound specific, therefore rotation of azoles has likely altered the frequency of some polymorphisms present within populations. Nonetheless, resistance to azoles has now become a serious problem in many destructive plant pathogens, probably due to over-reliance on one class of fungicide combined with a lack of durable alternative classes (Price et al. 2015).

The development of CYP51B mutations in wheat pathogen *Zymoseptoria tritici* is a well-documented example of resistance evolution in fungal plant pathogens (Cools and Fraaije 2013). Fungicide sensitivity assays have been used to determine the $EC_{50}$ value (effective dose of fungicide where 50% of the population is inhibited by the fungicide) of field isolates containing various amino acid polymorphisms compared with the wild type CYP51B present in the majority of *Z. tritici* populations. Combined with CYP51B sequence data, elevated $EC_{50}$ values have now been heavily linked to the presence and accumulation of CYP51B polymorphisms (Fraaije et al. 2012). Moreover, using a heterologous yeast expression system, the phenotypic response to fungicides has been linked to CYP51B polymorphisms within an isogenic background (Carter et al. 2014; Cools et al. 2010; Hawkins et al. 2014).

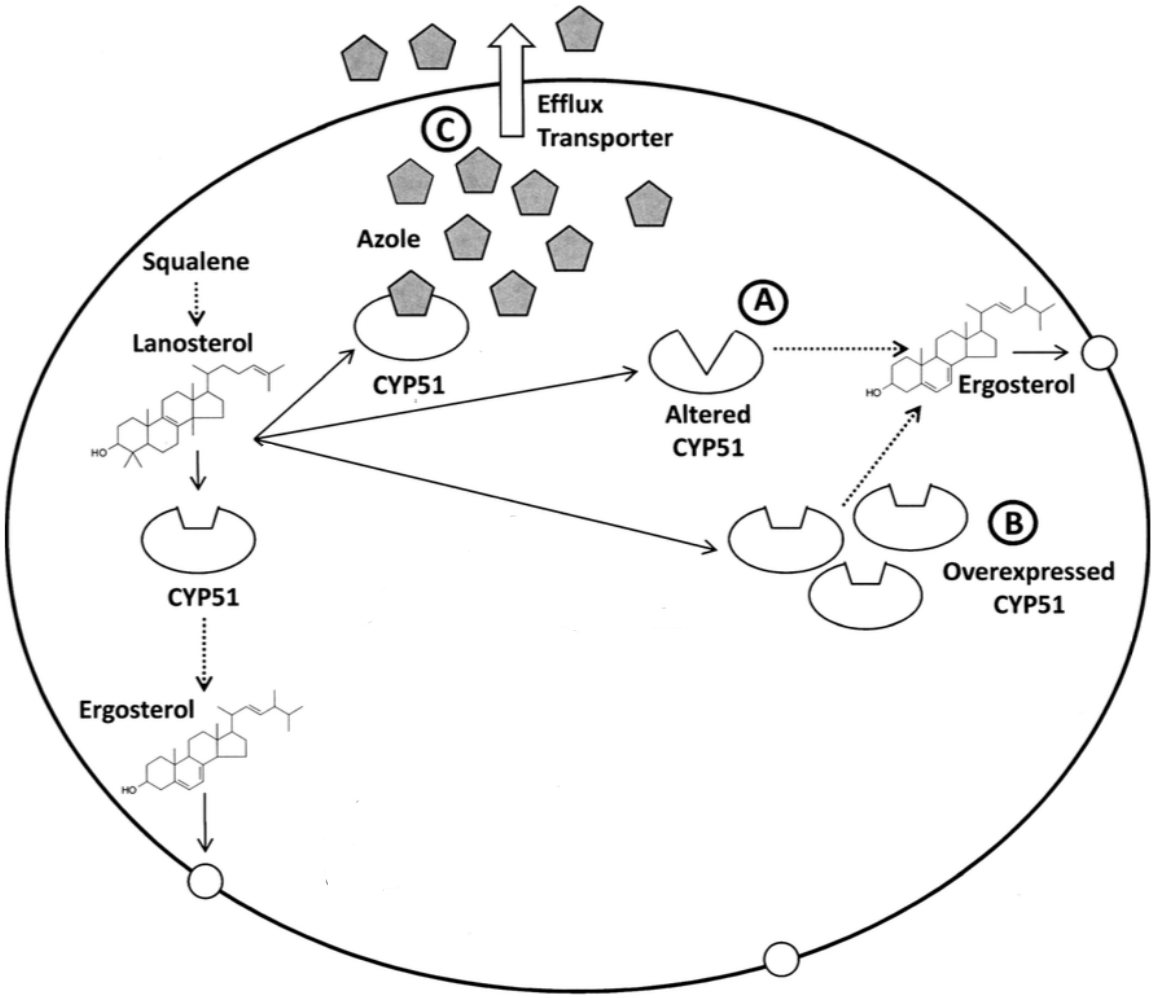



**Figure 1.11.** Mechanisms of azole resistance in medical and plant pathogenic fungi. Azole resistance can occur through a) point mutations in the target enzyme CYP51B (structural alterations), b) increased expression of target enzyme CYP51B, c) overexpression of efflux transporter proteins, removing antifungal from the cell. Schematic modified from Parker et al. (2014).

One polymorphism that has been identified in many species of fungi with resistance to azoles is the Y140F/H polymorphism (*S. cerevisiae* numbering). Insensitive species include human pathogen *Candida albicans* (Y132F) and plant pathogens *Z. tritici* (Y137F*), M. fijiensis* (Y136F)*, B. graminis* f.sp*. tritici* (Y136F) and *B. graminis* f.sp*. hordei* (Y136F) (Cañas-Gutiérrez et al. 2009; Cools and Fraaije 2013; Flowers et al. 2015; Wyand and Brown 2005; Yan et al. 2009). The heterologous yeast expression system has suggested that this polymorphism alters azole efficacy and spectral binding studies have identified changes in the interaction between azole and CYP51B when Y140F/H is present (Sagatova et al. 2016).

Determination of high-resolution crystal structures of CYP51B in *S. cerevisiae* has led to the identification of a water-mediated hydrogen bond network connecting the ligand azole, protein residues and heme cofactor; all possible due to the presence of water molecules buried within the azole binding site of the protein. One notable residue in the network is tyrosine at position 140 (Y140), forming a hydrogen bond with one of the water molecules (Sagatova et al. 2015, 2016). Substitution of this residue with phenylalanine (Y140F) or histidine (Y140H) disrupts the formation of a hydrogen bond, thus reducing binding efficiency.

These findings somewhat support a prior investigation into the structural effects of accumulating CYP51B polymorphisms in *Z. tritici* (Mullins et al. 2011), which identified that polymorphisms, including Y137F, altered the location of interacting residues, moving them away from the heme bound azole and increasing the size of the binding pocket, which in turn reduced the affinity of fungicides to the protein.

Over-expression of CYP51B, mediated by insertions in the promoter of the gene, has also been linked to a insensitive phenotype in some plant pathogens (Carter et al. 2014; Hamamoto et al. 2000). An increased cellular level of the protein reduces the inhibitory potential of the azoles due to increased binding sites (Parker et al. 2014). In the UK, the winter oilseed rape pathogen *Pyrenopeziza brassicae* has been reported to have developed a reduced sensitivity response to some triazole fungicides, and regulatory mechanisms together with the presence of polymorphisms in the coding sequence have been suggested as the causal mechanisms (Carter et al. 2014). Similarly, the presence of CYP51 paralogs in species such as *Rhynchosporium commune* (CYP51A and CYP51B) and *Fusarium* spp. (CYP51A*,* CYP51B and CYP51C) is also implicit in a lower intrinsic sensitivity to some azoles; mechanisms that can be attributed to increased levels of *CYP51* acting as a functional 14α- demethylases (Hawkins et al. 2014; Liu et al. 2011).

Resistance to azole fungicides via increased or improved efflux of antifungals out of the cell has been well documented in human pathogens but less so in agricultural plant pathogens. Nonetheless, increased expression of a gene (*BcatrD*) encoding an ABC transporter protein has been correlated to reduced oxpoconazole sensitivity in *Botrytis cinerea* (Hayashi et al. 2002). Similarly, an ABC transporter (*PMR1*) identified in citrus pathogen *Penicillium digitatum* has been shown to be a determinant of resistance to DMI fungicide triflumizole (Nakaune et al. 1998).

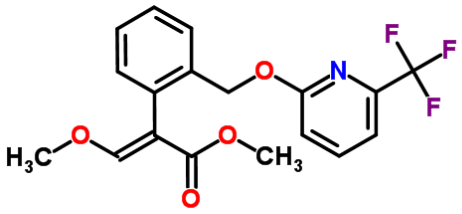
### 1.5.3 Quinone outside inhibitors

Quinone outside inhibitors (QoIs), commonly known as strobilurin fungicides, are an important class of commercially available agricultural fungicides (Figure 1.12), although in recent years their efficacy has been threatened by fast evolving resistance (Oliver and Hewitt 2014). Strobilurins were first discovered in a wood-rotting Basidiomycete fungus *Strobilurus tenacellus* as a naturally synthesised β-methoxyacrylic acid (Anke et al. 1977; Bartlett et al. 2002).

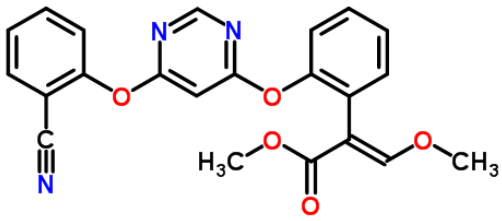
QoI fungicides are single site mitochondrial respiration inhibitors that bind to the quinol oxidation site ($Q_O$ site) of cytochrome b, which is one of three subunits that constitute the large (480 kDa) trans-membrane homodimer, cytochrome bc1 complex (Bartlett et al. 2002; Fisher and Meunier 2008). Cytochrome bc1 is positioned in the inner mitochondrial membrane of fungi another eukaryotic organisms and is fundamental to the mitochondrial electron transport chain (Figure 1.13). Bound QoI compounds block electron transfer between cytochrome b and cytochrome $c_1$, inhibiting the production of ATP and reducing fitness of the fungus.

#### 1.5.3.1 QoI resistance

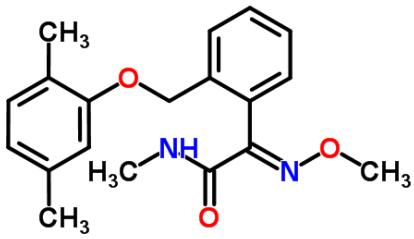
The initial prospect of QoI fungicides was soon hampered by resistance evolution, an occurrence that materialised within just 2 years of their inception (Gisi et al. 2002). In comparison to azole resistance, QoI resistance is less complex; just a single common polymorphism in the cytochrome b coding sequence (G143A) emerged in various pathogen populations where QoI efficacy had languished. The mutation is thought to affect the structural properties of cytochrome b, precluding the binding of QoI compounds, a mechanism that explains the large phenotypic difference in the presence of QoI fungicides. Additionally, the polymorphism grants complete cross-resistance with no notable fitness costs, so rotation of QoI fungicides does not alter selection pressure, which may explain the rapid evolution of resistance.



Picoxystrobin



Azoxystrobin



Dimoxystrobin

**Figure 1.12.** Chemical structures of three QoI compounds used for the control of plant pathogens in agriculture.

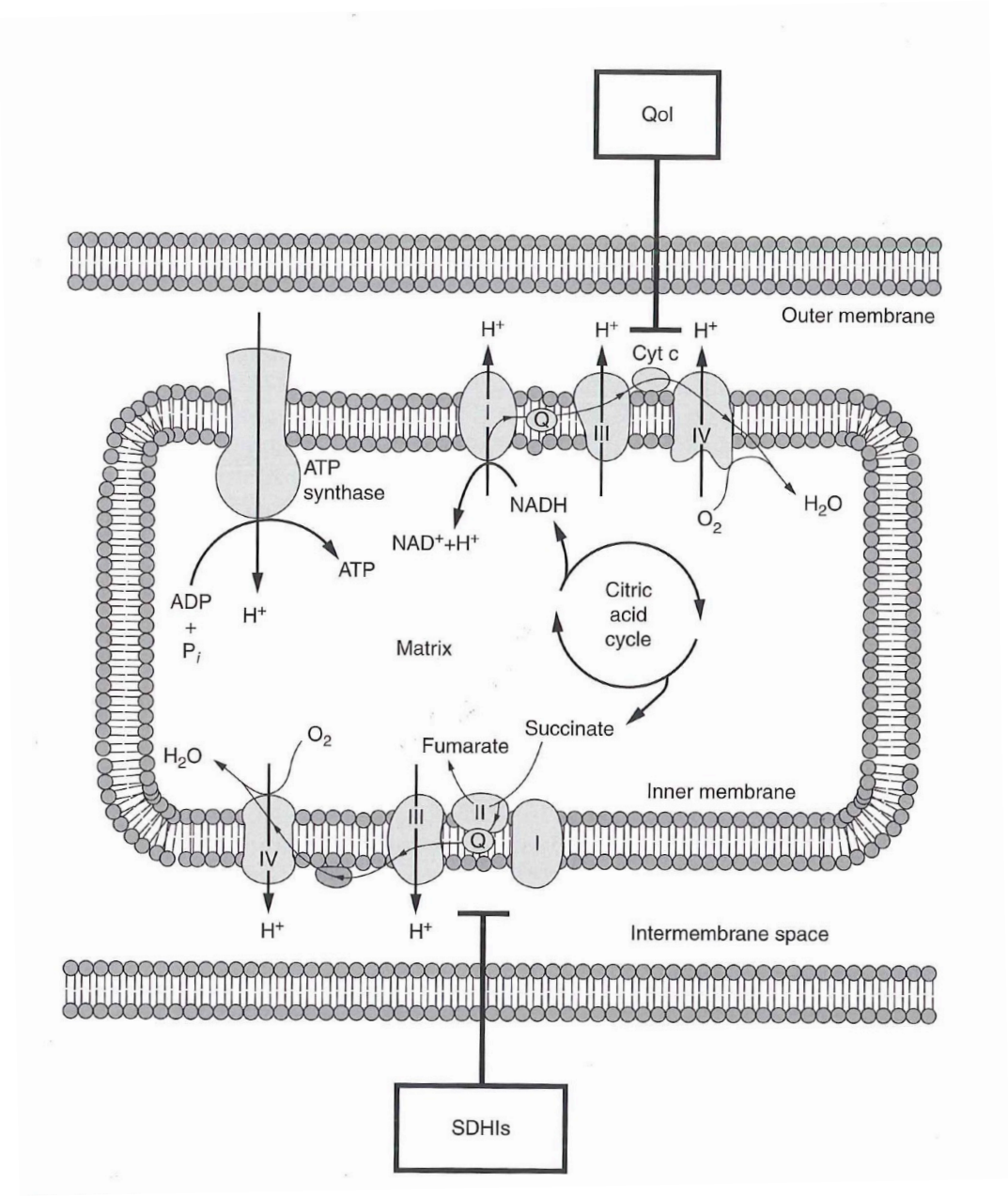



**Figure 1.13.** A representation of the inner and outer mitochondrial membrane, together with the protein complexes involved in mitochondrial respiration by action of the electron transport chain. The target sites of succinate dehydrogenase inhibitors (SDHIs) and quinone outside inhibitors are labelled. Schematic taken directly from Oliver and Hewitt (2014).

Interestingly, some plant pathogens have been found to be resistant to the G143A mutation due to the presence of an intron in the cytochrome b gene (Grasso et al. 2006; Sierotzki et al. 2007). The nucleotide polymorphism that denotes the substitution of glycine (G) to alanine (A) alters the splice site of the intron, which in turn alters mRNA synthesis. Therefore, occurrence of G143A mutation would be lethal in organisms where the intron is present due to the indispensable nature of cytochrome b. Another, less severe mutation, F129L has also appeared, although this is considered less effective at reducing QoI efficacy in crops due to lower $EC_{50}$ values (Kim et al. 2003).

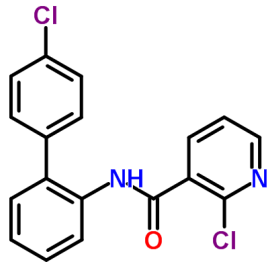
### 1.5.4 Succinate dehydrogenase inhibitors

Succinate dehydrogenase inhibitors (SDHIs) (Figure 1.14) are similar to QoIs in that they inhibit mitochondrial respiration (Avenot and Michailides 2010). SDHI compounds target succinate dehydrogenase (SDH), a large trans-membrane protein consisting of 4 subunits (SDHA-D) and a key component in the electron transport chain, which also has a role in the tricarboxylic acid cycle (Figure 1.13) (Horsefield et al. 2006). SDHIs inhibit succinate dehydrogenase by physically blocking the ubiquinone-binding site (UQ binding site) that is formed by residues of the SDHB, SDHC and SDHD subunits; an action that has a deleterious affect on the fungal cell by inhibiting ATP production (Avenot and Michailides 2010; Sierotzki and Scalliet 2013).

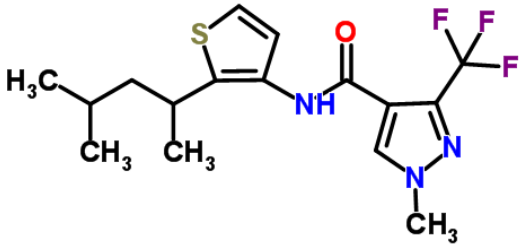
In recent years, a new generation of novel SDHI compounds has taken precedence over development of other fungicide classes (e.g. QoI, DMI and MBC) due to the development of resistance and legislation restraints (Oliver and Hewitt 2014; Sewell et al. 2016). SDHI compounds were first introduced in 1966 but were generally only effective against Basidomycetes, such as *P. striiformis* f. sp. *tritici*. New-generation SDHIs have an improved effectiveness against a larger range of fungal pathogens on many crops (Oliver and Hewitt 2014).

#### 1.5.4.1 SDHI resistance

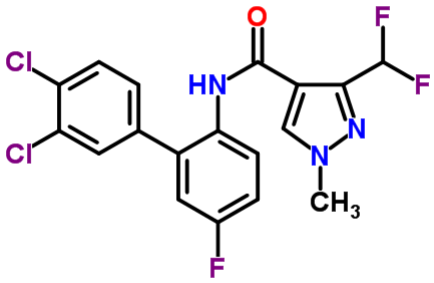
Resistance to new-generation SDHIs has been reported in many important crop pathogens, including *Z. tritici* (Dooley et al. 2016), *B. cinerea* (Stammler and Speakman 2006) and *Alternaria alternata* (Avenot and Michailides 2009). The molecular mechanism of resistance is intricate due to the multiple subunits that constitute the SDH complex. Initial studies using UV-irradiated mutant strains concluded that a resistance phenotype was associated with mutations in the SDH genes (Georgopoulos and Ziogas 1977). More recently, field strains with a similar insensitive phenotype were discovered.




Boscalid




Penthiopyrad




Bixafen

**Figure 1.14.** Chemical structures of three SDHI compounds used for the control of plant pathogens in agriculture.

The polymorphism at highest frequency is a histidine (H) to tyrosine (Y) substitution, which occurs in SDHB and is located at the UQ binding site. Molecular modelling determined that mutations located near to the UQ-binding site are expected to decrease SDHI binding affinity (Stammler et al. 2007). A decrease in binding affinity can be anticipated in the case of the histidine to tyrosine polymorphism due to its location at the UQ-binding site and its direct involvement in the binding of SDHIs via hydrogen-bonding (Horsefield et al. 2006). To increase the longevity of SDHI fungicides, anti-resistance strategies, such as investigations into cross-resistance and improved diagnostics, are being implemented (Fraaije et al. 2012).

### 1.5.5 Other crop protection strategies

Two other approaches are used combat the potential loss of yield from plant pathogenic fungi; these are cultural control and breeding strategies. Cultural methods target the amount of inoculum that could potentially cause disease. In the case of phoma stem canker, reducing exposure of the crop to potential disease-causing ascospores can reduce the severity of epidemics (Aubertot et al. 2006). This can be achieved by either rotating the crop, removing infected stubble at the end of each growing season or tilling the soil (West et al. 2001). Rotation dilutes the number of ascospores in the air that can potentially cause disease in the newly planted crops. Removing the stubble physically removes the developing ascospores from the crops, thus reducing potential inoculum for the next cropping season. Tilling can be used to bury the diseased stems and inhibit the number of ascospores released from them. It is thought that a minimum tillage system could result in increased disease severity, a scenario that has been hypothesised to explain the increased disease severity in Australia.

Resistance breeding strategies exploit the gene-for-gene interaction described in Chapter 1.1.2. Breeders artificially select favourable traits in crops, such as higher yield, improved drought tolerance or enhanced disease resistance (Sleper and Poehlman 2006). Breeders target specific alleles at certain crucial loci (Niks et al. 2011). Current crops now have an array of cultivars each with differing levels of resistance. This resistance can be either monogenic or polygenic and specific cultivars can contain single or several resistance genes. For example, *B. napus* cv. Jet Neuf carries *Rlm4* in its genome, which corresponds to the *L. maculans* population carrying *AvrLm4* (Huang et al. 2006). When this crop is planted in areas supporting a *L. maculans* population with a high frequency of *AvrLm4,* an avirulent response will occur and disease will be inhibited.

## 1.6 Aim and objectives

This project aims to further understand the role that fungicides have in controlling phoma stem canker by investigating their efficacy against *Leptosphaeria maculans* and *L. biglobosa* in crops, *in vitro* and *in planta*.

Objectives

1) To investigate the efficacy of a penthiopyrad (SDHI) + picoxystrobin (QoI) mixture, a novel non-azole based fungicide, for phoma stem canker control.

2) To investigate the sensitivity of *Leptosphaeria* spp. both *in vitro* and *in planta*, to DMI, SDHI and QoI fungicides.

3) To investigate the role of lanasterol 14α−demethylase (CYP51) in differences in azole sensitivity between *Leptosphaeria* spp.

# Chapter 2 General materials and methods

## 2.1 *L. maculans* and *L. biglobosa* isolation from phoma leaf lesions

Phoma leaf lesions were directly removed from a carefully cleaned leaf and placed inside a Petri dish lined with wetted filter paper (Whatman No. 1, GE Healthcare Life Sciences, UK). The lesions were left for 3-5 days inside an incubator set to 20 $^{O}$C on a 12/12 hour dark/light cycle to induce production of pycnidia. After incubation, the lesions were checked under a light microscope for single pycnidia with pink cirrhi. In a sterile HEPA-filtered laminar flow cabinet (Class II MSC, Envar, Rossendale, Lancs, UK) and using a sterile needle, the single pycnidia were selected and each placed into a water droplet pre-prepared on a Petri dish. Using the needle, each pycnidium was well mixed within the droplet before pipetting onto fresh PDA (Appendix A.1) agar plates amended with 50 µg/ml penicillin and 50 µg/ml streptomycin. The plates were then sealed using Parafilm (Bemis, NA, USA) and stored in darkness at 20 $^{O}$C. It was possible to identify species on potato dextrose agar (PDA, Appendix A.1) because *L. maculans* produces white flat mycelium with no pigmentation, whereas *L. biglobosa* produces fluffy mycelium with yellow-brown pigmentation.

## 2.2 *L. maculans* and *L. biglobosa* isolation from stem canker samples

Firstly, the stem canker was well washed under a tap and dried using paper towels. Necrotic canker samples were shaved from the affected stem cortex using a clean, sharp scalpel before being cut into smaller pieces (0.5 x 0.5 cm); this aimed to reduce the possibility of fungi developing from each piece and to reduce the chance of fast-growing saprophytes spoiling the samples. Using sodium hypochlorite solution (NaOCl) with 1% available chlorine, the surface of each sample was sterilised for 3 minutes before being immersed in sterile distilled water ($dH_2O$). The stem samples were left to dry on filter paper inside a sterile HEPA-filtered laminar flow cabinet. The samples were placed on fresh PDA plates amended with penicillin and streptomycin. The plates were left in darkness at 20 $^{O}$C for 5-7 days before colonies were selected and subcultured onto V8 medium amended with 50 µg/ml penicillin and 50 µg/ml streptomycin. The sub-cultured plates were transferred to an incubator set to 20 $^{O}$C on a 12/12 hour dark/light cycle to induce pycnidial production. Single pycnidium isolation was then done as described in Chapter 2.1.

Table 2.1. Collection of *Leptosphaeria* isolates used throughout this study

| Isolate | Species | Location | Year | Host crop | Cultivar | Isolation method | Provided by | CYP51B sequenced |
|---|---|---|---|---|---|---|---|---|
| ME24 | *L. maculans* | Yorkshire | 2002 | *Brassica napus* | Apex | Single pycnidium | Yongju Huang | |
| TS13-10 | *L. maculans* | Cambridge, UK | 2013 | *Brassica napus* | Catana | Single pycnidium | Thomas Sewell | x |
| TS13-11 | *L. maculans* | Cambridge, UK | 2013 | *Brassica napus* | Catana | Single pycnidium | Thomas Sewell | |
| TS13-12 | *L. maculans* | Cambridge, UK | 2013 | *Brassica napus* | Catana | Single pycnidium | Thomas Sewell | x |
| TS13-13 | *L. maculans* | Cambridge, UK | 2013 | *Brassica napus* | Catana | Single pycnidium | Thomas Sewell | x |
| TS13-19 | *L. maculans* | Cambridge, UK | 2013 | *Brassica napus* | Catana | Single pycnidium | Thomas Sewell | |
| TS13-2 | *L. maculans* | Cambridge, UK | 2013 | *Brassica napus* | Catana | Single pycnidium | Thomas Sewell | x |
| TS13-3 | *L. maculans* | Cambridge, UK | 2013 | *Brassica napus* | Catana | Single pycnidium | Thomas Sewell | |
| TS13-4 | *L. maculans* | Cambridge, UK | 2013 | *Brassica napus* | Catana | Single pycnidium | Thomas Sewell | x |
| TS13-5 | *L. maculans* | Cambridge, UK | 2013 | *Brassica napus* | Catana | Single pycnidium | Thomas Sewell | |
| TS13-6 | *L. maculans* | Cambridge, UK | 2013 | *Brassica napus* | Catana | Single pycnidium | Thomas Sewell | |
| TS13-7 | *L. maculans* | Cambridge, UK | 2013 | *Brassica napus* | Catana | Single pycnidium | Thomas Sewell | x |
| TS13-8 | *L. maculans* | Cambridge, UK | 2013 | *Brassica napus* | Catana | Single pycnidium | Thomas Sewell | |
| TS13-9 | *L. maculans* | Cambridge, UK | 2013 | *Brassica napus* | Catana | Single pycnidium | Thomas Sewell | |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| TS14-1 | *L. maculans* | Cambridge, UK | 2014 | *Brassica napus* | Catana | Single pycnidium | Thomas Sewell | | x |
| TS14-12 | *L. maculans* | Cambridge, UK | 2014 | *Brassica napus* | Catana | Single pycnidium | Thomas Sewell | | |
| TS14-13 | *L. maculans* | Cambridge, UK | 2014 | *Brassica napus* | Catana | Single pycnidium | Thomas Sewell | | x |
| TS14-14 | *L. maculans* | Cambridge, UK | 2014 | *Brassica napus* | Catana | Single pycnidium | Thomas Sewell | | |
| TS14-2 | *L. maculans* | Cambridge, UK | 2014 | *Brassica napus* | Catana | Single pycnidium | Thomas Sewell | | x |
| TS14-21 | *L. maculans* | Cambridge, UK | 2014 | *Brassica napus* | Catana | Single pycnidium | Thomas Sewell | | |
| TS14-4 | *L. maculans* | Cambridge, UK | 2014 | *Brassica napus* | Catana | Single pycnidium | Thomas Sewell | | |
| TS14-9 | *L. maculans* | Cambridge, UK | 2014 | *Brassica napus* | Catana | Single pycnidium | Thomas Sewell | | x |
| TS13-18 | *L. maculans* | Cambridge, UK | 2013 | *Brassica napus* | Catana | Single pycnidium | Thomas Sewell | | x |
| H Rox 12-2-1 | *L. maculans* | Cowlinge, UK | 2012 | *Brassica napus* | Roxet | Single pycnidium | Georgia Mitrousia | | |
| B2003.2.8 | *L. biglobosa* | Orston, UK | 2003 | *Brassica napus* | Recital | Single pycnidium | Yongju Huang | | |
| A Exc 12-10 | *L. biglobosa* | Banbury, UK | 2012 | *Brassica napus* | Excel | Single pycnidium | Georgia Mitrousia | | |
| D Rox 12-10 | *L. biglobosa* | Banbury, UK | 2012 | *Brassica napus* | Roxet | Single pycnidium | Georgia Mitrousia | | x |
| E Exc 12-9-21 | *L. biglobosa* | Bainton, UK | 2013 | *Brassica napus* | Excel | Single pycnidium | Georgia Mitrousia | | |
| F2 Exc 12-2-3 | *L. biglobosa* | Stockbridge, UK | 2013 | *Brassica napus* | Excel | Single pycnidium | Georgia Mitrousia | | x |
| F2 Exc 12-3-1 | *L. biglobosa* | Stockbridge, UK | 2013 | *Brassica napus* | Excel | Single pycnidium | Georgia Mitrousia | | |
| F2 Exc 12-6-1 | *L. biglobosa* | Stockbridge, UK | 2013 | *Brassica napus* | Excel | Single pycnidium | Georgia Mitrousia | | x |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| F2 Exc dm 11-5 | *L. biglobosa* | Stockbridge, UK | 2012 | *Brassica napus* | Excel | Single pycnidium | Georgia Mitrousia | |
| C Rox 12-8-1 | *L. biglobosa* | Morley, UK | 2013 | *Brassica napus* | Roxet | Single pycnidium | Georgia Mitrousia | x |
| H 12-6 | *L. biglobosa* | Cowlinge, UK | 2013 | *Brassica napus* | Drakkar | Single pycnidium | Georgia Mitrousia | |
| H Dr 12-12 | *L. biglobosa* | Cowlinge, UK | 2013 | *Brassica napus* | Drakkar | Single pycnidium | Georgia Mitrousia | |
| H Dr 12-2 | *L. biglobosa* | Cowlinge, UK | 2013 | *Brassica napus* | Drakkar | Single pycnidium | Georgia Mitrousia | x |
| H Exc 12-12-1 | *L. biglobosa* | Cowlinge, UK | 2013 | *Brassica napus* | Excel | Single pycnidium | Georgia Mitrousia | x |
| H Exc 12-12-31 | *L. biglobosa* | Cowlinge, UK | 2013 | *Brassica napus* | Excel | Single pycnidium | Georgia Mitrousia | |
| H Exc 12-2-2 | *L. biglobosa* | Cowlinge, UK | 2013 | *Brassica napus* | Excel | Single pycnidium | Georgia Mitrousia | x |
| K Rox 12-2-1 bc | *L. biglobosa* | Harpenden, UK | 2012 | *Brassica napus* | Roxet | Single pycnidium | Georgia Mitrousia | |
| K 12-33 | *L. biglobosa* | Harpenden, UK | 2013 | *Brassica napus* | Drakkar | Single pycnidium | Georgia Mitrousia | |
| K Exc 12-10-21 | *L. biglobosa* | Harpenden, UK | 2012 | *Brassica napus* | Excel | Single pycnidium | Georgia Mitrousia | x |
| K Exc 12-11 | *L. biglobosa* | Harpenden, UK | 2012 | *Brassica napus* | Excel | Single pycnidium | Georgia Mitrousia | |
| K Rox 12-6-31 | *L. biglobosa* | Harpenden, UK | 2013 | *Brassica napus* | Roxet | Single pycnidium | Georgia Mitrousia | |
| Lb68 | *L. biglobosa* | Welford, UK | 2002 | *Brassica napus* | N/A | Single pycnidium | Yongju Huang | |
| W10 | *L. biglobosa* | Wuxuw, China | 2010 | *Brassica napus* | N/A | Single pycnidium | Yongju Huang | |

## 2.3 Subculture, maintenance and storage of *L. maculans* and *L. biglobosa* isolates

Isolates were maintained throughout the project using a HEPA-filtered laminar flow cabinet. Using a sterile scalpel, a single square of agar was cut from the radial, actively growing edge of an existing culture and placed onto a fresh PDA plate amended with 50 µg/ml penicillin and 50 µg/ml streptomycin. The Petri plate was then sealed using Parafilm (Bemis) and colonies were left to grow at 20 $^{O}$C in darkness for 14 days.

For short-term storage (3 months), isolates were grown and stored on PDA plates amended with 50 µg/ml penicillin and 50 µg/ml streptomycin and stored at 4 $^{O}$C (Appendix A.1). For long-term storage, isolates were stored as 35 % glycerol spore suspension stocks at -80 $^{O}$C. On one occasion, 50 µg/ml tetracycline was added to the medium (Appendix A.1), due to a gram negative bacterial contaminant that was resistant to streptomycin.

## 2.4 Production of *L. maculans* and *L. biglobosa* conidial suspensions

Under a HEPA-filtered laminar flow cabinet, two large squares were cut from the radial, actively growing edge of an existing culture and placed mycelium-down on a fresh V8 plate amended with 50 µg/ml penicillin and 50 µg/ml streptomycin. Using forceps, the pieces were distributed around the plate ensuring that the mycelium had been distributed evenly over the medium. The agar squares were then sliced into smaller pieces and placed evenly around the plate. Small squares of sterile filter paper were placed on the plate for collection of pycnidia. The plates were sealed with Parafilm and incubated for 2 weeks at 20 $^{O}$C on a 12/12 hour dark/light cycle to induce pycnidial production. After 2 weeks, the small squares of filter paper were removed, placed inside a 1.5 ml tube (Greiner-Bio-One GmbH, Germany) and stored at -20 $^{O}$C. The plate, now clearly displaying visible pycnidia, had 5 ml of distilled water added to it before being fully agitated with a plastic sterile L spreader (Sterilin, Newport, UK). The liquid was then filtered through sterile Miracloth (CalBioChem, USA) into a 15 ml tube (Cellstar, Greiner-Bio-One GmbH, Germany), ensuring that no debris was transferred with it. Using a haemocytometer (Bright-line, Hausser Scientific, Horsham PA, USA), the conidial suspension was quantified and diluted accordingly to a target concentration of $10^6$ spores per ml. The samples were then

aliquoted into 2 ml tubes (Greiner-Bio-One GmbH, Germany) or 15 ml tubes for immediate experimentation or storage at -20 $^{O}$C (short-term) or -80 $^{O}$C (long-term).

## 2.5 Harvesting *L. maculans* and *L. biglobosa* mycelium

Under a HEPA-filtered laminar flow cabinet, a single cellulose disc was added to a prepared V8 plate amended with 50 µg/ml penicillin and 50 µg/ml streptomycin. Two large squares were cut from the radial, actively growing edge of an existing culture and placed mycelium-down on the surface of the cellulose disc. The squares were carefully spread over the surface of the disc, ensuring that the mycelium was evenly distributed; the squares were deposited in each half of the plate with any excess or loose agar removed to ensure that no agar was transferred into the mycelium samples. The plates were sealed with Parafilm and stored at 20 $^{O}$C in darkness. After 14 days, the plates were placed back under the HEPA-filtered laminar flow cabinet and, using a sterile scalpel, the mycelium was collected together. Care was taken throughout to ensure that no agar was transferred into the mycelium sample. The mycelium was then placed inside a 2 ml tube and either used immediately or stored at -20 $^{O}$C.

## 2.6 *L. maculans* and *L. biglobosa* DNA extraction using DNAMITE plant kit

Samples collected as mycelium, affected leaf tissue or directly from cankerous stem tissue were disrupted with liquid nitrogen before being freeze-dried for 24 hours. The dried samples were ground into a fine powder inside a 2 ml tube using a micro-pestle. Three sterile metal beads and approximately 0.03g of ground sample were placed in a fresh 2 ml screw-capped tube (Greiner-Bio-One GmbH, Germany). The DNA extraction process was done using a DNAMITE Plant DNA extraction Kit with a modified method (Microzone Limited, Sussex, UK). Solution LA (1 ml) was transferred into each sample and vortexed briefly before being homogenised (FastPrep-24 Instrument, MP Biomedicals, Santa Ana, CA, USA) at speed setting 4 for 40 sec. Aliquots of solution PA (100 µl) were added to the sample and vortexed briefly. The sample tubes were placed in a micro-centrifuge (Eppendorf Micro centrifuge 5415R, Hamburg, Germany) set to 11000rpm for 5 minutes. The supernatant (500 µl) was transferred into a new 1.5 ml tube containing 500 µl of

solution CA. Care was taken during this step to ensure that no debris from the sample was transferred into the new tube. The samples were vortexed briefly and stored for 5 min at 20 $^{O}$C. The samples were returned to the centrifuge set to 13000 rpm for 7 min; this deposited the DNA as a pellet. Finally, the supernatant was carefully removed and discarded and the tubes were centrifuged for 1 min. Any remaining supernatant was removed using a pipette. An aliquot of SD water (50-80 µl) was added to each sample before storing at 20 $^{O}$C for 30 min to allow the DNA to rehydrate. DNA concentrations were quantified using a Nanodrop spectrophotometer (Nanodrop Technologies, DE, USA) and diluted to working concentrations of 20 ng/µl. The extracted DNA samples were stored at -20 $^{O}$C until use.

## 2.7 Preparation of winter oilseed rape plants in controlled environment cabinets

Seeds of cvs Catana (Dekalb, Monsanto UK) were pre-germinated in a shallow rectangular germination tray (50 x 30 x 8 cm) containing a mixture of 50% enriched general-purpose compost (Miracle-Gro, UK) and 50% John Innes No 3 compost (JA Bower, UK) (Appendix A.3). Located in a glasshouse, the germination tray was watered regularly until seedlings had germinated and grown to approximately 1 cm in height. Selection of similar sized seedlings was then possible for further experimentation.

## 2.8 Preparation and application of commercial grade fungicides

Preparation of fungicides was done according to material safety data sheet (MSDS) and label recommendations. Under a HEPA filtered fume cupboard, commercial fungicide product was mixed into pre-sterilised water in a 100 ml Duran bottle and diluted accordingly. The mixture was then added to a clean plastic water-atomiser (WM Morrison plc, UK) and applied to the oilseed rape true leaves or cotyledons until first indications of run-off occurred.

## 2.9 Polymerase chain reaction parameters

Custom oligonucleotide primers (Table 2.2) were designed using Primer3 plugin

(Untergasser et al. 2012) in Geneious Pro R9.1.4 (Biomatters Limited, Auckland, New Zealand) and synthesised by Invitrogen (Thermo Fisher Scientific, MA, USA). Primers were checked for primer dimer formation using Primer3 plugin in Geneious Pro R9.1.4 (Biomatters Limited). All PCRs were completed in a Techne T3 thermocycler (Bibby Scientific, UK). Routine PCRs were achieved using REDTaq® ReadyMix™ PCR reaction mixture, which consisted of Taq DNA polymerase, 99% pure deoxynucleotides, reaction buffer and an inert red dye in a 2x concentrate (Sigma, MO, USA). Restriction cloning PCR was done using EasyA High Fidelity PCR cloning enzyme (Agilent, CA, USA). All PCR reaction mixes had a final primer concentration of 150 nM. PCRs were done using 10 – 200 ng of template DNA. Sterile water (1-2.5 µl ) was used as a negative control.

PCR products that were analysed using gel electrophoresis, and those that did not already have a dye introduced into the master mix were mixed with 20% GelPilot DNA Loading Dye, 5x (Qiagen, Hilden, Germany) or 15% Gel Loading Dye, Purple 6x (New England Biolabs, MA, USA). Molecular biology grade agarose (Sigma) was dissolved in 1 X TBE buffer (0.89 M Tris Borate, 20mM $Na_2$ EDTA) to make 0.7 – 2% (w/v) agarose gels. Gels were stained with Gel Red (Biotium Inc, CA, USA) at a final concentration of 1%. Either 5 or 10 µl of PCR product was loaded onto the gel for routine gel electrophoresis and 25 – 50 µl of the product was loaded if it was to be excised for purification. Each gel was loaded with 10 µl of either a 1 kb or a 100 bp DirectLoad DNA ladder (Sigma). Gels were electrophoresed at 70 - 90 V for 100 - 150 min. DNA fragments were visualised under a 302 nm UV-transilumminator (Syngene, MD, USA) and the complementary imaging software.

Where necessary, PCR products were gel-excised or directly purified using the MinElute gel extraction kit (Qiagen), Monarch DNA Gel Extraction Kit (New England Biolabs) or QIAquick PCR Purification kit (Qiagen). All methods were followed according to the manufacturer's protocols.

## 2.10 Quantitative real-time polymerase chain reaction parameter

Quantitative real-time polymerase reactions were prepared using Brilliant II QRT-PCR SYBR Green Low ROX Master Mix (Sigma) and executed in Agilent Technologies Stratagene Mx3005P real-time PCR machine (Agilent). The reaction mixture contained 2.5 µl of DNA (at a concentration of 25 µg/ml), 0.6 µl forward and

reverse primer (at a final concentration of 300 nM), 10 µL of Brilliant III Ultra-Fast SYBR Green QPCR Master Mix with low Rox (Agilent) and HPLC grade water (Sigma) to a total volume of 20 µl. Thermocycling parameters were as follows: initial denaturation at 95 °C for 2 min, followed by 40 cycles of 95 °C for 15 sec, 60 °C (*L. maculans*) or 55 °C (*L. biglobosa*) for 30 sec and 72 °C for 36 sec. A single dissociation curve cycle was performed following main thermocycling. A 10-fold standard curve ranging between 10,000 pg and 0.1 pg was prepared using high quality DNA (> 1.80 260/280 absorbance ratio) for either *L. maculans* or *L. biglobosa*. All reactions were prepared in duplicate and total DNA (pg) was automatically calculated using the MxPro QPCR software (Agilent).

## 2.11 DNA sequencing

Sequencing was executed by GATC Biotech AG (London, UK) using the Sanger dideoxy method on ABI 3730 XL sequencing machines. Purified PCR products were adjusted to 20 - 30 ng/µl, purified plasmid DNA was adjusted to 100 ng/µl and sequence-specific primers adjusted to a concentration of 10 mM (final concentration of 150 nM). Sequence output files were imported into Geneious R9.1.14 (Biomatters Limited). Sequence chromatograms were checked manually for poor sequence quality and trimmed appropriately. Sequences larger than 1kbp that were subsequently sequenced in fragments were assembled *de novo* using the Cap3 plugin (Huang and Madan 1999) in Geneious R9.1.4 (Biomatters Limited). Protein sequence open reading frames were predicted using ORFinder (NCBI 2016) and DNA sequences were translated in Geneious R9.1.4. When appropriate, nucleic acid and protein sequences were aligned using Clustal Omega (Sievers et al. 2011).

## 2.12 Statistical analysis

All statistical analysis was done using R programming language (R Development Core Team 2011) in the RStudio GUI (Boston, MA, USA). Specific statistical tests are referred to in corresponding experimental chapters.

**Table 2.2.** Custom oligonucleotide primers used throughout this study

| Primer name | Sequence (5" - 3") | Experiment |
|---|---|---|
| LmacF | CTTGCCCACCAATTGGATCCCCTA | *Leptosphaeria* spp. identification (Liu et al. 2006) |
| LmacR | GCAAAATGTGCTGCGCTCCAGG | |
| L.bigF | ATCAGGGGATTGGTGTCAGCAGTTGA | |
| L.bigR | GCAAAATGTGCTGCGCTCCAGG | |
| Lmac1_F | ACGTTGGTTTGATGGTCCGA | *Leptosphaeria* spp. sequencing |
| Lmac1_R | CCTCTTGTGTGAGGCCGAAT | |
| Lmac2_F | CCACTTACCACGCCCGTATT | |
| Lmac2_R | AGATCATGTCGTGCGAGTCC | |
| Lmac_3_F | CCGCTCCTTCGACTCCAAAT | |
| Lmac_3_R | CACAAATCCAAGCACGAGCC | |
| Lbig1_F | GTCGTCAGTTAGTGGTGCGA | |
| Lbig1_R | TTGTTGCCCGTCGTATCCAA | |
| L.big_2_F | TCCTCAAGCGCCACAAATCT | |
| L.big_2_R | GGGGTTGACGAAGTGGGAAT | |
| L.big_3_F | CTCCTGCTGAAGAAGCCGAA | |
| L.big_3_R | GAGGAGGATCCAGGCGATTG | |
| L.big_4_F | CATGATCGCCCTCCTCATGG | |
| L.big_4_R | AAATAA ATTGACGCTCTACTCCACC | |
| LmCYP51res_F | GCAAAGCTTATGGCTGTTCTTGCTACCGT | *Leptosphaeria* spp. restriction cloning (*HindIII* & *NotI*) |
| LmCYP51res_R | TTAGCGGCCGCCTACTCGACCTTCTCCCTCC | |
| LbCYP51res_F | GCAAAGCTTATGGGTGTTCTTGCTACCATTG | |
| LbCYP51res_R | TTAGCGGCCGCCTACTCCACCTTTTCTCTGCGC | |
| M13F | TGTAAAACGACGGCCAGT | pGEM-T-easy sequencing primers |
| M13R | CAGGAAACAGCTATGACC | |

# Chapter 3 Effects of a penthiopyrad and picoxystrobin fungicide mixture on control of phoma stem canker (*Leptosphaeria* spp.) on UK winter oilseed rape

## 3.1 Introduction

Phoma stem canker is a disease of oilseed rape, which is caused by closely related fungal species *Leptosphaeria maculans* and *L. biglobosa* (Fitt, et al. 2006a; Stonard et al. 2010). Both pathogens follow a monocyclic disease cycle in the UK, with phoma leaf spotting symptoms in autumn/winter and stem base canker in spring/summer. Severe cankers inhibit the flow of water and nutrients to the seed, and thus decrease seed yield and quality. Oilseed rape is the third most valuable arable crop grown in the UK and has a total annual value of > £600 M and an average on-farm yield of 3.5-4.0 t/ha (AHDB Cereals & Oilseeds 2015). Globally, phoma stem canker has been calculated to annually cause approximately £700M worth of losses, making it a significant threat to worldwide oilseed rape production and food security (Fitt et al. 2006b).

Generally, *L. maculans* forms damaging stem base cankers and *L. biglobosa* forms less damaging upper stem lesions on UK winter oilseed rape (Fitt et al. 2006a; Huang et al. 2011). This difference is considered a result of differences in timing of ascospore release, with *L. maculans* spores released in early/mid-autumn and *L. biglobosa* spores released in early/mid-winter (Fitt et al. 2006b). More recently, however, *L. biglobosa* has been shown to cause severe upper stem lesions and lodging of crops in some growing seasons (Huang et al. 2014a). If this occurs regularly, *L. biglobosa* could become a more important threat to winter oilseed rape yield.

Together with conventional plant breeding strategies that adopt effective resistance genes (Delourme et al. 2006), fungicides are commonly used in the UK to control phoma stem canker on winter oilseed rape. In 2014, 98.1 % of the total area of oilseed rape (674,580 ha) received fungicide treatment for control of diseases including phoma stem canker because growers generally expect such treatments to give a yield response (Garthwaite et al. 2014). UK winter oilseed rape experiments have often shown a yield response from fungicide application against phoma stem canker, although an increase in yield was registered only when canker severity in unsprayed plots was ≥ 3 on a 0-5 disease severity scale (West et al. 2002). Typically,

azole fungicides have been applied because of their effective action against *L. maculans* as well as their relatively low cost compared to alternatives. Examples include flusilazole, prothioconazole and tebuconazole (Eckert et al. 2010; Huang et al. 2011). Other fungicides are available to growers; these include quinone outside inhibitor (QoI) fungicides and succinate dehydrogenase inhibitor (SDHI) fungicides, both of which disrupt energy production in the fungal cell (Avenot and Michailides 2010; Bartlett et al. 2002); however, their efficacy against phoma stem canker has not been evaluated.

Legislation from the European Union has forced the withdrawal of some fungicides used to control fungal pathogens in arable crops (Marx-Stoelting et al. 2014). An example is the withdrawal of flusilazole, a chemical widely used for phoma stem canker control in the UK until 2014. Despite concluding that flusilazole fulfils safety requirements set by Member States, on review the European Commission withdrew usage of Flusilazole across the entire European Union (European Commission 2007). Withdrawal of flusilazole reduced options available to growers for control of phoma stem canker, along with other crop diseases. It is thus imperative to obtain a complete understanding of the effects that novel fungicide mixtures have on phoma stem canker in winter oilseed rape crop.

This chapter describes work investigating the efficacy of a new fungicide mixture Refinzar® (a.i. penthiopyrad plus picoxystrobin, an SDHI plus QoI, respectively) to reduce phoma leaf spotting, decrease phoma stem canker severity and improve oilseed rape yield.

It should be noted that field data collected during cropping seasons 2011/2012 and 2012/13 were collected by Steven Moloney with assistance from Dr Avice Hall.

## 3.2 Materials and methods

### 3.2.1 Weather conditions at the field site

Weather data for the 2011/12, 2012/13, 2013/14 and 2014/15 winter oilseed rape growing seasons were collected at Boxworth, Cambridgeshire, UK (52.259814, -0.025437); near the winter oilseed rape field experiments and the Burkard spore sampler in the 2014/15 cropping season and approximately 15 km from the site of the Burkard spore sampler in 2011/12, 2012/13 and 2013/14. Temperature and rainfall data were collected daily using an automated weather station (Campbell Scientific, UK).

### 3.2.2 Ascospore numbers

The numbers of *Leptosphaeria* ascospores in the air were estimated using a 7-day volumetric spore sampler (Burkard Manufacturing Co. Ltd, UK). For the 2011/12, 2012/13 and 2013/14 cropping seasons, the spore sampler was located at Whittlesford, Cambridgeshire, UK (52.109299, 0.156023). For the 2014/15 cropping season, the spore sampler was located at Boxworth, Cambridgeshire (52.270127, -0.027112). The spore sampler accommodated a rotating drum (2 mm per hour) that held a strip of Melinex tape. The tape was lined with a thin layer of petroleum jelly and hexane paste mixture (10 g petroleum jelly, 20 ml hexane). After 7 days of sampling, the rotating drum was removed and the Melinex tape was divided into seven 24-hour segments. Each segment was then cut horizontally, with one half mounted for microscopy to count spore numbers and one half stored at -20 $^{o}$C for future molecular analysis. The slide-mounted tape was stained with trypan blue solution (0.4% w/v in water, Sigma-Aldrich, UK) so that the ascospores were visible under a light microscope (100x total magnification). Counting was done in three longitudinal traverses across the slide and the number of ascospores recorded for each traverse. The concentration of ascospores in the air was calculated according to the equation described by Lacey and West (2006).

### 3.2.3 Winter oilseed rape field experiments

Field experiments were established near Boxworth, Cambridgeshire, UK for the 2011/12, 2012/13, 2013/14 and 2014/15 cropping seasons (Appendix C.1-C.4). The winter oilseed rape cultivar Catana (Dekalb, UK) was used because of its susceptibility to *L. maculans* (resistance rating of 4 in the UK North region on a 1-9 scale; where 9 is very resistant) but good resistance against *Pyrenopeziza brassicae* the cause of light leaf spot (AHDB Cereals & Oilseeds 2015).

In each growing season, seeds of cv. Catana were sown in mid/late August at a seed rate of 5 kg/ha and a drilling depth of 1 cm. To test the efficacy of a new fungicide mixture (penthiopyrad + picoxystrobin), by comparison to existing fungicides (flusilazole or prothioconazole), for control of phoma stem canker (*Leptosphaeria* spp.) and impact on winter oilseed rape yield, experiments were arranged in a randomised block design with three replicates. Each plot received one of 14 treatments (four different fungicides applied under three different timing regimes (T1, T2 or T1 and T2 combined), one untreated throughout the cropping season, one

treated with a spring spray only, T3), thus totalling 42 plots (Table 3.1). The fungicide Refinzar® (DuPont UK Ltd; a.i. penthiopyrad 160 g/l plus picoxystrobin 80 g/l) was used in all four cropping seasons. The product has been marketed as a potential alternative to the azole fungicides that are used widely in the UK on winter oilseed rape. Sanction® (DuPont UK Ltd; a.i. flusilazole 250g/l) was used for the first two cropping seasons before its active ingredient flusilazole was withdrawn. It was replaced by another azole fungicide, Proline 275® (Bayer Crop Science UK Ltd; a.i. prothioconazole 275 g/l), for the 2013/14 and 2014/15 cropping seasons. To represent the components of Refinzar®, Galileo® (DuPont UK Ltd; a.i. picoxystrobin 250 g/l) and LEM17® (DuPont UK Ltd; a.i. penthiopyrad 200 g/l) were also applied but these data are not presented. The fungicide spray timings differed from season to season, with the first application (T1) taking place in autumn when 10% of plants were affected with phoma leaf spots. The second application (T2) was made 8 weeks after T1 in the 2011/2012 season and 4 weeks after T1 in the 2012/13, 2013/14 and 2014/15 seasons. All plots except the untreated control received a spring-flowering spray (T3) against the pathogen *Sclerotinia sclerotiorum*, the causal agent of sclerotinia stem rot.

### 3.2.4 Phoma leaf spotting, stem canker severity and yield assessment

Phoma leaf spotting was assessed by randomly sampling ten plants per plot in the 2011/12 and 2012/13 cropping seasons and 15 plants per plot in the 2013/14 and 2014/15 cropping seasons; as described in Steed et al. (2007). The sampling was done regularly between November and February each cropping season. The total numbers of *L. maculans* (large grey lesions with pycnidia) and *L. biglobosa* (small dark lesions with few or no pycnidia) leaf spots on each leaf were recorded, together with the growth stage of the plant (Appendix C.5).

Phoma stem canker severity assessment was done once in the 2011/12 and 2012/13 cropping seasons (25 July 2012 and 9 July 2013), twice in the 2013/14 cropping season (27 May and 1 July 2014) and twice in the 2014/15 cropping season (1 June and 29 June 2015). A random sample of either 10 (2011/12 and 2012/13), 25 (2013/14) or 15 (2014/15) plants was collected from each of the 42 plots using the method described in Steed et al. (2007). The severity of basal cankers was assessed by cutting the stem at the base of each sampled plant and scoring the cross-sectional area of necrotic tissue according to a 0-6 scale (Huang et al. 2011), modified from Lô-Pelzer et al. (2009). Upper stem lesions were cut at the centre point of the lesions

**Table 3.1.** Treatment list giving fungicides and spray timings used in field experiments at Boxworth, Cambridge over four winter oilseed rape (cv. Catana) cropping seasons. Experiments were arranged in a randomised block design with three replicates. T1 spray was applied in the autumn when 10% of the plants had phoma leaf spotting. T2 spray was applied in the autumn/winter 4 or 8 weeks after T1. A third fungicide spray (T3) targeting sclerotinia stem rot was applied to all treatments except treatment 1, which remained untreated throughout the cropping season. In 2011/12 and 2012/13 cropping seasons, prothioconazole was used as the flowering spray (T3) and in 2013/14 and 2014/15 picoxystrobin was used.

| Spray timing | T1 (10% leaf spotting) | | T2 (T1 + 4 or 8 weeks) | |
|---|---|---|---|---|
| Treatment number | Chemical | Rate g a.i/ha | Chemical | Rate g a.i/ha |
| 1 | Untreated | - | Untreated | - |
| 2* | Untreated | - | Untreated | - |
| 3^ | Flusilazole or Prothioconazole | 200 or 176 | Untreated | - |
| 4 | Penthiopyrad | 160 | Untreated | - |
| 5 | Picoxystrobin | 80 | Untreated | - |
| 6 | Penthiopyrad + Picoxystrobin | 160 + 80 | Untreated | - |
| 7^ | Untreated | - | Flusilazole or Prothioconazole | 200 or 176 |
| 8 | Untreated | - | Penthiopyrad | 160 |
| 9 | Untreated | - | Picoxystrobin | 80 |
| 10 | Untreated | - | Penthiopyrad + Picoxystrobin | 160 + 80 |
| 11^ | Flusilazole or Prothioconazole | 200 or 176 | Flusilazole or Prothioconazole | 200 or 176 |
| 12 | Penthiopyrad | 160 | Penthiopyrad | 160 |
| 13 | Picoxystrobin | 80 | Picoxystrobin | 80 |
| 14 | Penthiopyrad + Picoxystrobin | 160 + 80 | Penthiopyrad + Picoxystrobin | 160 + 80 |

* Received T3 flowering spray and therefore differs from treatment 1 which was untreated throughout cropping season.

^ Flusilazole was applied in 2011/12 and 2012/13 until its withdrawal and was replaced by prothioconazole in 2013/14 and 2014/15

and assessed on the same scale (Appendix C.6). Desiccated plots were harvested using a small plot harvester and yield (t/ha) recorded. Presence of light leaf and sclerotinia on stems was also noted.

### 3.2.5 Stem canker subsampling, DNA extraction and species-specific PCR

To investigate whether the phoma stem cankers were caused by *L. maculans* and/or *L. biglobosa*, stems with basal stem canker or upper stem lesion symptoms were subsampled for DNA extraction and *Leptosphaeria* species-specific PCR. Approximately three stems per plot were selected from basal stem canker and upper stem lesion samples from all 42 plots of the 2013/14 field experiment. Using a scalpel, thin shavings of the basal canker or upper stem lesion tissue were cut away from each stem and placed in 2 ml Eppendorf tubes (Sigma-Aldrich Co LLC, UK). The subsamples were stored at -20 $^{o}$C after freeze-drying for 24 hours. The sub-samples were then ground into a powder using a mortar and pestle. Sub-samples of the powdered stem material were transferred into 2 ml Eppendorf tubes and DNA was extracted using a DNA extraction kit (DNAMITE Plant kit; Microzone Ltd, UK) and quantified using a Nanodrop ND-1000 spectrophotometer (Labtech International, UK). Identification of species was done using end-point PCR with species-specific PCR primers LmacF/LmacR for *L. maculans* and LbigF/LmacR for *L. biglobosa* (Liu et al. 2006). Gel electrophoresis was done to identify the presence of *L. maculans* and/or *L. biglobosa* DNA.

### 3.2.6 Statistical analysis

The R software was used for statistical analyses of data (R Development Core Team 2011). Linear mixed effects models were done on phoma leaf spotting, canker severity and yield data. Two-way mixed effect ANOVA was done on fungicide spray treatments. One-way mixed effect ANOVA was done independently on spray timing treatment and then fungicide treatment. Residuals were tested for normality using the Shapiro-Wilk test of normality.

## 3.3 Results

### 3.3.1 Weather conditions at the field site

Rainfall patterns differed between the four seasons during autumn/winter (phoma leaf spot development stage) and summer (phoma stem canker development stage). In the 2011/12 cropping season, the autumn and winter months were dry compared with the 2013/14 cropping season. In August and September, 73 mm of rainfall was recorded. Periods of prolonged rainfall did not commence until December 2011 and there were never periods of heavy rainfall. In the summer (2012), it was predominantly wet, with heavy rainfall in April (101 mm), June (103 mm) and July (115 mm) (Figure 3.1b). In the 2012/13 cropping season, prolonged rainfall occurred much earlier, with periods of substantial rainfall commencing in mid-September and continuing to mid-February with the occasional short dry period. In August and September, 70 mm of rainfall was recorded. The spring and summer were dry with occasional periods of short-term rainfall (Figure 3.1d). In the 2013/14 cropping season, the rainfall pattern was similar to that of the 2012/13 growing season in the autumn/winter. Rainfall started in early autumn, with increases in August and September over a few days and then continued for a period between October and mid-November. In August and September 2013, 91 mm of rainfall was recorded. A period of prolonged rainfall occurred between December and February (202 mm over 88 days) (Figure 3.1f). In the 2014/15 cropping season high rainfall commenced early (8 August) with a period of very heavy rainfall (112.6 mm) causing flash floods in the region. In August and September 2014, 192 mm of rainfall was recorded although 58 % of this was on 8 August. Rainfall in the winter months was more sporadic than in the previous seasons, with no periods of particularly prolonged rainfall between December and February (Figure 3.1h).

Across the four seasons, average temperature followed a typical pattern, with temperature decreasing to ≤ 0 $^{o}$C in December, January and February. Periods of particularly low temperatures differed between seasons. In the 2011/12 cropping season, a low temperature (-7.1 $^{o}$C) occurred on 10 and 11 of February. Average temperature between 1 October and 31 May was 7.8 $^{o}$C (Figure 3.1b). In 2012/13, a similar pattern was observed, but low temperature (-4.4 $^{o}$C) occurred a month earlier on 14 January. One notable difference in this cropping season was an uncharacteristic period of cold weather in mid/late March. Snowfall and temperatures < 0 $^{o}$C were recorded during this period. Average temperature between 1 October and 31 May was 5.7 $^{o}$C (Figure 3.1d). In 2013/14, there was no period of particularly

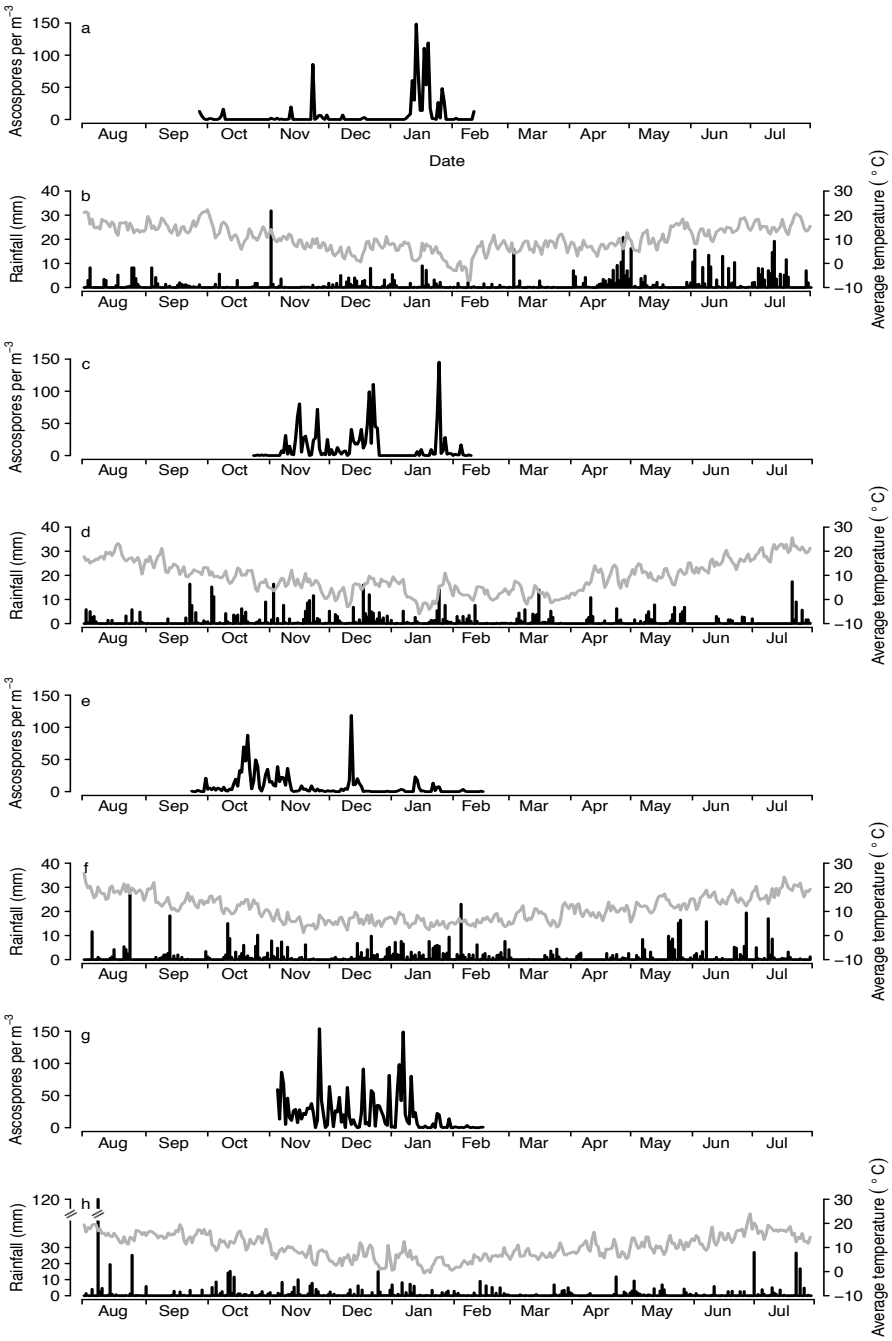



**Figure 3.1.** Numbers of ascospores of *Leptosphaeria* spp. (a, c, e, g), average temperature and daily rainfall (b, d, f, h) monitored over four cropping seasons. a-b) 2011/12 cropping season; c-d) 2012/13; e-f) 2013/14; g-h) 2014/15. Weather data were collected at Boxworth, Cambridgeshire, using a day interval automated weather station. The grey line represents average temperature ($^{o}$C) and black bars represent total daily rainfall (mm). Airborne ascospores (number $m^{-3}$) were collected using a Burkard spore sampler that was situated at Whittlesford, Cambridgeshire (15 km from site of the field experiment) in 2011/12, 2012/13 and 2013/14 and Boxworth, Cambridgeshire in 2014/15.

cold weather, with average daily temperature never < 0 $^{o}$C. Average temperature between 1 October and 31 May was 8.3 $^{o}$C (Figure 3.1f). The 2014/2015 cropping season was similar to the previous season in that there was no period of particularly cold weather, with average daily temperature only < 0 $^{o}$C on two occasions (-0.7 $^{o}$C and -0.4 $^{o}$C on 19 January and 22 January, respectively). Average temperature between 1 October and 31 May was 7.2 $^{o}$C (Figure 3.1h).

### 3.3.2 Ascospore numbers

The numbers of ascospores in the air and the period in which most ascospores were released differed between growing seasons. In 2011/12 and 2012/13, there was a major discharge of spores in November and a large discharge of spores in January; the discharge in November was longer in 2012/13 (Figure 3.1a, c). In 2013/14, the spore release pattern was similar to 2012/13 but differed in timing; ascospore dispersal occurred over a longer period in the autumn, with a large release in the winter of both seasons; however, in 2013/14, the autumn release of spores was a month before the equivalent release in 2012/13 (November in 2012/13 and October in 2013/14). Similarly, a large release of spores in the winter occurred a month earlier in 2013/14 than 2012/13 (January in 2012/13 and December in 2013/14) (Figure 3.1c, e). Due to accessibility issues in 2014/2015 cropping season, spore release data commenced at the start of November. Nonetheless, two large releases were recorded at the end of November and mid/late January (Figure 3.1g). A common pattern among all four seasons was the relationship between rainfall and spore release. In most seasons, spore release in large numbers commenced after a period of prolonged or heavy rainfall. For example, heavy rainfall at the start of November 2011 was associated with ascospore release later that month. However, some spores were also released after periods of light rainfall, such as in December 2013.

### 3.3.3 Winter oilseed rape field experiments

In all four cropping seasons, the spring flowering spray had no affect on leaf spotting, canker severity or yield compared to the control; therefore, the untreated control data presented are a mean of untreated plots and spring spray only (T3) plots. Penthiopyrad alone produced similar results to penthiopyrad + picoxystrobin and picoxystrobin alone produced similar results to the untreated control. As penthiopyrad

+ picoxystrobin is the commercially available product (Refinzar), the single components have been excluded from the analysis and data are not presented.

### 3.3.4 Phoma leaf spotting

In the 2011/12, 2012/13 and 2014/15 cropping seasons, incidence of phoma leaf spotting in unsprayed plots did not increase in severity on winter oilseed rape leaves until March and phoma leaf spotting was never severe during the autumn/winter; therefore, data are not shown. In 2013/14, the phoma leaf spotting started earlier and incidence (% plants affected) was much greater in unsprayed plots in the autumn/winter months compared to the previous two winter oilseed rape cropping seasons (Figure 3.2). Experimental plots treated with penthiopyrad + picoxystrobin or prothioconazole had significantly less *L. maculans* type leaf lesions per plant when compared with the untreated control, except when fungicides had only just been applied (T2 only plots at December 2013 assessment) or when their activity had decreased over time (T1 application at February 2014 assessment). The penthiopyrad alone treatment was statistically similar to the picoxystrobin + penthiopyrad treatment.

The two fungicides significantly decreased number of *L. biglobosa* type lesions, compared with the untreated control, in December 2013 on T1-only and on T1-plus-T2 plots, and in February 2014 on T2 only treated plots. When comparing the efficacy of the two fungicides, there was no significant difference in the numbers of *L. maculans* type lesions present between penthiopyrad + picoxystrobin and prothioconazole treated plots (Figure 3.2a, c, e). Furthermore, there was no significant difference in the numbers of *L. biglobosa* type leaf lesions present between penthiopyrad + picoxystrobin and prothioconazole treated plots (Figure 3.2b, d, f).

### 3.3.5 Stem canker severity

In the 2011/12, 2012/13 and 2014/15 cropping seasons, stem canker was not severe (Figure 3.3). Severity was never more than 1.5 on a 0-6 scale for either upper stem lesions or basal stem cankers in these three cropping seasons. Fungicide application did not significantly decrease stem canker severity in 2011/12 and only prothioconazole at the combined T1/T2 application timing significantly reduced

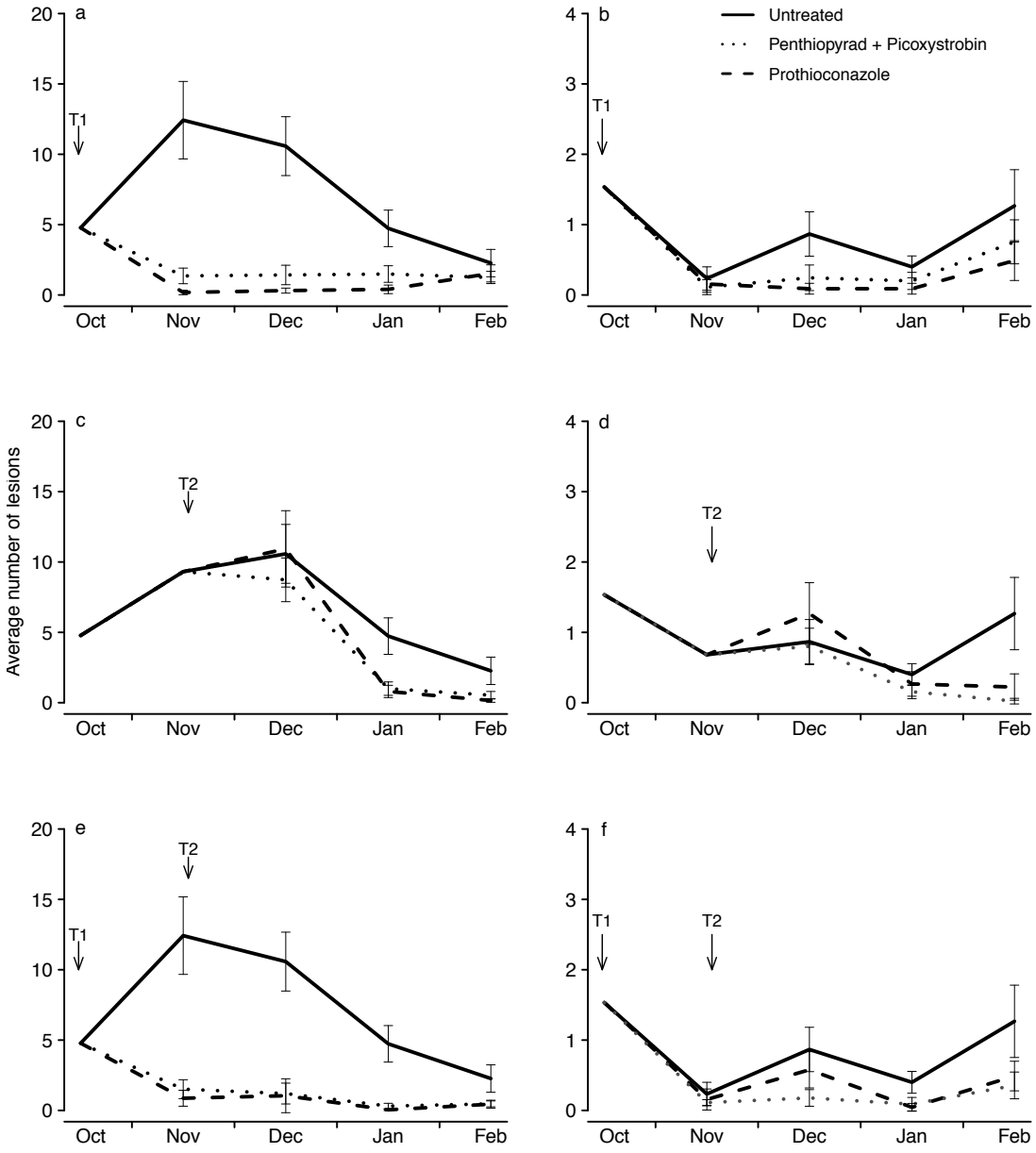



**Figure 3.2.** Incidence of phoma leaf spotting associated with *Leptosphaeria maculans* (a, c, e) or *L. biglobosa* (b, d, f) type leaf lesions on winter oilseed rape (cv. Catana) plots sprayed with fungicide at T1 (early) (a, b), T2 (late) (c, d) or T1 & T2 (combined) (e, f) in the 2013/14 cropping season near Boxworth, Cambridgeshire. Fifteen winter oilseed rape plants were collected from each plot and assessed for incidence of *L. maculans* and *L. biglobosa* type leaf lesions. Plots were treated with penthiopyrad + picoxystrobin (dotted line), prothioconazole (dashed line) or untreated (solid line). Average number of leaf lesions per leaf was calculated. Standard errors of the means are represented as error bars. Details of spray timings are given in Table 3.1.

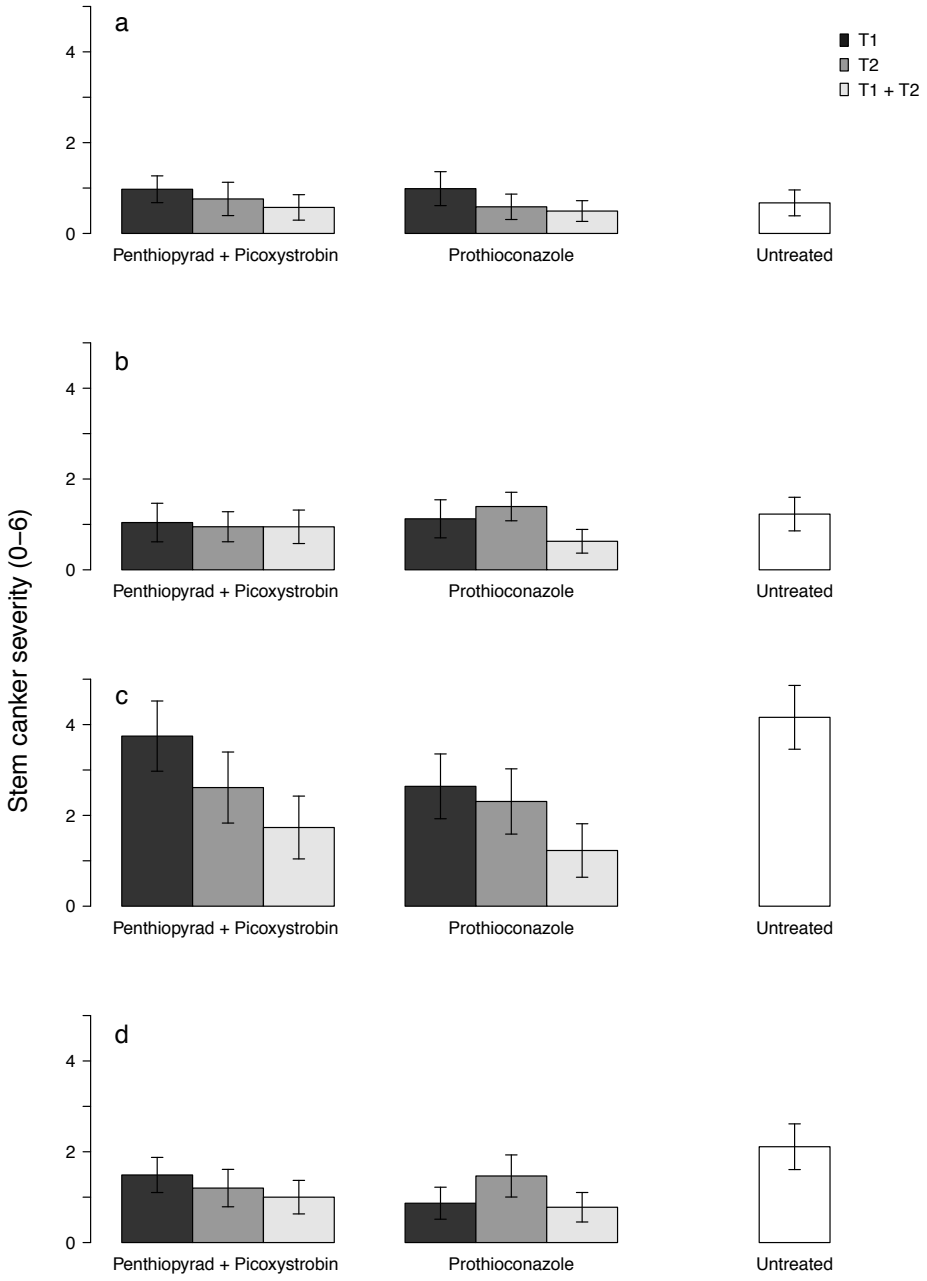



**Figure 3.3.** Basal stem canker severity on experimental winter oilseed rape (cv. Catana) plots in a) 2011/12, b) 2012/13, c) 2013/14 and d) 2014/15 cropping seasons near Boxworth, Cambridgeshire. Plots received sprays of penthiopyrad + picoxystrobin or prothioconazole at T1 (early), T2 (late) or T1 & T2 (combined). Basal stem canker severity (scale 0-6; Lô-Pelzer et al., 2009) was scored on 25 plant stems sampled from each plot. Standard errors of the means are represented as error bars (6 df). Details of spray timings are given in Table 3.1.

severity compared to the control in 2012/13. In the 2013/14 cropping season (Figure 3.3c), canker was more severe than in other seasons. There were significant differences in the severity of basal stem cankers between fungicide treatments and between timings ($P < 0.05$, 12 df); however, there was no significant difference in upper stem lesion severity between fungicide treatments or timings (data not shown). Unlike prothioconazole, penthiopyrad + picoxystrobin did not decrease the severity of basal stem cankers when applied at the T1 spray timing only when compared to untreated ($P < 0.05$, 4 df). Nonetheless, at T2 and T1/T2 timings, both penthiopyrad + picoxystrobin and prothioconazole reduced severity equally. Penthiopyrad + picoxystrobin at T1/T2 and prothioconazole at T1/T2 performed similarly, reducing basal stem canker severity more than if they were applied at T1 only or T2 only. Although there were significant differences between fungicide treatments and between timings, the interactions were not significant and were removed from the final model.

No other diseases were severe in the field experiments across all four growing seasons; however, in 2014/15 cabbage stem flea beetle affected winter oilseed rape establishment in the Cambridgeshire region and may have had an affect on the field experiments. Light leaf spot was present but not severe and sclerotinia was present on just one sampled stem.

### 3.3.6 Yield

Improvement in yield of fungicide-treated plots was sometimes positive and sometimes negative when compared with the control over the four cropping seasons (Figure 3.4). Despite effects of treatment on stem canker severity across all cropping seasons, there was no significant effect of fungicide treatment on yield in any season.

### 3.3.7 Stem canker subsampling, DNA extraction and species-specific PCR

A total of 133 basal stem canker samples and 74 upper stem lesion samples was analysed by PCR. The proportions of upper stem lesions and basal stem cankers with *L. maculans* DNA detected in the sample was much greater than those with *L. biglobosa* DNA detected (Table 3.2). Out of 74 samples of upper stem lesions, 45 had only *L. maculans* DNA detected; two samples had only *L. biglobosa* DNA

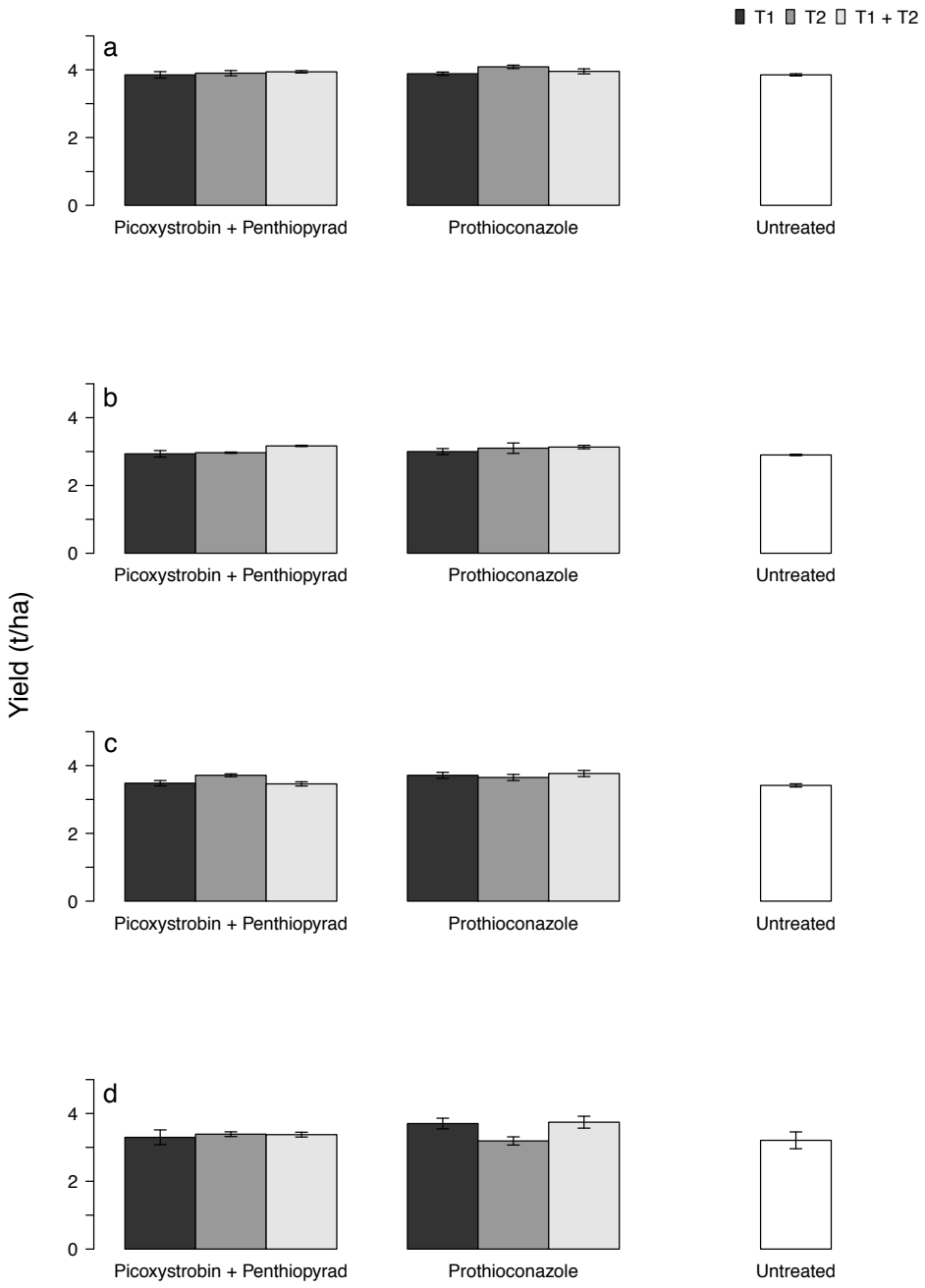



**Figure 3.4.** Average yield (t/ha) from experimental winter oilseed rape (cv. Catana) plots in a) 2011/12, b) 2012/13, c) 2013/14 or d) 2014/15 cropping seasons near Boxworth, Cambridgeshire. Plots received sprays of penthiopyrad + picoxystrobin or prothioconazole at an early (T1), late (T2) or combined (T1 & T2) timings. Desiccated plots were harvested using a small plot harvester and yield was calculated. Standard errors of the means are represented as error bars (6 df). Details of spray timings are given in Table 3.1.

**Table 3.2.** Numbers (percentage) of winter oilseed rape (cv. Catana) phoma stem canker subsamples with *L. maculans* or *L. biglobosa* DNA present determined by species-specific PCR for *L. maculans* and *L. biglobosa* (subsamples collected from stem base cankers* or upper stem lesions* sampled from all plots on 1 July 2014 were ground into a powder before DNA was extracted).

| Stem sample | Number (%) of stem canker subsamples with | | | |
|---|---|---|---|---|
| | *L. maculans* only | *L. biglobosa* only | Both | Neither |
| Upper stem lesion (n = 74) | 45 (60.8 %) | 2 (2.7 %) | 11 (14.9 %) | 16 (21.6 %) |
| Basal stem canker (n = 133) | 102 (77 %) | 0 | 4 (2.7 %) | 27 (20.3 %) |

* three stem base cankers or upper stem lesions per plot

detected and 11 samples had DNA of both species detected. No *L. maculans* or *L. biglobosa* DNA was detected in 16 upper stem samples. Of 133 basal stem canker samples, 102 had only *L. maculans* DNA detected and four samples had both species detected. No samples had only *L. biglobosa* DNA recorded. No *L. maculans* or *L. biglobosa* DNA was detected in 27 basal stem canker samples.

## 3.4 Discussion

These results suggest that in winter oilseed rape cropping seasons when there are moderately severe phoma stem canker epidemics, penthiopyrad + picoxystrobin and prothioconazole are both effective at reducing phoma stem canker severity. Severe canker results in yield loss because transport of water and nutrients up the stem is decreased by girdling, thus resulting in premature ripening and shrivelled seed pods (West et al. 2002). These results show that penthiopyrad + picoxystrobin or prothioconazole both prevent the formation of severe cankers, potentially allowing good pod development.

Furthermore, they show that foliar application of penthiopyrad + picoxystrobin or prothioconazole in the autumn reduced the number of *L. maculans* type leaf lesions that formed on leaves. Application of either fungicide when incidence of *L. maculans* leaf spotting reached 10% plants affected (T1) significantly reduced the number of lesions; a further application one or two months later (T2) appears to have had a smaller but still significant effect on the number of lesions. Work with GFP-labelled *L. maculans* has shown that if the phoma leaf spot stage is prevented, the pathogen does not grow along the leaf petiole to form stem cankers (Huang et al. 2009). Thus, this early stage inhibition stops the later development of cankers; exemplified here by the T1 and T2 application of either penthiopyrad + picoxystrobin or prothioconazole, which significantly reduced the number of lesions on leaves in November 2013 and December 2013 and significantly reduced stem canker severity in the following July (2014).

By contrast, in seasons when there is little early phoma leaf spotting (e.g. 2011/12 and 2012/13), the data suggest that fewer fungicide sprays are needed since canker severity was very low and it did not affect yield. The timing and severity of basal stem cankers and upper stem lesions has previously been reported to affect the potential yield of winter oilseed rape crops (Zhou et al. 1999). Early, severe basal cankers or upper stem lesions are more likely to cause yield loss than later/slight basal stem

cankers or upper stem lesions. The development of later, less severe stem cankers can be associated with a later release of ascospores, as shown by the 2011/12 and 2012/13 cropping seasons, when a large release of ascospores occurred later in the season compared to 2013/14; when there was less rainfall in August and September the release of ascospores was delayed, resulting in a later onset of phoma leaf spotting. Disease severity has previous been linked to yield loss in winter oilseed rape; only when disease severity is high (≥ 3 on a 0 – 5 severity scale) does a yield response occur in fungicide-treated plots (West et al. 2002).

The results for timing of ascospore release and leaf spotting suggest that the optimum fungicide application regime differs between seasons. In 2013/14, ascospore release was earlier, due to greater rainfall in August/September, than in the previous two seasons, thus resulting in a more severe canker prior to harvest. These observations are in general agreement with the UK phoma stem canker disease model published by Evans et al. (2008), based on many seasons of data, since the model predicts an earlier date for 10% phoma leaf spotting when rainfall and/or temperature are high during summer. Furthermore, the model predicts the date of onset and severity of canker using thermal time, with greater thermal time between 10% phoma leaf spotting and harvest resulting in more severe cankers. This explains why canker severity was less in 2011/2012 and 2012/13, when winter temperatures were less than in 2013/14.

The low incidence of *L. biglobosa* leaf spots, and small amount of *L. biglobosa* DNA in stem canker samples suggests that the disease was caused predominantly by *L. maculans* in these experiments. It has been suggested that *L. maculans* and *L. biglobosa* have a north-south distribution in England (Stonard et al. 2010), so a smaller amount of *L. biglobosa* in these southern sites was not unexpected. A multiple site study over several years is required to establish more information on the threat that *L. biglobosa* poses to UK oilseed rape production.

# Chapter 4 Investigating fungicide-sensitivity differences between *Leptosphaeria maculans* and *L. biglobosa*

## 4.1 Introduction

Phoma stem canker, a significant disease of oilseed rape, is caused by sibling pathogens species *Leptosphaeria maculans* and *L. biglobosa*. Sibling species are defined as one or two species that closely resemble each other but are unable to interbreed (Mayr 1942; Steyskal 1972). Usually, they have diverged from a recent common ancestor and often share many environmental, biochemical, behavioural and genetic characteristics (Fitt et al. 2006b; Gudelj et al. 2004; Mayr 1970).

In the light of these similarities, historically *L. maculans* and *L. biglobosa* had been considered to be just one species. Nonetheless, recent application of molecular and taxonomic techniques have aided in the true identification of two or potentially more species, which is now referred to as the *Leptosphaeria* species-complex (Mendes-Pereira et al. 2003; Shoemaker and Brun 2001). Some sibling species, such as *L. maculans* and *L. biglobosa* or *Oculimacula yallundae* and *O. acuformis,* the causal agents of eyespot in cereals, occupy similar niches but can exist contemporaneously, avoiding competitive exclusion because of slight differences in the space and time of their epidemics, meaning that they occupy slightly different niches (Fitt et al. 2006b).

Together with the exploitation of plant resistance, fungicides are commonly used to control the devastating effects of plant pathogens. In the UK, azole fungicides are often used to control *L. maculans*. Other fungicide types, such as QoI and SDHI fungicides, are also used but to a lesser extent or in combination with a triazole fungicide (Oliver and Hewitt 2014).

A uniqueness of coexisting species is that they cause similar disease symptoms on their host and are therefore subjected to similar agronomical practices, such as fungicide application. Any phenotypic difference in fungicide sensitivity, therefore, could affect competition between two coexisting pathogens, resulting in a population shift in accordance with the two-species competition model (Hassell and Comins 1976).

Previous reports have shown that populations of the closely related sibling pathogens *O. yallundae* and *O. acuformis* differ in sensitivity to the DMI fungicide prochloraz *in vitro* (Bateman et al. 1995). Prochloraz was used to replace several methyl

benzimidazole (MBC) fungicides that were used to control the disease before being rendered ineffective because of an increase in less sensitive *O. yallundae* and *O. acuformis* populations.

Identification of MBC-insensitive isolates resulted in the construction of a long-term field experiment using MBC-sensitive and MBC-insensitive isolates of both *O. acuformis* and *O. yallundae* (Bierman et al. 2002). The field sites were treated with carbendazim (MBC), prochloraz (DMI) or carbendazim/prochloraz. After five years, in plots treated with prochloraz mixture, the proportion of *O. acuformis* in the population had increased to >80% compared to the untreated control, a shift suggesting that prochloraz affects mycelium of *O. acuformis* and *O. yallundae* differently.

Previous studies have suggested that *L. maculans* and *L. biglobosa* isolates also have a difference in sensitivity to some azole fungicides *in vitro* and *in planta* (Eckert et al. 2010; Huang et al. 2011). With this in mind, it is imperative that a further understanding of this sensitivity difference, both *in planta* and *in vitro*, is investigated to ensure continued effective chemical control of phoma stem canker. Moreover, understanding of the efficacy of novel fungicide mixtures is needed to help diversify fungicide choices and prevent the selection of insensitive populations.

This chapter describes work investigating differential azole sensitivity in *Leptosphaeria maculans* and *L. biglobosa* populations both *in vitro* and *in planta*, and provides knowledge on the efficacy of the novel fungicide mixture, penthiopyrad plus picoxystrobin.

## 4.2 Materials and methods

### 4.2.1 Fungicide sensitivity of *L. maculans* and *L. biglobosa* mycelial growth on solid medium using technical grade fungicides

The method for studying fungicide inhibition of mycelial growth on solid-media was modified from that of Eckert et al. (2010) and involved potato dextrose agar (PDA, Appendix A.1) amended with decreasing concentrations of technical grade prothioconazole-desthio (Sigma), penthiopyrad (DuPont UK Ltd, Hertfordshire, UK), picoxystrobin (DuPont UK Ltd) or a penthiopyrad plus picoxystrobin mixture (2:1, penthiopyrad:picoxystrobin) (DuPont UK Ltd) (Figure 4.1).

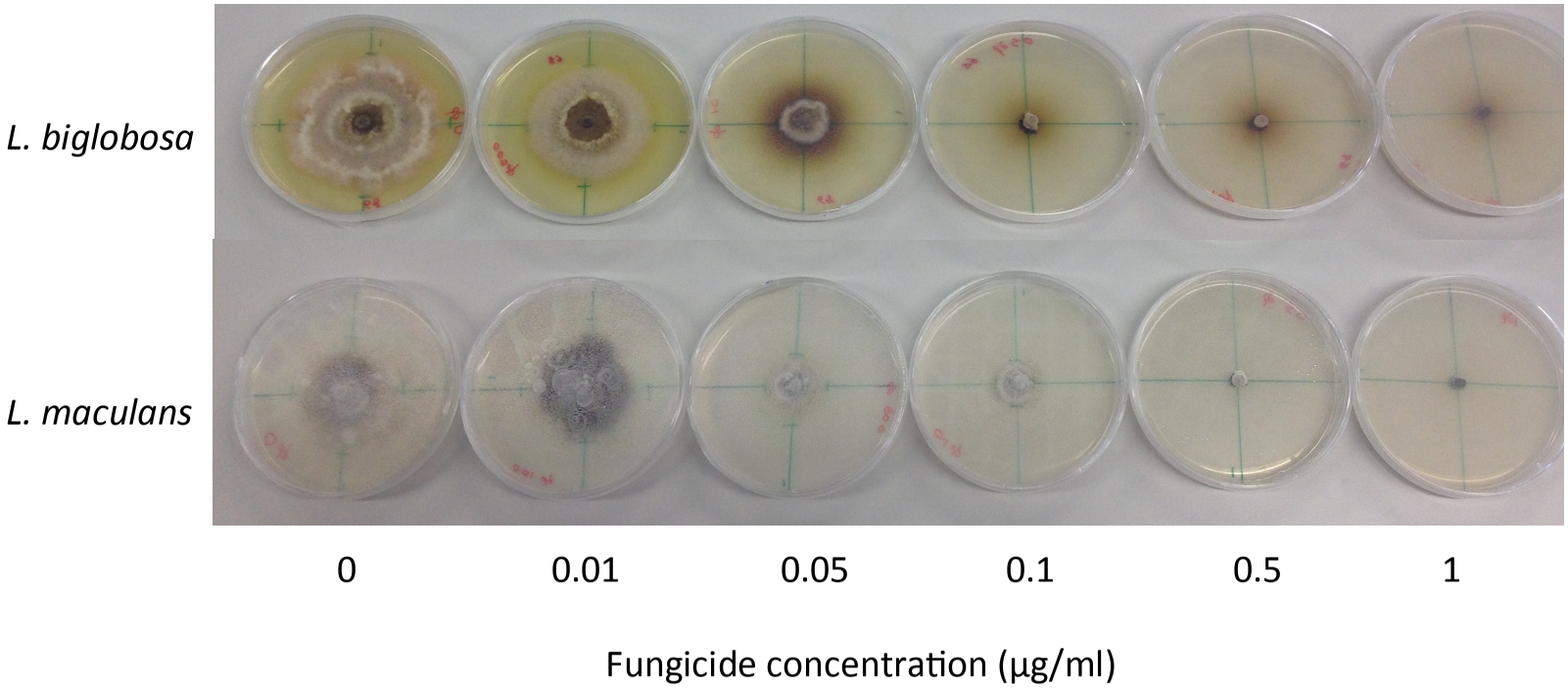



**Figure 4.1.** Example of the Petri plate layout used in the mycelium inhibition assay on fungicide-amended media. Using a modified version of the method described in Eckert et al. (2010), PDA was amended with increasing concentrations of fungicide. A pre-cultured plug of mycelium (4 mm diameter) was applied to the centre of the agar. Colony diameter (mm) was recorded regularly.

Stock fungicide solutions (6 mg/ml) were prepared in acetone before inoculation of plates and working solutions were subsequently prepared in water and diluted, depending on the required final concentration in the medium (Table 4.1). A final acetone concentration to match that in the fungicide-amended PDA plates was added to the control media. Molten fungicide-amended agar (15 ml) was then transferred to 90mm diameter Petri plates and left to solidify before inoculation.

Each Petri plate was inoculated with a single plug of mycelium taken from a prepared culture of *L. maculans* or *L. biglobosa* isolates (Chapter 2.1). All isolates had been confirmed to contain the species-specific predicated wild-type Lanosterol 14α-demethylase allele (Chapter 5). The mycelial plugs were taken from the radial, actively growing edges of the cultures using a flame-sterilised cork borer (4mm diameter) and placed mycelium-down into the centre of the fungicide-amended agar plates. All plates were individually sealed using Parafilm (Bemis, NA, USA) and incubated at 20 $^{O}$C for 22 days. The radial growth of the colony was measured at 4, 7, 11, 15, 18 and 22 days post inoculation (DPI). Using a mark at the centre of the colony, average diameter (mm) was calculated by measuring the length and width of the colony and calculating an average. The diameter of the initial mycelial plug (4mm) was subtracted from these measurements.

A Holling's type III non-linear regression model was fitted to the data for growth of *L. maculans* and *L. biglobosa* on all fungicide-amended media and at all fungicide concentrations. The equation (Eq. 4.1) can be described by writing:

$$y = \frac{at^2}{b^2+t^2} \qquad \text{(Equation 4.1)}$$

where *y* is diameter, *t* is DPI, *a* is asymptote and *b* is DPI at half of *a*.

The two parameters, asymptote and time at half of asymptote, were estimated from the equation and used for a separate ANOVA. If the non-linear regression model did not fit the data, a linear regression was applied but excluded from the ANOVA.

### 4.2.2 Fungicide-sensitivity of *L. maculans* and *L. biglobosa* isolates *in vitro*

Fungicide-sensitivity assays, modified from those of Fraaije et al. (2007) and Carter et al. (2014) consisted of 2x sabouraud dextrose media (Appendix A.1) amended with increasing concentrations of technical grade flusilazole, tebuconazole, prothioconazole, prothioconazole-desthio, penthiopyrad or picoxystrobin (Table 4.2).

**Table 4.1.** Fungicide concentrations used to generate fungicide-amended media. Using a method modified from Eckert *et al.* (2010), final concentrations were calculated. The required volume of stock fungicide solution was added to 15 ml of distilled water to produce a working solution, which was then added to molten agar to produce the fungicide-amended media.

| Final concentration (µg/ml) | [Δ]Working concentration (µg/ml) | *Required volume of stock solution (µl) |
|---|---|---|
| 0 | 0 | 0 |
| 0.01 | 0.2 | 0.5 |
| 0.05 | 1 | 2.5 |
| 0.1 | 2 | 5 |
| 0.5 | 10 | 25 |
| 1 | 20 | 50 |

* Volume of stock fungicide solution (6 mg/ml) added to 15ml distilled water to produce working solution

[Δ] 15 ml of working solution added to 300 ml of molten agar

.

**Table 4.2.** Final concentrations of fungicide in media used to determine sensitivity of *L. maculans* and *L. biglobosa* to various fungicides.

| | 96 well plate number (1-12) | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| Fungicide | Fungicide concentration (µg/ml) | | | | | | | | | | | |
| Flusilazole | 0 | 0.02 | 0.039 | 0.078 | 0.156 | 0.313 | 0.63 | 1.25 | 2.5 | 5 | 10 | 20 |
| Tebuconazole | 0 | 0.02 | 0.039 | 0.078 | 0.156 | 0.313 | 0.63 | 1.25 | 2.5 | 5 | 10 | 20 |
| Prothioconazole | 0 | 0.2 | 0.39 | 0.78 | 1.56 | 3.13 | 6.25 | 12.5 | 25 | 50 | 100 | 200 |
| Prothioconazole-desthio | 0 | 0.00034 | 0.001 | 0.003 | 0.0091 | 0.027 | 0.082 | 0.25 | 0.74 | 2.22 | 6.67 | 20 |
| Penthiopyrad | 0 | 0.00034 | 0.001 | 0.003 | 0.0091 | 0.027 | 0.082 | 0.25 | 0.74 | 2.22 | 6.67 | 20 |
| Picoxystrobin | 0 | 0.00034 | 0.001 | 0.003 | 0.0091 | 0.027 | 0.082 | 0.25 | 0.74 | 2.22 | 6.67 | 20 |

Technical grade fungicides were prepared in acetone as 10 mg/ml (flusilazole, tebuconazole, prothioconazole-desthio, penthiopyrad and picoxystrobin) or 50 mg/ml (prothioconazole) stock solutions 2 hours before inoculation of plate. A dilution series using 2x sabouraud dextrose media and the prepared fungicide stock was done aseptically. Aliquots (100 µl) of fungicide-amended media were added to wells of flat-bottomed 96-well microtitre plates (NUNC™, Thermo Fisher). A control well with no fungicide added to the growth media was used for all isolates (Figure 4.2).

Conidia of 23 *L. maculans* isolates and 22 *L. biglobosa* isolates were harvested 24 hours before plate inoculation and adjusted to a concentration of 1x $10^6$ conidia/ml (Table 2.2). Aliquots (100 µl) of conidial suspensions were added to each well of a single row and in duplicate. Plates were incubated at 20 $^{O}$C for 4 days. Fungal growth was measured by absorbance using a FLUOstar OPTIMA microplate reader (BMG Labtech, Offenburg, Germany) set to wavelength 630 nm. Fungicide-sensitivity for each isolate was calculated as 50% effective concentration ($EC_{50}$) using a dose-response relationship curve generated by the FLUOstar OPTIMA microplate software.

### 4.2.3 Fungicide-sensitivity of *L. maculans* and *L. biglobosa in planta*

Preparation of oilseed rape plants was done according to Chapter 2.7. Plants were arranged in an alternate block design, which consisted of two replicate blocks, each containing three treatments (eight plants per treatment).

Plants were grown from the seedling stage in a plant growth chamber (Conviron Europe, UK). The chamber was set to a 12-hour light/12-hour dark cycle with a light intensity of 210 µe/$m^2$ during the light regime. Under regular growing conditions, relative humidity in the chamber was set at 70% and temperature was set at 20 $^{O}$C when light and 18 $^{o}$ C when dark.

Throughout the experiment, true leaves were removed after emergence to increase the longevity of the two cotyledons. When they reached approximately 5 cm in width, the cotyledons were inoculated with the conidial suspension of one *L. maculans* ($H_{ROX}$ 12-2-1) or one *L. biglobosa* ($F_2$ Exc dm 11-5) isolate by wounding. Using a pre-sterilised needle, a single wound site was made slightly to the left of the main vein of the cotyledon. Conidial suspension (10 µl), at a concentration of 1x$10^6$ conidia/ml, was applied directly to each wound site, forming a droplet that covered the wound.

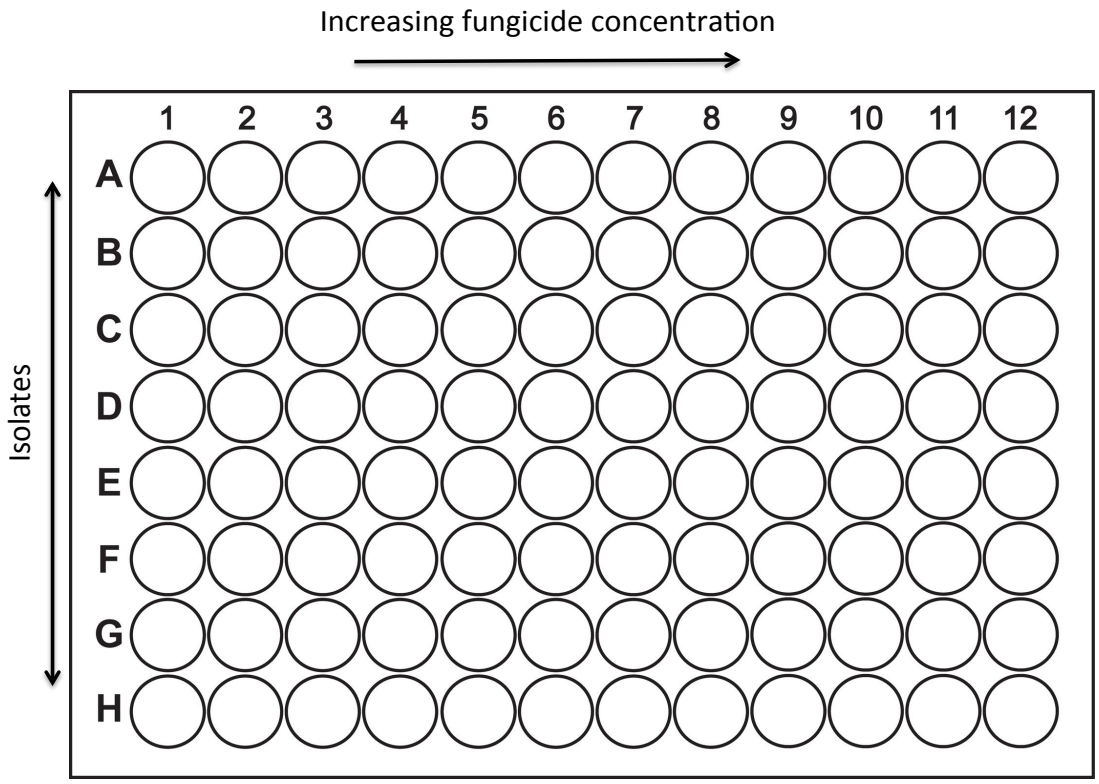



**Figure 4.2.** Microtitre plate layout for *in vitro* fungicide sensitivity assay. Increasing concentrations of fungicide-amended Sabouraud dextrose broth were added to successive wells (100 µl). Following this, 100 µl of $10^6$ spores/ml suspension was also added. Each row (lettering) on the plate contained an individual *L. maculans* or *L. biglobosa* isolate and each column (numbering) contained a specific fungicide concentration. Each plate had a control column (1) which had no added fungicide. Modified from Carter et al. (2014).

After inoculation, the plants were lightly sprayed with water and the tray was covered with a humidity lid. The controlled environment cabinet was then adjusted to 100 % relative humidity in complete darkness; the plants were left for 24 hours before re-establishing normal growing conditions and removing the humidity lid. Throughout the experiment, cotyledons were watered daily with a foliar water spray and at the base when necessary.

After 6 DPI, commercial grade prothioconazole or penthiopyrad + picoxystrobin were prepared (according to Chapter 2.8) and applied at a concentration of 2 µg/ml or 20 µg/ml using a water atomiser (Morrison's, UK). Untreated control (0 µg/ml) cotyledons were sprayed with water at this time.

Disease assessments, evaluated by measuring lesion diameter (mm) were done at 2, 4, 6, 8, 10, 12 and 14 DPI. At 14 DPI, the cotyledons were removed, instantly frozen in liquid nitrogen and stored at -80 $^{O}$C for qPCR analysis.

QPCR was completed according to Chapter 2.10. Briefly, leaves were instantly frozen in liquid nitrogen and ground into a fine powder. DNA was extracted and diluted to a concentration of 25 ng/µl. The PCR reactions were done using Brilliant III Ultra-fast SYBR Green QPCR master mix with low Rox at a final volume of 20 µl and a primer concentration of 0.3 µM were used. PCR parameters were as follows: initial denaturation at 95 °C for 2 min, followed by 40 cycles of 95 °C for 15 sec, 60 °C (*L. maculans*) or 55 °C (*L. biglobosa*) for 30 sec and 72 °C for 36 sec.

A logistic non-linear regression model was fitted to the lesion diameter data for *L. maculans* and *L. biglobosa* on all fungicide-amended agar at all concentrations (R Development Core Team 2011). The SSlogis equation (Eq. 4.2) can be described by writing:

$$y = \frac{a}{1+e^{\frac{b-t}{c}}} \qquad \text{(Equation 4.2)}$$

where *y* is diameter, *t* is DPI, *a* is asymptote, *b* is point of inflection and *c* is scale (adjustment of starting value).

The three parameters asymptote, point of inflection and scale were estimated from the equation (Eq. 4.2) and used for a separate ANOVA. If the non-linear regression model did not fit the data, a linear regression was applied but excluded from the ANOVA.

### 4.2.4 Understanding the curative or preventative properties of a penthiopyrad + picoxystrobin mixture on colonisation by *Leptosphaeria* spp. *in planta*.

Preparation of oilseed rape plants was done according to Chapter 2.7. Plants were arranged in a randomised block design, which consisted of three replicate blocks, each containing nine treatments (three plants per treatment).

Conidial suspensions of selected isolates were prepared according to Chapter 2.4. Inoculation was done using a modified version of the wound-inoculation method described by Huang et al. (2011).

Plants were grown from the seedling stage in a plant growth chamber. The chamber was set to a 12-hour light/12-hour dark cycle with a light intensity of 270 µe $m^{-2}$ during the light regime. Under standard growing conditions, relative humidity in the chamber was set at 70% and temperature was set at 20 $^{o}$ C when light and 18 $^{o}$ C when dark.

Fungicides were prepared and sprayed according to Chapter 2.8. On this occasion, the fungicides were prepared at one tenth of the recommended field rate, according to that used by Eckert et al. (2010). Therefore, a final concentration (in water) of 87 µg/ml commercial grade prothioconazole and 80 µg/ml of commercial grade penthiopyrad + picoxystrobin (40 µg/ml) was applied directly to oilseed rape true leaves until run off.

Once the plants had reached the two/three true leaf stage (Appendix C.5.), plants that received the fungicide treatment prior to pathogen inoculation (T1) were sprayed. After 2 days, *L. maculans* ($H_{ROX}$ 12-2-1) or *L. biglobosa* ($F_2$ Exc dm 11-5) isolates were inoculated onto the second and third leaves that had been previously wounded. Before wounding and inoculation, the targeted wound sites were gently rubbed using a soft tissue (Kleenex brand, UK). This was done to stop the conidial suspension from rolling off the waxy leaf surface. Using a pre-sterilised needle, four wounds were made in each of the second and third leaves. Two wounds were made either side of the main leaf vein close to the bottom of the leaf. Using an automated pipette (StarLab, UK), 10 µl of conidial suspension was applied directly to each wound site. This formed a conidial-suspension droplet that covered the wound site.

The trays of inoculated plants were then covered by miniature polyethylene-tunnels (WM Morrison plc, UK), which acted as humidity chambers. The internal walls of the polyethylene-tunnels were sprayed with water using a spray bottle set at ‘gentle misting’ (WM Morrison plc, UK). Using black polyethylene bags, the polyethylene-

tunnels were covered so that no light could enter. After 48 hours, the polyethylene-tunnels were removed and the plants were sprayed once more to guarantee leaf moisture retention. After 6 DPI, all plants receiving the late fungicide treatment (T2) were sprayed. Untreated control plants (T3) were sprayed with water at both the T1 and T2 spray timings.

During the experiment, soil moisture content was maintained by watering at the base. Disease assessment was done by measuring lesion diameter at 4, 12, 16, 20 DPI.

## 4.3 Results

### 4.3.1 Fungicide sensitivity of *L. maculans* and *L. biglobosa* mycelial growth on solid medium using technical grade fungicides

The growth rates of *L. biglobosa* and *L. maculans* on media amended with four different fungicides and at five different concentrations were determined (Figure 4.3 – 4.6). After 22 days on the untreated control plates, where no fungicide had been included, *L. biglobosa* consistently grew to a larger colony diameter than *L. maculans* (average 74.4 mm and 50.3 mm, respectively).

Difference in total growth between the two species was determined by calculating the difference in area the under the growth curve, generated from changes in the *L. biglobosa* and *L. maculans* colony diameters over 22 days. Growth of *L. biglobosa* was greater than that of *L. maculans* on all media with fungicides at low concentrations (0.00, 0.01 and 0.05 µg/ml). At higher concentrations, however, the difference was less, with *L. maculans* and *L. biglobosa* growing similarly on prothioconazole-desthio (0.1, 0.5 and 1 µg/ml), penthiopyrad + picoxystrobin (0.1, 0.5 and 1 µg/ml) and penthiopyrad (0.1, 0.5 and 1 µg/ml) plates. There was a correlation between concentration and growth difference between the two species, with a significant response recorded (5 df, $P$ <0.01). However, fungicide type did not affect the difference in growth between the two species.

An ANOVA was done using the asymptote and rate parameters obtained from Holling's type II non-linear regression (Table 4.3). The asymptote values, representative of maximum growth, differed significantly between fungicide types (3 df, $P$ < 0.01), concentrations (5 df, $P$ < 0.01) and *Leptosphaeria* species (1 df, $P$ < 0.05). Analysis post-hoc determined that picoxystrobin differed from penthiopyrad (1 df, $P$ < 0.05), prothioconazole-desthio (1 df, $P$ < 0.01) and the penthiopyrad +

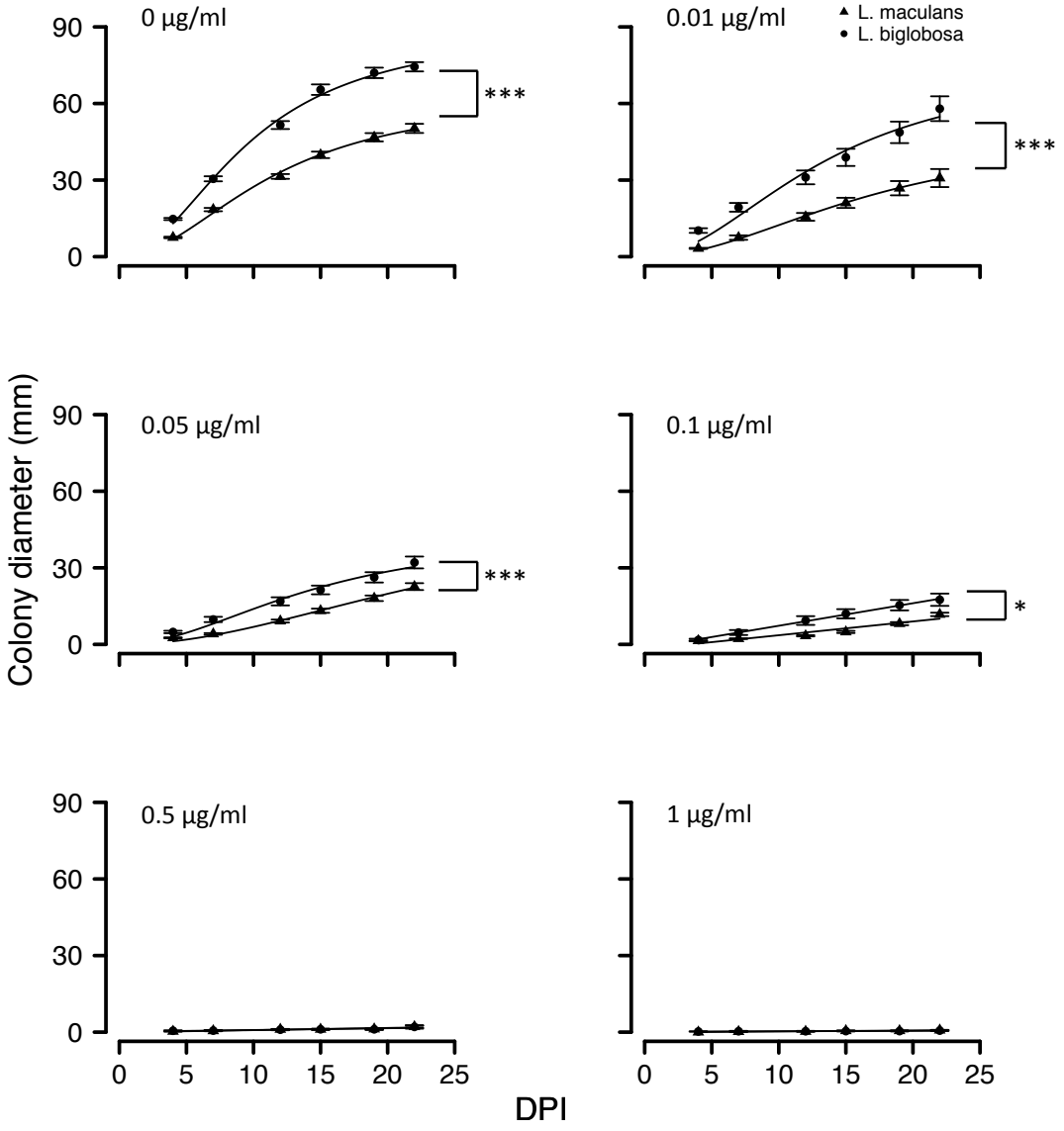



**Figure 4.3.** Effects of the fungicide mixture penthiopyrad plus picoxystrobin on the growth of *L. maculans* and *L. biglobosa* mycelium. a) 0 µg/ml (control); b) 0.01 µg/ml; c) 0.05 µg/ml; d) 0.1 µg/ml; e) 0.5 µg/ml; f) 1 µg/ml. Plugs of actively growing mycelium were harvested from fungal colonies and transferred onto fungicide-amended media at six different concentrations. Vertical and horizontal diameters of the fungal colonies were measured regularly post inoculation (DPI) over a period of 22 days. An average diameter for each treatment was calculated. Asterisks denote significant difference between species after 22 DPI (* $P < 0.05$, ** $P < 0.01$ *** $P < 0.001$).

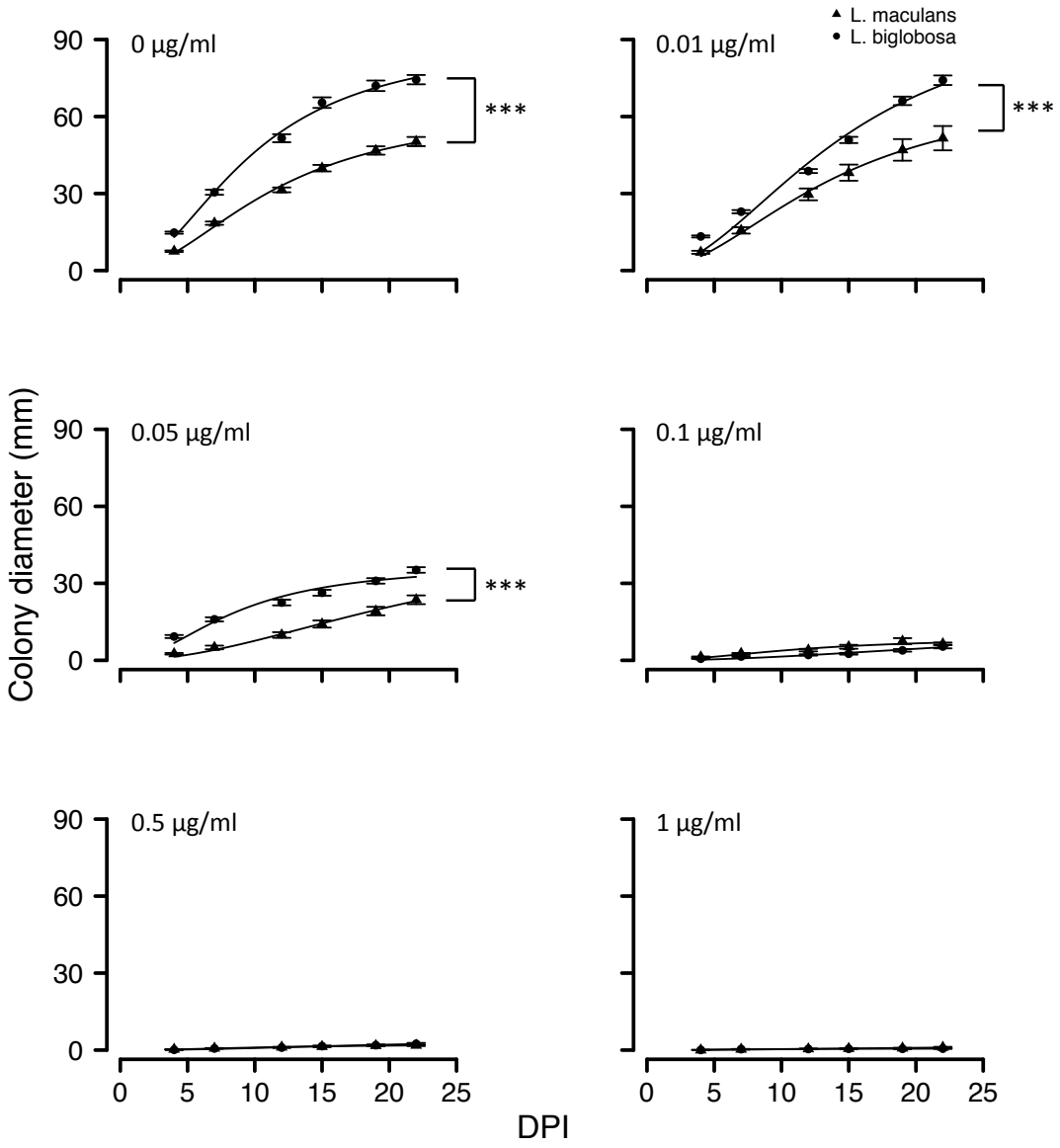



**Figure 4.4.** Effects of the fungicide prothioconazole-desthio on the growth of *L. maculans* and *L. biglobosa* mycelium. a) 0 µg/ml (control); b) 0.01 µg/ml; c) 0.05 µg/ml; d) 0.1 µg/ml; e) 0.5 µg/ml; f) 1 µg/ml. Plugs of actively growing mycelium were harvested from fungal colonies and transferred onto fungicide-amended media at six alternate concentrations. Vertical and horizontal diameters of the fungal colonies were measured regularly post inoculation (DPI) over a period of 22 days. An average diameter for each treatment was calculated. Asterisks denote significant difference between species after 22 DPI (* $P < 0.05$, ** $P < 0.01$ *** $P < 0.001$).

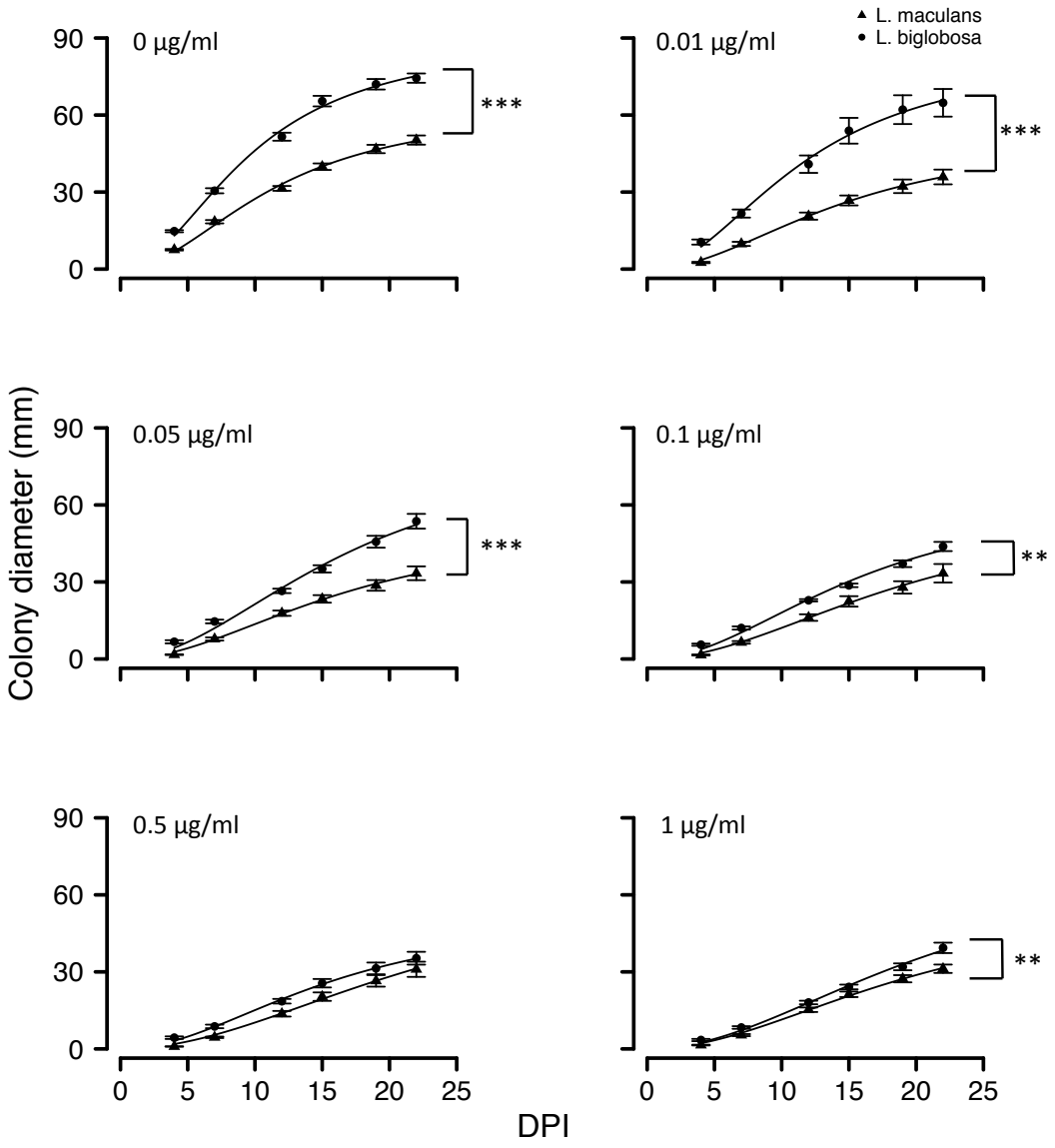



**Figure 4.5.** Effects of the fungicide penthiopyrad on the growth of *L. maculans* and *L. biglobosa* mycelium. a) 0 µg/ml (control); b) 0.01 µg/ml; c) 0.05 µg/ml; d) 0.1 µg/ml; e) 0.5 µg/ml; f) 1 µg/ml. Plugs of actively growing mycelium were harvested from fungal colonies and transferred onto fungicide-amended media at six alternate concentrations. Vertical and horizontal diameters of the fungal colonies were measured regularly post inoculation (DPI) over a period of 22 days. An average diameter for each treatment was calculated. Asterisks denote significant difference between species after 22 DPI (* $P < 0.05$, ** $P < 0.01$ *** $P < 0.001$).

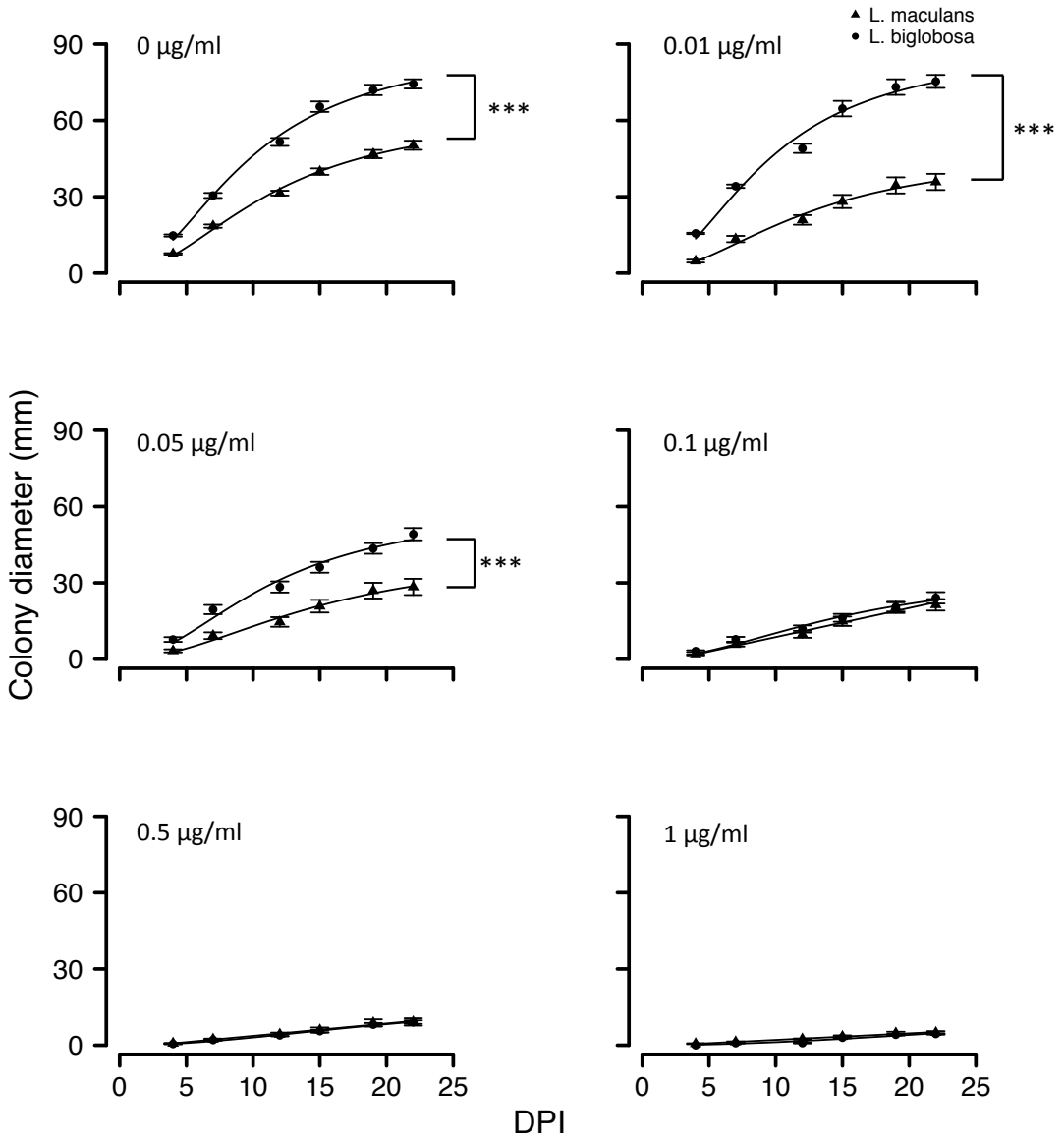



**Figure 4.6.** Effects of the fungicide picoxystrobin on the growth of *L. maculans* and *L. biglobosa* mycelium. a) 0.00 µg/ml (control); b) 0.01 µg/ml; c) 0.05 µg/ml; d) 0.1 µg/ml; e) 0.5 µg/ml; f) 1 µg/ml. Plugs of actively growing mycelium were harvested from fungal colonies and transferred onto fungicide-amended media at six alternate concentrations. Vertical and horizontal diameters of the fungal colonies were measured regularly post inoculation (DPI) over a period of 22 days. An average diameter for each treatment was calculated. Asterisks denote significant difference between species after 22 DPI (* $P < 0.05$, ** $P < 0.01$ *** $P < 0.001$).

**Table 4.3.** Model parameters from Holling's type III non-linear regression on the inhibition of *L. maculans* and *L. biglobosa* mycelial growth using technical grade fungicide. Plugs of pre-cultured mycelium were applied to agar plates with increasing concentration of fungicide and growth in diameter (mm) was recorded.

| | | *L. maculans* | | *L. biglobosa* | |
|---|---|---|---|---|---|
| Fungicide | Concentration (µg/ml) | Asymptote (mm) | Time at half of asymptote (DPI) | Asymptote (mm) | Time at half of asymptote (DPI) |
| Untreated | 0 | 63.90 | 11.62 | 91.46 | 9.88 |
| Prothioconazole-desthio | 0.01 | 71.25 | 13.74 | 104.59 | 14.66 |
| | 0.05 | 49.52 | 23.47 | 37.43 | 8.54 |
| | 0.1 | 8.82 | 11.58 | 14.51 | 29.88 |
| | 0.5 | 2.30 | 11.37 | 4.79 | 22.18 |
| | 1 | 1.96 | 21.89 | 0.61 | 7.17 |
| Penthiopyrad | 0.01 | 47.50 | 12.39 | 88.94 | 9.45 |
| | 0.05 | 42.08 | 15.03 | 59.94 | 11.52 |
| | 0.1 | 37.75 | 18.65 | 37.43 | 15.48 |
| | 0.5 | 16.87 | 19.53 | 19.32 | 23.04 |
| | 1 | 8.45 | 17.81 | 19.39 | 38.77 |
| Picoxystrobin | 0.01 | 52.12 | 14.79 | 84.85 | 11.85 |
| | 0.05 | 52.84 | 17.01 | 84.03 | 17.14 |
| | 0.1 | 60.15 | 19.90 | 65.40 | 16.19 |
| | 0.5 | 67.24 | 23.57 | 53.90 | 16.03 |
| | 1 | 58.21 | 20.24 | 74.49 | 21.31 |
| Penthiopyrad + picoxystrobin | 0.01 | 49.44 | 17.32 | 75.60 | 13.56 |
| | 0.05 | 53.68 | 26.14 | 44.01 | 14.72 |
| | 0.1 | n/a | n/a | n/a | n/a |
| | 0.5 | 3.06 | 18.17 | 2.35 | 14.26 |
| | 1 | 1.47 | 21.89 | 0.48 | 4.27 |

n/a = non-determinable using statistical model

picoxystrobin mixture (1 df, $P < 0.01$). Increasing the concentration had an effect on the asymptote, with a clear difference in response determined *post hoc* between the higher (0.5, 1 µg/ml) and lower concentrations (0, 0.01 µg/ml). The asymptote value for *L. biglobosa* isolates was significantly greater than that of *L. maculans*.

The predicted rate at which the diameter of the *Leptosphaeria* colonies increased to their asymptote, according to the non-linear Holling's type II regression model, did not differ between *L. biglobosa* and *L. maculans* for all four fungicides at all six concentrations.

### 4.3.2 Fungicide-sensitivity of *L. maculans* and *L. biglobosa* isolates *in vitro*

Fungicide-sensitivity assays were done to determine the sensitivity of *L. maculans* and *L. biglobosa* isolates to several fungicides with different modes of action.

#### 4.3.2.1 Sensitivity of *Leptosphaeria* species to azole fungicides

A number of *L. maculans* and *L. biglobosa* isolates were assessed for their sensitivity to four azole fungicides: prothioconazole, flusilazole, tebuconazole and prothioconazole-desthio. Both species were very sensitive to prothioconazole-desthio, flusilazole and tebuconazole, although they were less sensitive to prothioconazole (Table 4.4 – 4.5).

In comparison between *L. maculans* (n = 23) and *L. biglobosa* (n = 21) isolates, there was a significant difference between them in sensitivity to flusilazole (Figure 4.7), prothioconazole-desthio (Figure 4.8) and prothioconazole (Figure 4.9) treatments. There was no significant difference in sensitivity to tebuconazole (Figure 4.10).

The largest difference between the two species was with prothioconazole, where *L. biglobosa* isolates were 16.8 times less sensitive than *L. maculans* isolates *(*Figure 4.9). Alternatively, with prothioconazole-desthio, a metabolite of prothioconazole, there was a change between the two species, with *L. maculans* isolates 2.1 times less sensitive than *L. biglobosa* isolates (Figure 4.10). There was less of a difference with flusilazole; *L. biglobosa* was 1.3 times less sensitive than *L. maculans* (Figure 4.8).

**Table 4.4.** Sensitivities of *L. biglobosa* isolates ($EC_{50}$ (µg/ml)) to demethylation inhibitors flusilazole, tebuconazole, prothioconazole, prothioconazole-desthio; succinate dehydrogenase inhibitor penthiopyrad and quinone outsider inhibitor picoxystrobin. $EC_{50}$ (µg/ml) determined using decreasing concentration of fungicide in a 96 well plate assay. $EC_{50}$ values were calculated as means of two independent replicates.

| | Fungicide sensitivity $EC_{50}$ (µg/ml) | | | | | |
|---|---|---|---|---|---|---|
| Isolate | Flusilazole | Tebuconazole | Prothioconazole | Prothioconazole -desthio | Penthiopyrad | Picoxystrobin |
| 2003.2.8 | 0.19 | 0.92 | 61.21 | 0.06 | 0.34 | 0.03 |
| A Exc 12-12-10 | 0.19 | 0.65 | 65.60 | 0.05 | 0.11 | 0.10 |
| D Rox 12-10 | 0.20 | 0.70 | 84.14 | 0.04 | 0.06 | 0.02 |
| Exc 12-9-21 | 0.26 | 0.98 | 62.18 | 0.07 | 0.17 | 0.03 |
| F2 Exc 12-2-3 | 0.20 | 0.79 | n/a | 0.07 | 0.12 | 0.03 |
| F2 Exc 12-3-1 | 0.19 | 0.72 | 47.47 | 0.05 | 0.18 | 0.02 |
| F2 Exc 12-6-1 | 0.17 | 0.70 | 58.34 | 0.08 | 0.13 | 0.02 |
| F2 Exc dm 11-5 | 0.16 | 0.92 | 83.41 | 0.07 | 0.06 | 0.02 |
| G Rox 12-8-1 | 0.18 | 0.69 | 50.10 | 0.05 | 0.15 | 0.02 |
| H 12-6 | 0.18 | 0.69 | 64.35 | 0.04 | 0.11 | 0.02 |
| H Dr 12-12 | 0.21 | 0.81 | 47.73 | 0.06 | 0.12 | 0.02 |
| H Dr 12-2 | 0.18 | 0.67 | 63.83 | 0.06 | 0.46 | 0.03 |
| H Exc 12-12-1 | 0.21 | 0.75 | 62.59 | 0.06 | 0.06 | 0.02 |
| H Exc 12-12-31 | 0.22 | 0.72 | 56.27 | 0.06 | 0.07 | 0.02 |
| H Exc 12-2-2 | 0.18 | 0.72 | 50.85 | 0.06 | 0.14 | 0.03 |
| K 12-33 | 0.17 | 0.66 | 79.60 | 0.05 | 0.09 | 0.02 |
| K Exc 12-10-21 | 0.19 | 0.69 | 66.24 | 0.05 | 0.26 | 0.03 |
| K Exc 12-11 | 0.21 | 0.76 | 100.63 | 0.05 | 0.07 | 0.02 |
| K Rox 12-6-31 | 0.19 | 0.76 | 61.38 | 0.07 | 0.35 | 0.02 |
| W10 | 0.17 | 0.68 | 76.16 | 0.06 | 0.19 | 0.04 |
| Lb68 | 0.08 | 0.36 | 20.35 | 0.18 | 0.16 | 0.04 |

n/a = determination not possible

**Table 4.5.** Sensitivities of *L. maculans* isolates ($EC_{50}$ (µg/ml)) to demethylation inhibitors flusilazole, tebuconazole, prothioconazole, prothioconazole-desthio; succinate dehydrogenase inhibitor penthiopyrad and quinone outside inhibitor picoxystrobin. $EC_{50}$ (µg/ml) determined using decreasing concentration of fungicide in a 96 well plate assay. $EC_{50}$ values were calculated as means of two independent replicates.

| | Fungicide sensitivity $EC_{50}$ (µg/ml) | | | | | |
|---|---|---|---|---|---|---|
| Isolate | Flusilazole | Tebuconazole | Prothioconazole | Prothioconazole -desthio | Penthiopyrad | Picoxystrobin |
| TS14-9 | 0.17 | 0.96 | 3.89 | 0.10 | 0.05 | 0.06 |
| TS14-4 | 0.16 | 1.04 | 3.84 | 0.12 | 0.09 | 0.10 |
| TS14-21 | 0.17 | 1.00 | 2.93 | 0.13 | 0.17 | 0.10 |
| TS14-2 | 0.10 | 0.61 | 3.26 | 0.13 | 0.12 | 0.09 |
| TS14-14 | 0.12 | 0.62 | 0.91 | 0.12 | 0.07 | 0.10 |
| TS14-13 | 0.17 | 1.12 | 3.79 | 0.12 | 0.09 | 0.06 |
| TS14-12 | 0.12 | 0.72 | 1.89 | 0.14 | 0.46 | n/a |
| TS14-1 | 0.10 | 0.59 | 1.36 | 0.12 | 0.11 | 0.13 |
| TS13-9 | n/a | n/a | n/a | 0.11 | 0.18 | 0.10 |
| TS13-8 | 0.08 | 0.48 | 1.64 | n/a | 0.13 | n/a |
| TS13-7 | 0.18 | 0.98 | 3.98 | n/a | 0.16 | n/a |
| TS13-6 | 0.35 | 1.79 | 34.81 | 0.10 | 0.16 | 0.11 |
| TS13-5 | 0.13 | 0.81 | 3.11 | n/a | 0.15 | n/a |
| TS13-4 | 0.14 | 0.85 | 3.72 | 0.13 | 0.05 | n/a |
| TS13-3 | 0.37 | 1.89 | 30.84 | 0.11 | 0.14 | 0.05 |
| TS13-2 | 0.20 | 0.97 | 31.81 | 0.12 | 0.09 | 0.20 |
| TS13-19 | 0.13 | 0.61 | 2.11 | 0.14 | 0.18 | n/a |
| TS13-18 | 0.11 | 0.45 | 1.86 | 0.10 | 0.39 | 0.03 |
| TS13-13 | 0.16 | 1.08 | 6.62 | 0.13 | 0.14 | 0.06 |
| TS13-12 | 0.12 | 1.11 | 3.72 | 0.05 | 0.06 | 0.12 |
| TS13-11 | 0.23 | 1.40 | 9.27 | 0.11 | 0.26 | 0.10 |
| TS13-10 | 0.08 | 0.60 | 2.21 | 0.12 | 0.13 | 0.15 |
| ME24 | 0.18 | 0.92 | 6.19 | 0.12 | 0.26 | 0.06 |
| Hrox 12-2-1 | 0.06 | 0.39 | 2.00 | 0.13 | n/a | 0.08 |

n/a = determination not possible

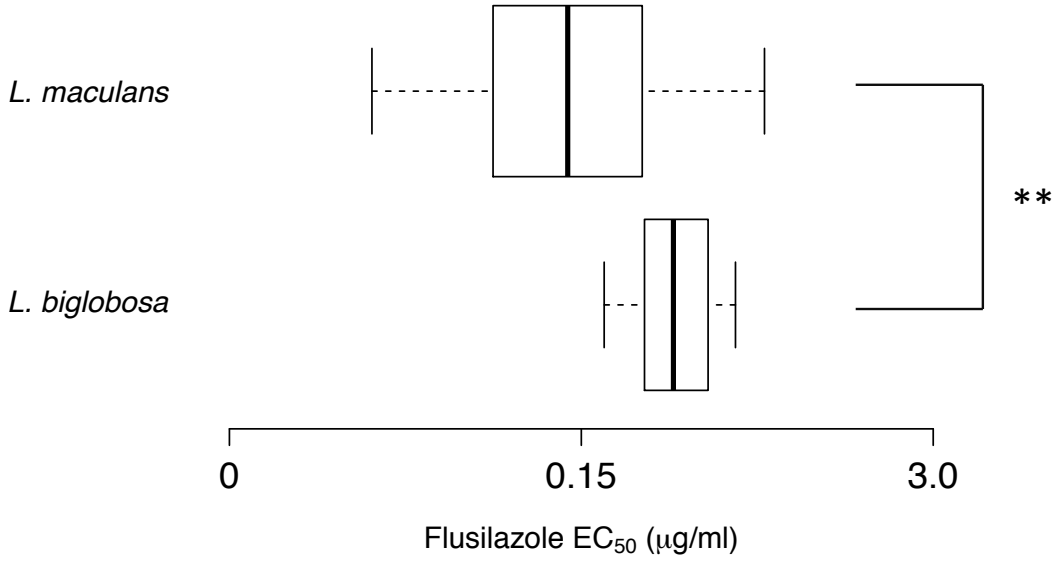



**Figure 4.7.** Box plot depicting sensitivities of *Leptosphaeria maculans* isolates (n = 23) and *L. biglobosa* isolates (n = 21) to flusilazole. Thick line denotes median value, box edges signify lower and upper quartiles and error bars represent minimum and maximum values. Asterisks denote significant difference (* $P < 0.05$, ** $P < 0.01$ *** $P < 0.001$).

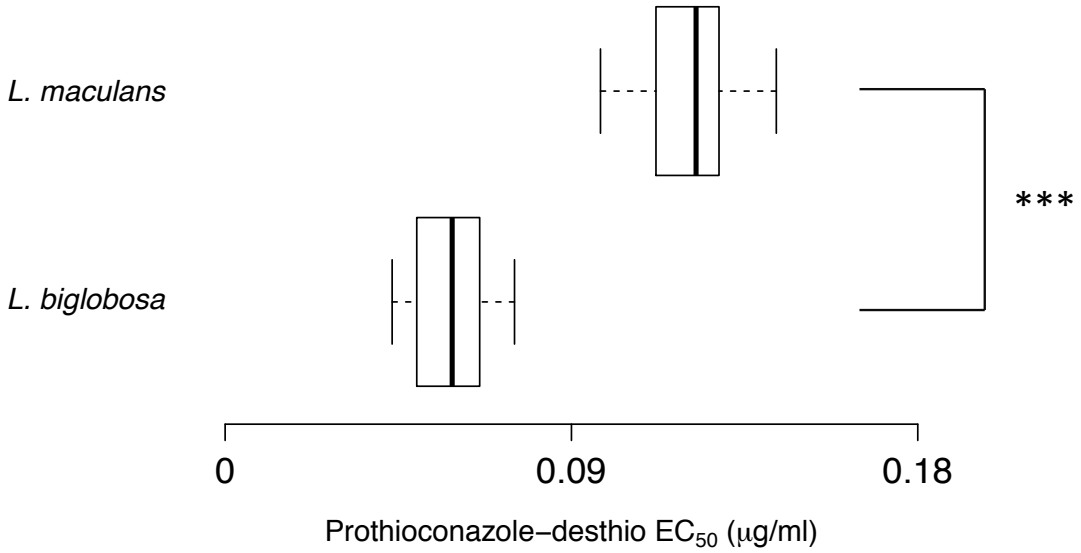



**Figure 4.8.** Box plot depicting sensitivities of *Leptosphaeria maculans* isolates (n = 22) and *L. biglobosa* isolates (n =21) to prothioconazole-desthio. Thick line denotes median value, box edges signify lower and upper quartiles and error bars represent minimum and maximum values. Asterisks denote significant difference (* $P < 0.05$, ** $P < 0.01$ *** $P < 0.001$).

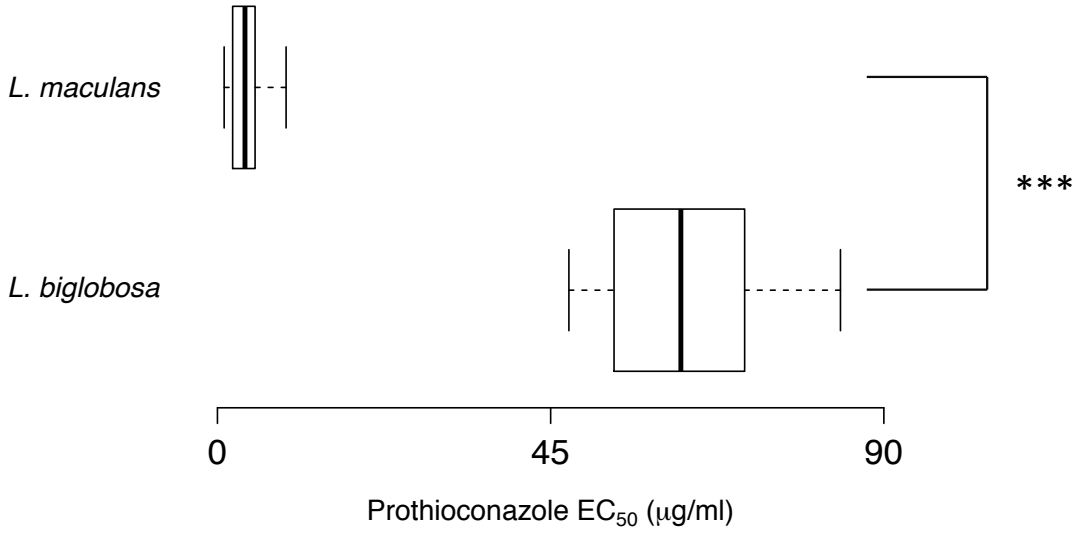



**Figure 4.9.** Box plot depicting sensitivities of *Leptosphaeria maculans* isolates (n = 23) and *L. biglobosa* isolates (n = 20) to prothioconazole. Thick line denotes median value, box edges signify lower and upper quartiles and error bars represent minimum and maximum values. Asterisks denote significant difference (* $P < 0.05$, ** $P < 0.01$ *** $P < 0.001$).

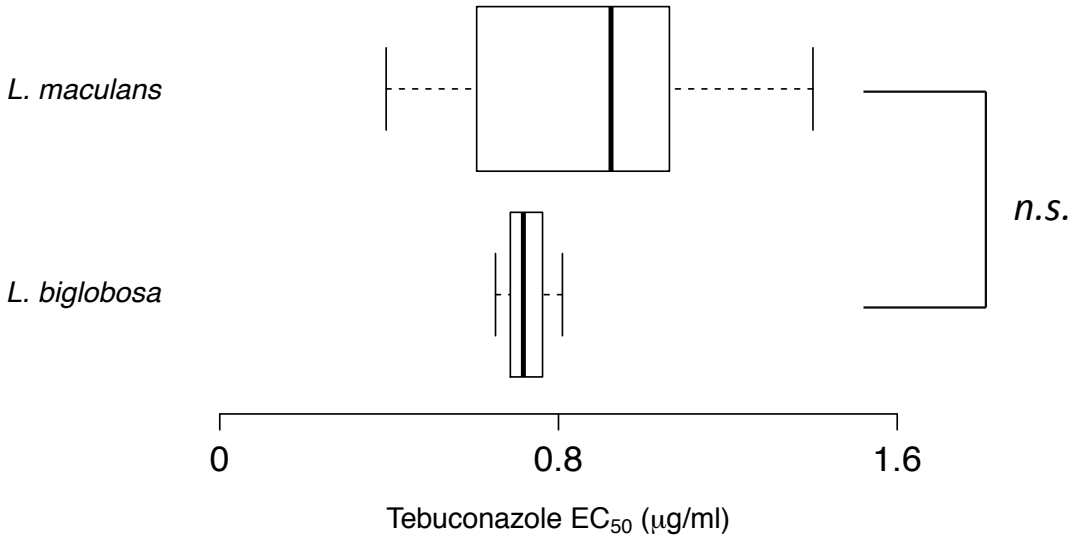



**Figure 4.10.** Box plot depicting sensitivities of *Leptosphaeria maculans* isolates (n = 23) and *L. biglobosa* isolates (n = 21) to tebuconazole. Thick line denotes median value, box edges signify lower and upper quartiles and error bars represent minimum and maximum values. Asterisks denote significant difference (* $P < 0.05$, ** $P < 0.01$ *** $P < 0.001$; *n.s.* not significant).

#### 4.3.2.2 Sensitivity of *Leptosphaeria* species to QoI fungicide

The collection of *L. maculans* (n = 21) and *L. biglobosa* (n = 21) isolates was assessed for their sensitivity to the QoI fungicide picoxystrobin. Both species were highly sensitive to the fungicide (Table 4.4 – 4.5). *L. maculans* was significantly less sensitive to picoxystrobin than *L. biglobosa*, which had a 2.28 fold increase in sensitivity (Figure 4.11).

#### 4.3.2.3 Sensitivity of *Leptosphaeria* species to SDHI fungicide

The collection of *L. maculans* (n = 23) and *L. biglobosa* (n = 21) isolates was assessed for their sensitivity to the SDHI fungicide penthiopyrad. Both species were highly sensitive to the fungicide (Table 4.4 – 4.5). Both species were very similar in their sensitivity to the fungicide; there was no significant difference in sensitivity between them (Figure 4.12).

### 4.3.3 Fungicide-sensitivity of *L. maculans* and *L. biglobosa in planta*

Fungicide-sensitivity assays *in planta* were used to determine whether *L. maculans* and *L. biglobosa* differed in their sensitivity to fungicides whilst colonising leaves.

Over the 14 days after leaves were inoculated with conidial suspension, pathogen colonisation patterns fitted a sigmoidal growth model for both species (Figure 4.13), at all concentrations, except for *L. maculans* when treated with 20 µg/ml penthiopyrad plus picoxystrobin (Table 4.6).

The value of the asymptote predicted by the non-linear regression model was significantly affected by fungicide treatment (2 df, $P$ <0.01); the values for penthiopyrad plus picoxystrobin and prothioconazole both differed from that of the untreated control, although they did not differ between each other. Concentration of fungicide (2 or 20 µg/ml) did not affect the asymptote and there was no significant difference between *Leptosphaeria* spp. The parameter value representing the inflection point of the curve was significantly different between species, with *L. biglobosa* having a smaller inflection point value compared to *L. maculans* treated with either fungicide (at all three concentrations) (1 df, $P$ < 0.05). The scale parameter was not significantly different between species, between concentrations or between fungicides.

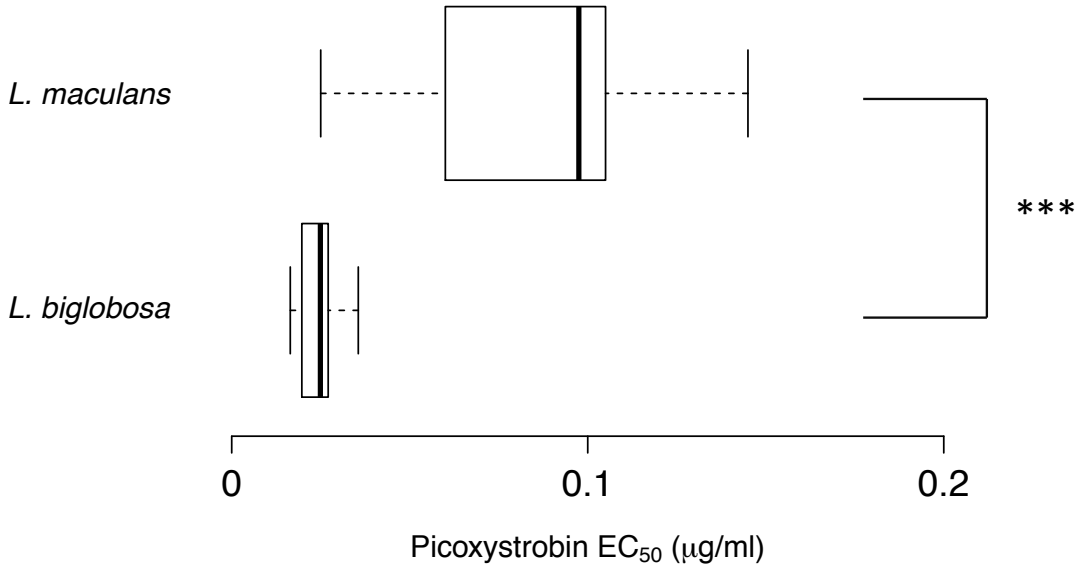



**Figure 4.11.** Box plot depicting sensitivities of *Leptosphaeria maculans* isolates (n = 21) and *L. biglobosa* isolates (n = 21) to picoxystrobin. Thick line denotes median value, box edges signify lower and upper quartiles and error bars represent minimum and maximum values. Asterisks denote significant difference (* $P < 0.05$, ** $P < 0.01$ *** $P < 0.001$).

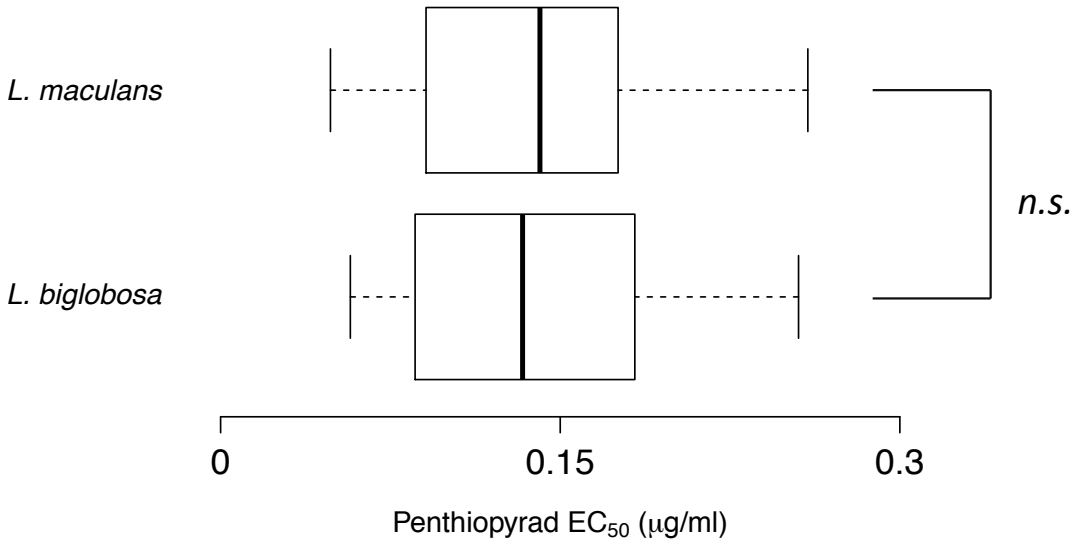



**Figure 4.12.** Box plot depicting sensitivities of *Leptosphaeria maculans* isolates (n = 23) and *L. biglobosa* isolates (n = 21) to penthiopyrad. Thick line denotes median value, box edges signify lower and upper quartiles and error bars represent minimum and maximum values. Asterisks denote significant difference (* $P < 0.05$, ** $P < 0.01$ *** $P < 0.001$; *n.s.* not significant).

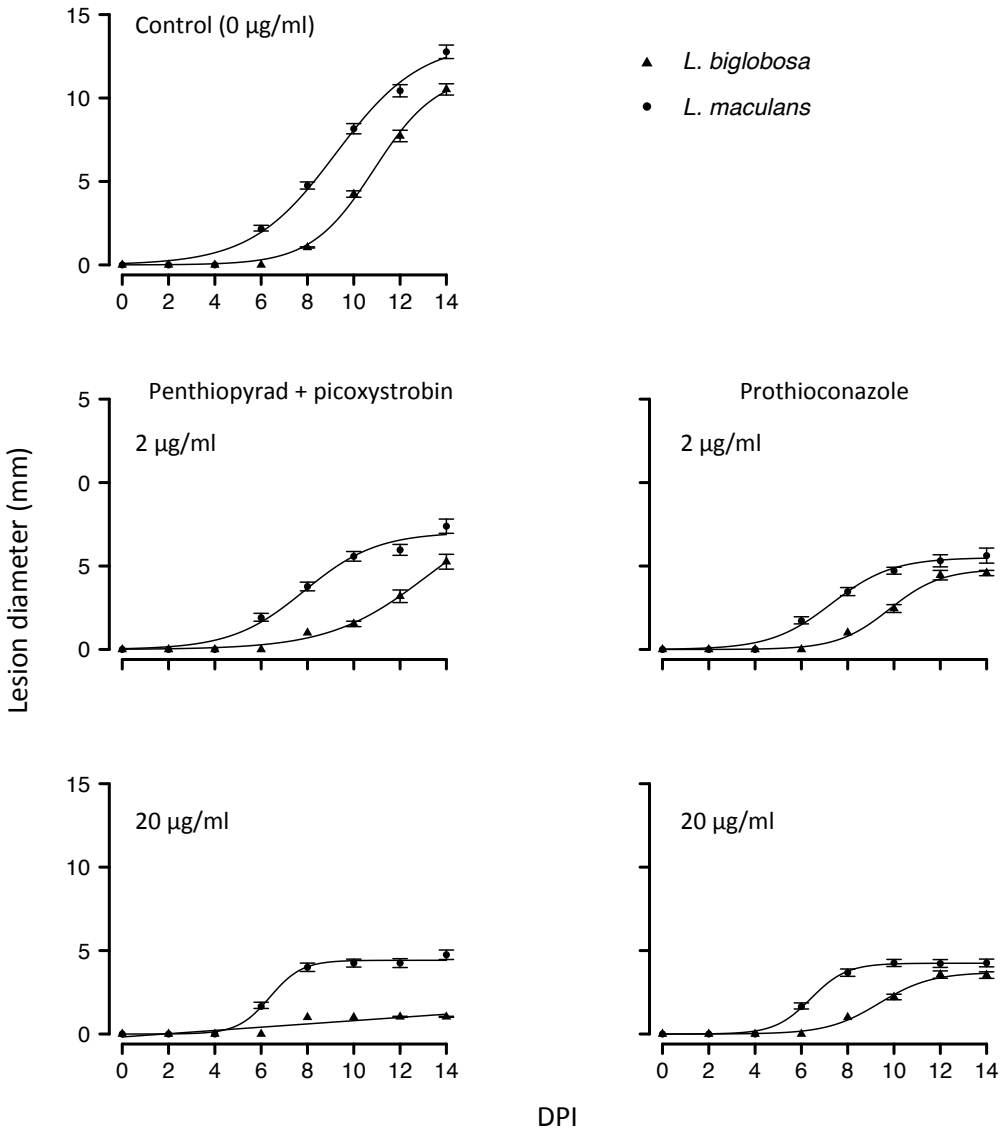



**Figure 4.13.** Effects of penthiopyrad plus picoxystrobin and prothioconazole on *Leptosphaeria maculans* and *L. biglobosa* lesion diameters *in planta*. Oilseed rape cotyledons (cv. Catana) were inoculated with either *L. maculans* or *L. biglobosa* conidial suspension before being sprayed with fungicide. Lesion diameter was recorded on alternate days post inoculation (DPI). Average diameter for each treatment was calculated and a logistic non-linear regression model was fitted to the data. Error bars denote standard error of the mean.

**Table 4.6.** Model parameters from logistic non-linear regression on the growth (diameter, mm) of *L. maculans* and *L. biglobosa* lesions on winter oilseed rape (cv. Catana) cotyledons treated with commercial grade prothioconazole, penthiopyrad + picoxystrobin or no fungicide (control). Cotyledons were inoculated in a growth cabinet and sprayed with increasing concentration of fungicide and growth in diameter (mm) was recorded.

| | | *L. maculans* | | | *L. biglobosa* | | |
|---|---|---|---|---|---|---|---|
| Fungicide | Concentration (µg/ml) | Asymptote (mm) | Time of inflection (DPI) | Scale* | Asymptote (mm) | Time of inflection (DPI) | Scale* |
| Untreated | 0 | 11.46 | 10.88 | 1.36 | 13.37 | 9.23 | 1.83 |
| Prothioconazole-desthio | 2 | 4.82 | 9.80 | 1.11 | 5.50 | 7.30 | 1.31 |
| | 20 | 3.70 | 9.36 | 1.12 | 4.24 | 6.41 | 0.77 |
| Penthiopyrad + picoxystrobin | 2 | 8.75 | 13.15 | 2.10 | 7.03 | 7.86 | 1.57 |
| | 20 | n/a | n/a | n/a | 4.42 | 6.38 | 0.69 |

* adjusts value of initial diameter (y) at time 0

After 14 DPI, *L. maculans* and *L. biglobosa* lesion diameter (mm) was not significantly different between prothioconazole-treated leaves and penthiopyrad plus picoxystrobin-treated leaves (Figure 4.14). Analysing prothioconazole independently at 14 DPI, concentration showed a significant response (Kruskal-Wallis chi-squared = 124.4, 2 df, $P < 0.05$). Lesion diameter decreased as concentration increased, with 20 µg/ml prothioconazole reducing both *L. maculans* and *L. biglobosa* lesion diameter by 3-fold compared to the untreated control. Nevertheless, after 14 DPI, *L. biglobosa* and *L. maculans* lesions were statistically similar in their diameter on prothioconazole-treated leaves. Decreasing penthiopyrad plus picoxystrobin concentrations also showed a significant response (Kruskal-Wallis chi-squared = 125.8, 2 df, $P < 0.01$); 20 µg/ml reduced *L. maculans* lesion diameter by 10-fold and *L. biglobosa* lesion diameter by 2.7-fold when compared to the untreated controls. Therefore, there was difference between species, with *L. biglobosa* lesions developing to a significantly larger diameter after 14 DPI (Kruskal-Wallis chi-squared = 18.3, 1 df, $P < 0.01$).

DNA quantity for each treatment was determined using qPCR to assess the effect of fungicide treatment on pathogen biomass (Figure 4.15). Increased concentration of fungicide decreased the amount of DNA for both *L. maculans* and *L. biglobosa*. A concentration of 20µg/ml prothioconazole reduced the amount of *L. maculans* DNA by 23 times and the amount of *L. biglobosa* DNA by six times compared to the untreated controls. A concentration of 20 µg/ml penthiopyrad plus picoxystrobin reduced the amount of *L. maculans* DNA by 174 times and amount of *L. biglobosa* DNA by six times compared to the untreated controls.

### 4.3.4 Understanding the curative or preventative properties of penthiopyrad + picoxystrobin mixture on colonisation by *Leptosphaeria* spp.

An alternate spray timings *in planta* fungicide application experiment was done to ascertain whether the fungicide penthiopyrad plus picoxystrobin demonstrated curative or preventative properties on *L. maculans* or *L. biglobosa* colonisation of oilseed rape true leaves (Figure 4.16a-d).

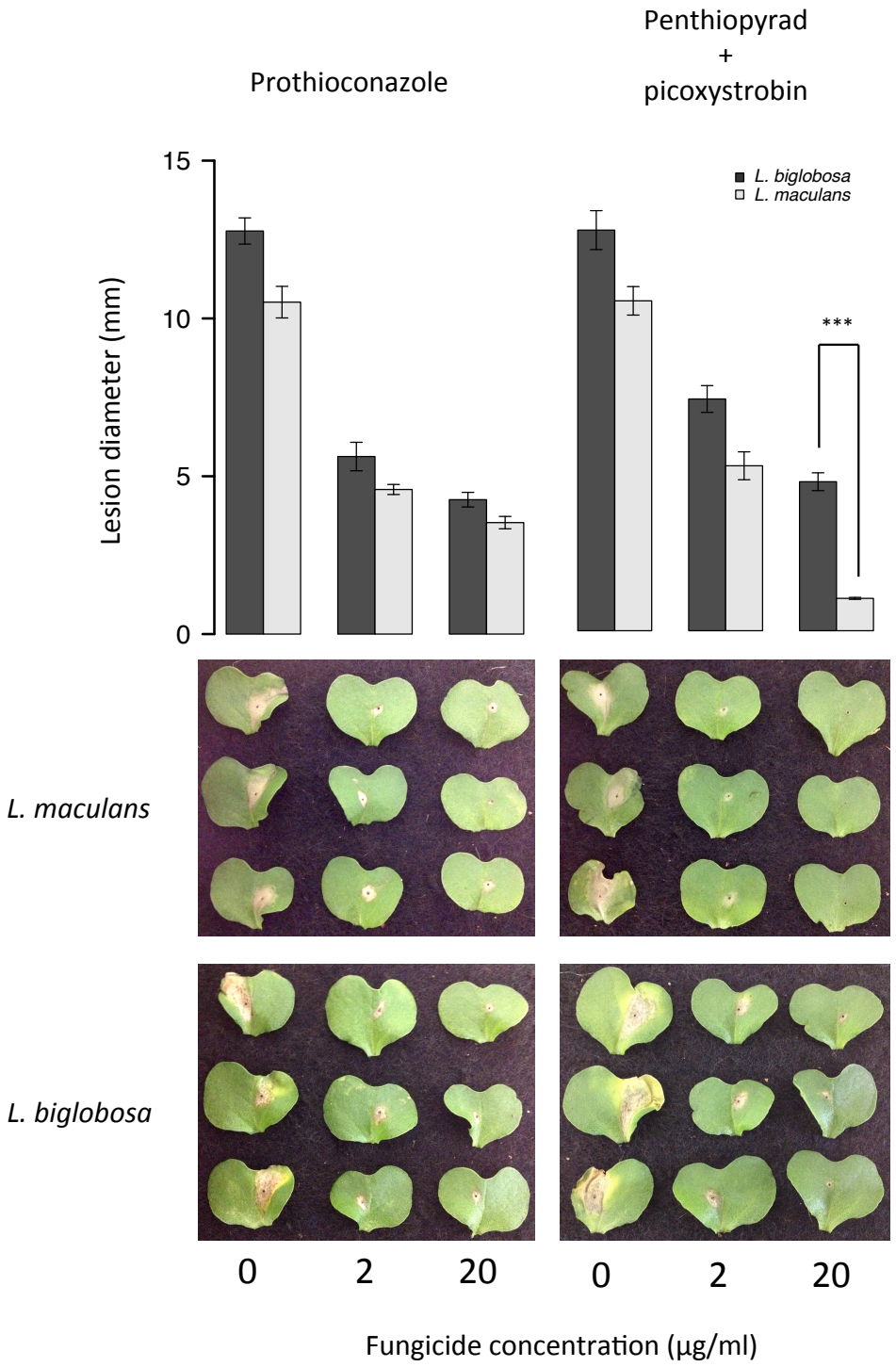



**Figure 4.14.** Sensitivity of *Leptosphaeria maculans* and *L. biglobosa* isolates to prothioconazole or penthiopyrad plus picoxystrobin 14 days after inoculation of winter oilseed rape cotyledons (cv. Catana). Cotyledons were inoculated with either *L. maculans* or *L. biglobosa* conidial suspension before being sprayed with fungicide at either 2 µg/ml or 20 µg/ml concentration. Lesion diameter was recorded and an average diameter for each treatment was calculated. Asterisks denote significant difference (* $P < 0.05$, ** $P < 0.01$ *** $P < 0.001$ and error bars denote standard error of the mean.

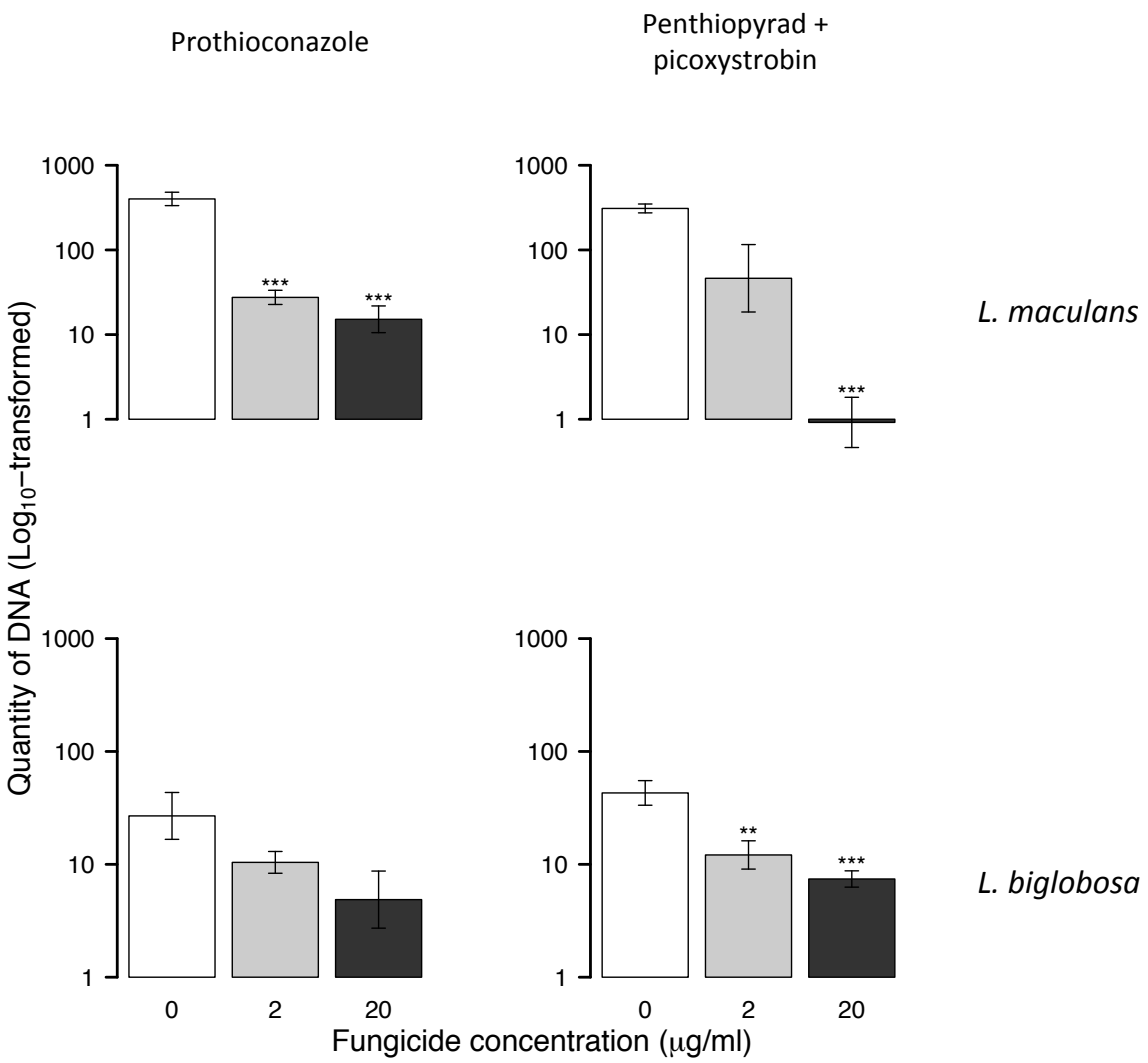



**Figure 4.15.** Quantification of DNA from prothioconazole and penthiopyrad plus picoxystrobin treated cotyledons inoculated with either *Leptosphaeria maculans* or *L. biglobosa*. Winter oilseed rape cotyledons (cv. Catana) were sampled at 14 DPI and DNA was extracted. QPCR was done using *L. maculans*- or *L. biglobosa*-specific primers. Data were $\log_{10}$-transformed. Asterisks denote significance when compared with the control (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$) and error bars denote standard error of the mean.

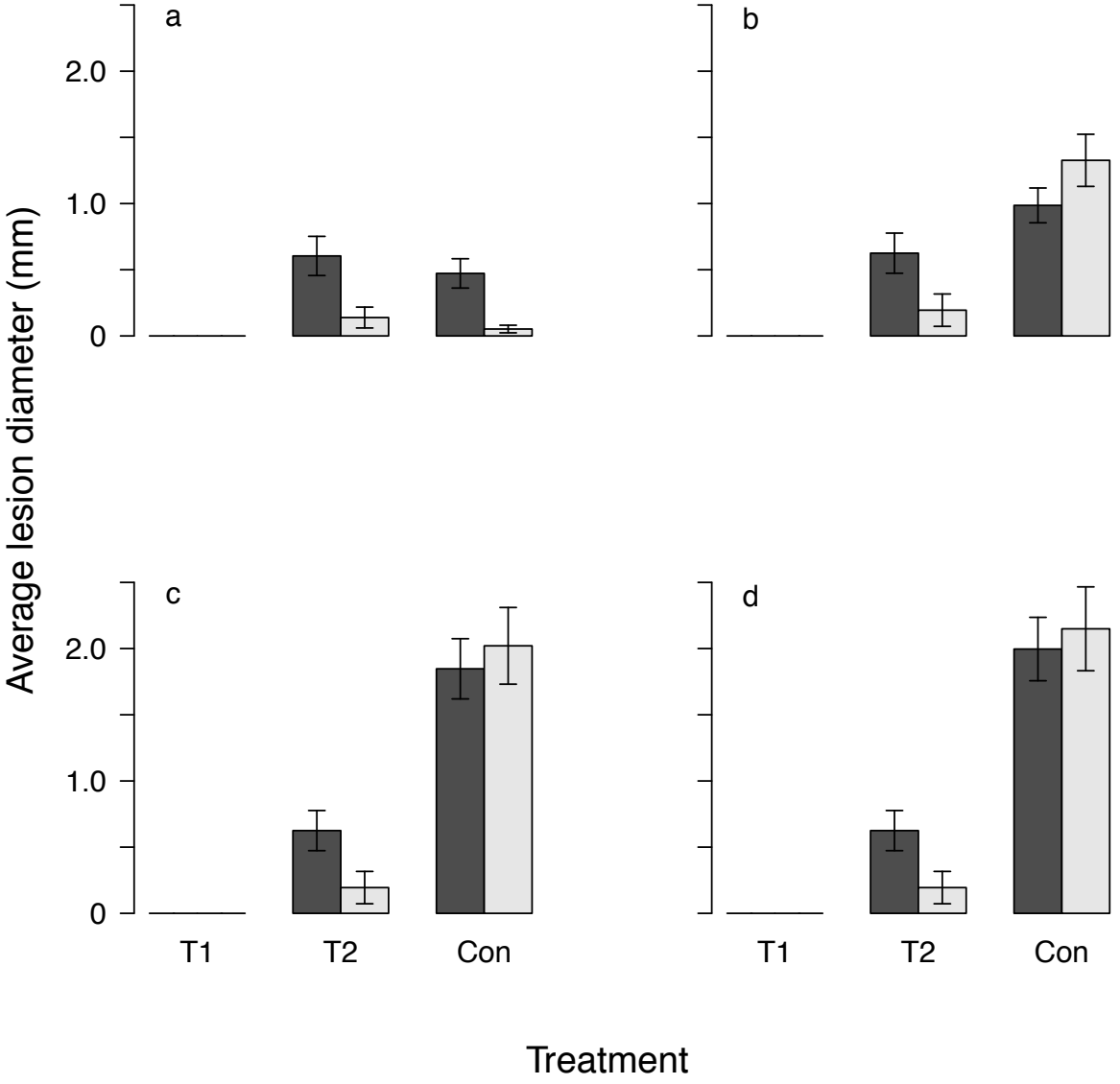



**Figure 4.16.** Efficacy of penthiopyrad plus picoxystrobin as a preventative and/or curative fungicide for the control of *Leptosphaeria maculans* (black) or *L. biglobosa* (grey) colonisation. Fungicide was applied to winter oilseed rape plants (three-leaf stage) either 2 days before (T1) or 6 days after (T2) inoculation with *L. maculans* or *L. biglobosa* conidial suspension. Control (Con) plants received no fungicide. Lesion diameter (cm) was recorded at 7 (a), 12 (b), 16 (c) and 20 (d) days post inoculation (DPI). Error bars denote standard error of the mean.

At 7, 12, 16 and 20 DPI, plants sprayed at the T1 timing, which was before pathogen inoculation, displayed no disease symptoms. Symptoms did appear on plants sprayed at the T2 (post-inoculation) and T3 (untreated) timings. Lesion diameter on T2-treated plants did not increase significantly with time for *L. maculans* (Kruskal-Wallis chi-squared = 0.004, 3 df, $P > 0.05$) or *L. biglobosa* (Kruskal-Wallis chi-squared = 0.032, 3 df, > 0.05). However, plants left untreated developed lesions that increased in diameter significantly with time for *L. maculans* (Kruskal-Wallis chi-squared = 41.3, 3 df, $P < 0.01$) and *L. biglobosa* (Kruskal-Wallis chi-squared = 38.5, 3 df, $P < 0.01$).

At 7 DPI (Figure 4.16a), *L. maculans* and *L. biglobosa* leaf lesion diameter differed significantly when plants had been sprayed at T2. Additionally, at 12, 16 and 20 DPI, there was also a notable difference (Kruskal-Wallis chi-squared = 6.55, 1 df, $P < 0.05$; at each time point). There was no difference between *L. maculans* and *L. biglobosa* in lesion diameter on plants treated at T3, except at the initial 7 DPI (Kruskal-Wallis chi-squared = 9.32, 1 df, $P < 0.05$).

## 4.4 Discussion

### 4.4.1 Fungicide sensitivity

These results suggest that *L. maculans* and *L. biglobosa* populations from the south-east of the UK are sensitive to DMI, SDHI and QoI antifungal agents, all of which are currently used to control fungal plant pathogens of winter oilseed rape.

For example, it would appear from these data that, for the isolates studied, neither *L. maculans* or *L. biglobosa* have developed resistance to azole fungicides flusilazole, tebuconazole or prothioconazole-desthio. The distributions of $EC_{50}$ values for each fungicide were constrained; suggesting that no isolate had developed increased resistance to fungicide treatment distinctive from that of the sampled population. This evidence supports previous historical findings that proposed there to be no emerging risk of azole-resistance among populations of either *L. maculans* or *L. biglobosa* (Eckert et al. 2010; Huang et al. 2011).

The evolutionary potential for resistance differs between plant pathogens due to the nature of the disease cycle of the pathogen (McDonald and Linde 2002; Oliver and Hewitt 2014). Species at high risk of evolving resistance to fungicides, such as *Zymoseptoria tritici*, have polycyclic disease cycles, where infection occurs following both sexual and asexual reproduction (Fones and Gurr 2015). In the UK, both *Leptosphaeria* spp. infect oilseed rape

with a monocyclic disease cycle, where ascospores that develop following sexual reproduction infect leaves to form leaf lesions that eventually result in stem base cankers (Fitt et al. 2006a; West et al. 2002). Asexual reproduction, which does occur on the leaves, does not go on to cause secondary disease symptoms, thus reducing the evolutionary potential of the pathogens. In addition, it has been predicted that the distribution of receptor genes (*R*-genes) that correspond to pathogen effector genes (*Avr*-genes) in resistant oilseed rape cultivars disrupts selection, protecting the selective influence of fungicide application, which in turn decreases the potential of the pathogens to evolve resistance to chemical control (Van den Bosch et al. 2014). For many years, *Brassica napus* cultivars have been bred to resist evolving *L. maculans* populations (Delourme et al. 2006). This well-managed distribution of major gene resistance could explain why *L. maculans* has yet to develop resistance to azole fungicides.

By contrast, there is little known about *R*-gene mediated resistance to *L. biglobosa*, since the pathogen has always been considered less damaging than *L. maculans* and less predominant in the UK due to the invasion of more dominant *L. maculans* strains (Fitt et al. 2006a; Fitt et al. 2006b). Pathogens with small effective population sizes have also been identified as having a smaller risk of evolving resistance, which might explain why *L. biglobosa* has yet to develop fungicide-resistance (McDonald and Linde 2002).

A unique feature of coexisting plant pathogen species is that they are two individual organisms which cannot interbreed that cause similar disease symptoms on the plant; their environment is therefore similar but their evolutionary pathways different (Fitt et al. 2006b). With this in mind, it is not unexpected that an intrinsic differential response to a selective pressure, such as fungicide application, would exist between the two closely related coexisting species (Bierman et al. 2002; Fitt et al. 2006b). Previous reports have suggested that *L. maculans* and *L. biglobosa* differ in their sensitivity to azole fungicides. For example, *L. maculans* had been identified as more sensitive to tebuconazole and flusilazole than *L. biglobosa* (Eckert et al. 2010; Huang et al. 2011). Data presented here supports this differential sensitivity for flusilazole but not for tebuconazole. Additionally, unlike in any previous studies, *Leptosphaeria* spp. isolates were tested for sensitivity to both prothioconazole and prothioconazole-desthio. Prothioconazole does not appear to have a very strong antifungal effect on *L. maculans* or *L. biglobosa* when compared with other azoles. Prothioconazole has previously been shown to have little antifungal activity on CYP51 - the target protein that is inhibited by azole fungicides (Parker et al. 2013). The metabolite prothioconazole-desthio has a more potent antifungal activity. With this in mind, the difference in sensitivity between the two species is most probably due to differences in

uptake and metabolism of prothioconazole rather than to alterations in antifungal sensitivity (Parker et al. 2011).

Prothioconazole-desthio has been shown here to be very effective at reducing the growth of both *Leptosphaeria* species, with *L. maculans* being less sensitive to the fungicide than *L. biglobosa*. The commercial form of prothioconazole has been used since 2004 and since the withdrawal of flusilazole in 2014 the chemical has been widely used on winter oilseed rape (Oliver and Hewitt 2014; Sewell et al. 2016). Consequently, contact between prothioconazole and *Leptosphaeria* spp. in the crop is likely to be significant; together with *L. maculans* being the more dominant *Leptosphaeria* species in the south of England (Stonard et al. 2010), increased resistance is more likely to have evolved in *L. maculans* populations than in *L. biglobosa* populations (McDonald and Linde 2002).

This concept does not explain the difference in sensitivity to flusilazole between the two species. As *L. maculans* and *L. biglobosa* fungicide sensitivities are not drastically different for both flusilazole and prothioconazole-desthio, differences between isolates could be the explanation for differential sensitivity. *L. maculans* isolates were predominantly obtained from Boxworth, UK, the site of the field experiments discussed in Chapter 3. Due to the lack of symptoms caused by *L. biglobosa* in field experiments in all cropping seasons, *L. biglobosa* isolates obtained had a wider UK distribution. A more detailed survey of $EC_{50}$ values from a large set of isolates collected across the UK is needed to fully investigate differential sensitivity in *Leptosphaeria* spp.

Both *Leptosphaeria* spp. were sensitive to penthiopyrad and sensitivity did not differ between the two species. Additionally, the distribution of $EC_{50}$ values was very similar, suggesting that the two pathogens have an analogous response to SDHI treatment. Penthiopyrad and other modern SDHI fungicides have not been applied widely on winter oilseed rape for phoma stem canker control (Garthwaite et al. 2014). Evolution of resistance is therefore unlikely to have developed in a monocyclic disease cycle, such as that of *L. maculans* and *L. biglobosa*; the data presented here support this current lack of evolved resistance. SDHI fungicides are classified as medium-high risk for resistance evolution (Oliver and Hewitt 2014). It is therefore imperative that further, continuous monitoring is implemented, similar to that for *Zymoseptoria tritici* (*Mycosphaerella graminicola*) populations (Fraaije et al. 2012), especially with the introduction of new SDHI-based fungicides, such as the novel mixture penthiopyrad plus picoxystrobin marketed for phoma stem canker control (Sewell et al. 2016).

*L. maculans* and *L. biglobosa* were both very sensitive to the strobilurin fungicide picoxystrobin, with *L. maculans* being less sensitive to the fungicide than *L. biglobosa*.

Unlike the modern broad-spectrum SDHI fungicides, which have been a more recent introduction into fungal disease control on winter oilseed rape, QoI antifungal agents have been used extensively for controlling other pathogens of the crop, such as *Sclerotinia sclerotiorum* during the flowering stage of the crop (Derbyshire and Denton-Giles 2016). Interaction between *Leptosphaeria* spp. and QoI fungicides is therefore possible through indirect exposure later in the life cycles of the pathogens. Continued monitoring is required to ensure that development of resistance, which is conferred by a single amino acid alternation in cytochrome bc1 (G143A), has not already occurred (Gisi et al. 2002). Furthermore, characterisation of the cytochrome bc1 gene in both *L. maculans* and *L. biglobosa* would help to predict the risk of resistance evolution, which is mitigated by the presence of an intron at codon 143 (Sierotzki et al. 2007).

### 4.4.2 Growth of *Leptosphaeria* spp. on fungicide-amended media

Over time, growth of mycelium of *Leptosphaeria* spp. on media was reduced by penthiopyrad and prothioconazole-desthio. Moreover, at the lower end of the concentration range analysed, as on the control, *L. biglobosa* grew better than *L. maculans*, whereas at the higher end of the concentration range analysed, both *Leptosphaeria* spp. were equally affected. This increased growth in the absence of fungicide and the presence of non-lethal doses may be explained because *L. biglobosa* is a better, more efficient saprophyte than *L. maculans*, growing faster on winter oilseed rape stubble over the summer months (Hammond and Lewis 1987). Nevertheless, these data suggest that, over time, both *Leptosphaeria* spp. are equally affected by the presence of prothioconazole-desthio or penthiopyrad.

Picoxystrobin had little activity on the mycelial growth of either *L. maculans* or *L. biglobosa*. This reduced activity supports current understanding, which suggests that QoI fungicides are more active on fungal spores than they are on mycelium (Bartlett et al. 2002). Spore germination is more dependant on high concentrations of ATP than mycelial growth. Considering that QoI fungicides reduce ATP levels, due to their activity at the electron transport chain, it is reasonable to suggest that the limited efficacy of picoxystrobin on *L. maculans and L. biglobosa* mycelium could be due to this phenomenon.

Moreover, some fungal organisms have an alternate oxidase pathway (AOX), which re-establishes the potential of electrons across the mitochondrial membrane after a quinone inhibitor, such as a QoI fungicide, blocks normal electron chain pathway via ubiquinone (Wood and Hollomon 2003). Inhibition of the AOX pathway, using an inhibitor such as salicylhydroxamic acid (SHAM), might explain whether this is the reason why established, actively growing mycelium maintained radial growth in the presence of a QoI fungicide.

### 4.4.3 *Leptosphaeria* spp. fungicide sensitivity *in planta*

The observation that oilseed rape cotyledon colonisation by *L. maculans* and *L. biglobosa* was reduced by the foliar application of prothioconazole or penthiopyrad plus picoxystrobin was anticipated, as both fungicides have been shown to be active against mycelium and conidia of both *Leptosphaeria* spp. *in vitro* (Chapter 4.3.3 – 4.3.5) and decrease phoma leaf spotting and stem canker symptoms in winter oilseed rape crops (Sewell et al. 2016).

This is the first study *in planta* to investigate differences between *L. maculans* and *L. biglobosa* in prothioconazole or penthiopyrad + picoxystrobin sensitivity. Analysis over time showed that the two fungal species initiated disease symptoms at different times, even when treated with fungicide, with *L. biglobosa* colonisation occurring earlier than *L. maculans* colonisation. Superior fitness during colonisation could give *L. biglobosa* an advantage over *L. maculans,* owing to the coexisting nature of the two pathogens (Fitt et al. 2006b). Nonetheless, in crops *L. maculans* leaf spots usually occur before those of *L. biglobosa* due to an earlier release of airborne ascospores, meaning that epidemics are more likely to be started by *L. maculans* in UK winter oilseed rape crops (Huang et al. 2011).

Further work, combining inoculum of both *L. maculans* and *L. biglobosa*, would provide more information on the dynamics of differing fungicide sensitivity of both species under a selective pressure such as that of fungicide application.

### 4.4.4 Preventative and curative effects of penthiopyrad + picoxystrobin mixture on colonisation by *Leptosphaeria* spp. *in planta*.

The commercial form of the fungicide-mixture penthiopyrad plus picoxystrobin appears to have both preventative and curative properties on winter oilseed rape leaves colonised by *Leptosphaeria* spp. Analysis *in planta* on mature winter oilseed rape leaves shows complete inhibition of both *Leptosphaeria* spp. when sprayed before pathogen inoculation; moreover, when fungicide was applied after the development of symptoms, the growth of the pathogens was inhibited.

Analysis of the individual components, unavailable at the time of experimentation, is required to distinguish which of the two fungicides is protecting the plant from pathogen development and which fungicide is stopping the continued development of an already established pathogen.

# Chapter 5 Azole-sensitivity in *Leptosphaeria* species: the role of lanosterol 14-α demethylase

## 5.1 Introduction

Antifungal demethylation inhibitors (DMIs), e.g. triazoles and imidazoles, target lanosterol 14α-demethylase (*erg11* (gene), CYP51 (protein)), a member of the cytochrome P450 superfamily and key regulatory enzyme in the ergosterol biosynthetic pathway (Kelly et al. 1995; Lepesheva and Waterman 2007) (Appendix F).

Ergosterol is an essential structural component of the fungal plasma membrane and is required to maintain membrane integrity, fluidity and permeability. Inhibition of CYP51 blocks the synthesis of ergosterol, which subsequently increases cytotoxic sterol precursors. The lack of membrane-bound ergosterol and the accumulation of toxic molecules in the cytosol have a combined fungistatic effect on the cell, decreasing membrane integrity and permeability (Marichal et al. 1999).

Azole fungicides are used to control phoma stem canker, a damaging disease of winter oilseed rape, which is caused by coexisting fungal plant pathogens *Leptosphaeria maculans* and *L. biglobosa*. Recent studies suggest that the two pathogens differ in the sensitivity to azole fungicides, both *in vitro* (Eckert et al. 2010) and *in planta* (Huang et al. 2011), with *L. biglobosa* exhibiting a less sensitive phenotype in the presence of some azole fungicides. Differences in sensitivity between coexisting plant pathogens have previously been shown to affect population structure (Bierman et al. 2002) and may explain the recent increase in *L. biglobosa* incidence in some UK locations (Huang et al. 2014a).

Increased exposure to azole fungicides has intensified the prominence of evolved resistance in some plant pathogen populations. Currently, three mechanisms of resistance to DMIs have been identified in agricultural crop pathogens; these are 1) target site polymorphisms (amino acid) 2) CYP51 overexpression or presence of CYP51 paralogs (CYP51A, CYP51B, CYP51C) and 3) increased expression of efflux pump genes (Parker et al. 2014; Price et al. 2015).

Missense mutations in the coding sequence of *erg11* (gene), and subsequent alterations in the secondary structure of CYP51B (protein), correlate with a resistance to DMI fungicides and are currently the most common mechanism of

azole-resistance (Carter et al. 2014; Cools and Fraaije 2008, 2013; Parker et al. 2014; Price et al. 2015). Using predictive homology modelling, polymorphisms in the CYP51B coding sequence have been shown to increase the size of the azole binding pocket, thus decreasing the affinity of the bound anti-fungal and reducing its affects as a fungicide (Mullins et al. 2011).

This chapter describes work investigating the role of lanosterol 14α-demethylase in the phenotypic fungicide-sensitivity differences shown between *Leptospaheria maculans* and *L. biglobosa*.

## 5.2 Materials and methods

### 5.2.1 Identification of *Lberg11/*CYP51B

*L. biglobosa* CYP51B (LbCYP51B) characterisation commenced with a BLASTp search of GenBank using the published *L. maculans* CYP51B (LmCYP51B) protein sequence (AAN28927.1) and a Clustal Omega multiple sequence alignment was generated using six protein sequences with similar percentage identity: *Pyrenophora tritici-repentis* (XP_001939023.1), *Alternaria alternata* (OAG21175.1), *Stagonosporopsis caricae* (AMM76225.1), *Botrytis cinerea* (CCD54835.1), *Sclerotinia sclerotiorum* (XP_001594997.1) and *Aspergillus nidulans* (XP_681552.1) (NCBI 2016; Sievers et al. 2011).

Using the alignment, a 13 amino acid motif conserved across five out of the six aligned species (DVVYDCPNSKLME) was selected for a custom BLASTp search in Geneious R9.1.4 (Biomatters Limited), which was executed against a database of translated open reading frames generated from published *L. biglobosa* genome data provided by INRA-Bioger, France (Grandaubert et al. 2014b). The corresponding nucleotide sequence was manually identified using an annotations text search; BLASTn search was performed to corroborate the putative LbCYP51B nucleotide sequence.

#### 5.2.1.1 LmCYP51B and LbCYP51B sequencing

Extraction of total DNA from 11 *L. maculans* isolates and eight *L. biglobosa* isolates

(Table 2.1) was done in accordance with Chapter 2.6. Briefly, mycelium of each isolate was disrupted in liquid nitrogen and freeze-dried before being ground into a powder. DNA extraction was done using the DNAMITE Plant DNA extraction kit. PCR amplification was completed (Chapter 2.9) using sequencing primers for both species (Table 2.2). Amplification conditions for both species were 1 cycle at 95 °C for 2 min; 35 cycles at 95 °C for 40 sec, 53.5 °C for 30 sec and 72 °C for 1 min; followed by a final extension at 72 °C for 5 min.

PCR products were purified using QIAquick PCR Purification kit (Qiagen) according to the manufacturer's guidelines. Sequencing procedure was followed in accordance with Chapter 2.11. Briefly, samples were prepared to a final concentration of 20 - 30 ng/µl; primer concentrations were 10mM. Samples were sent to GATC Biotech for Sanger sequencing; sequence data were analysed in Geneious R9.1.4 (Biomatters Limited) and contigs assembled using Cap3 (Huang and Madan 1999), with low quality ends trimmed manually. Constructed contigs were aligned alongside the published LmCYP51B gene sequence as the reference sequence (AY142146). Sequences were manually trimmed to the start (ATG) and stop codon (TAG). The recognised location of a single intron in LmCYP51B was manually removed. The predicted location of a single intron in LbCYP51B, inferred from the alignment with *L. maculans* reference sequence, was also removed. Finally, nucleotide sequences were checked using ORF Finder (NCBI 2016) and translated into amino acid sequence in Geneious R9.14 (Biomatters Limited).

#### 5.2.1.2 Phylogenetic analysis

Phylogenetic analysis was done using Molecular Evolutionary Genetics Analysis (MEGA7) software (Kumar et al. 2016). Firstly, several notable CYP51B orthologs were obtained using a BLASTn search, which was executed on the published LmCYP51B nucleotide coding sequence. Sequences were translated and a Clustal Omega alignment performed on the translated CYP51 protein sequences from *L. maculans*, *L. biglobosa*, *Pyrenophora tritici-repentis, Alternaria alternata, Phaeosphaeria nodorum, Stagonosporopsis cucurbitacearum, Botrytis cinerea, Sclerotinia sclerotiorum, Aspergillus fumigatus* (CYP51A and CYP51B)*, A. flavus, A. niger* and *Saccharomyces cerevisiae* with *Homo sapiens* as an outgroup. The corresponding coding nucleotide alignment was back-generated and phylogenetic relations were inferred by maximum-likelihood with 1000 bootstrap replications.

### 5.2.2 Comparative homology modelling

Predictive homology protein modelling was processed in Chimera (Yang et al. 2012) using Modeller v9.14 (Šali and Blundell 1993) (Figure 5.1). Firstly, to identify similar CYP51B sequences that have been structurally solved, a BLASTp search of the protein data bank (PDB) database was done individually on LmCYP51B and LbCYP51B protein sequences, both of which had been determined through cloning of complementary DNA, Sanger sequenced and translated in Geneious (Chapter 5.2).

A Clustal Omega multiple sequence alignment was done on the three most homologous CYP51B protein sequences, together with either LmCYP51B or LbCYP51B. For both *Leptosphaeria* spp., the closest solved CYP51B structures were from *A. fumigatus, S. cerevisiae* and *H. sapiens* (Figure 5.2 and Figure 5.3). The three homologous CYP51B structures used for modelling were automatically loaded into the software using annotations attached to the multiple sequence alignment files. Bound ligands within these structures were manually removed and the heme cofactor, which is a feature of CYP51B, was manually removed from the *S. cerevisiae* and *H. sapiens* structures but conserved from *A. fumigatus*, as this was the most similar CYP51B sequence.

The advanced options in Modeller v9.14 were used to build the model with hydrogens and the number of output models was set to five. Selection of the most reliable model was done using ModFOLD4, which predicts the quality of 3D protein models and assigns a *P*-value and a global model quality score (0-1 scale where a score of >0.4 indicates a confident model prediction) to each output model (McGuffin et al. 2013).

After the selection of the most reliable LmCYP51B and LbCYP51B structures, prothioconazole-desthio, in the binding confirmation of recently solved *S. cerevisiae* CYP51B protein PDB: 5EAD, was superimposed into the predicted structures using Matchmaker in Chimera with parameters set to default.

To test structural similarities between the two predicted CYP51B models, a structural alignment (TM- align) using a residue-to-residue comparison was generated with a 0 to 1 scoring system where 1 indicates a perfect match between the two structures (Zhang and Skolnick 2005). According to strict statistical analyses of structures in the PDB, scores less than 0.2 correspond to unrelated proteins whereas scores greater

than 0.5 generally assume the same fold.

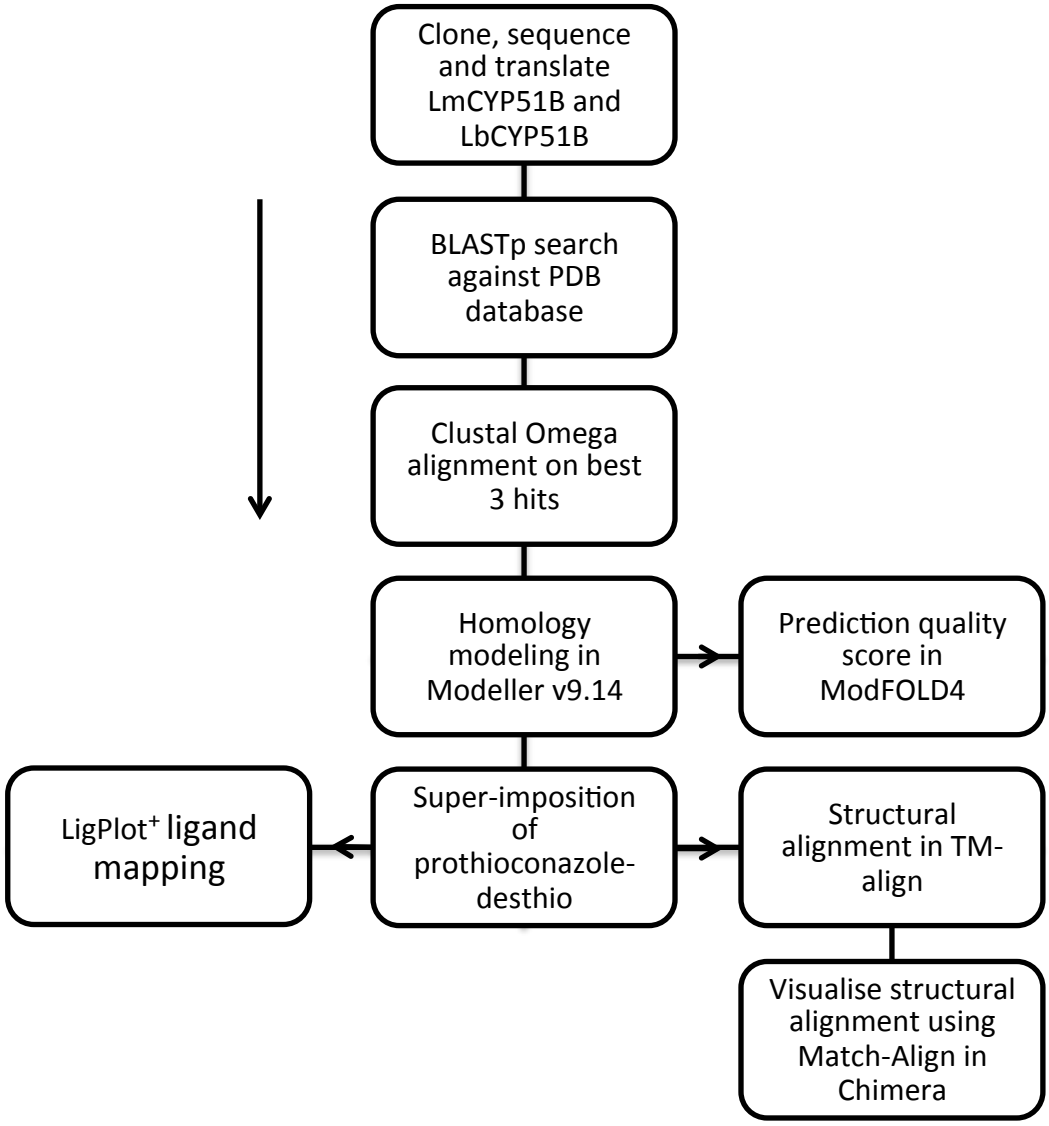



**Figure 5.1.** Homology modelling workflow for the generation of structural predictions of LmCYP51B and LbCYP51B. Protein sequences were aligned against CYP51B homologs from *Aspergillus fumigatus, Saccharomyces cerevisiae* and *Homo sapiens* and structural predictions were formed using Modeller v9.14; prediction quality was analysed using ModFOLD4. The antifungal chemical prothioconazole-desthio (*S. cerevisiae* docking confirmation) was superimposed using Match-Align in Chimera and comprehensive structures were aligned and compared using TM-align and Match-Align in Chimera.

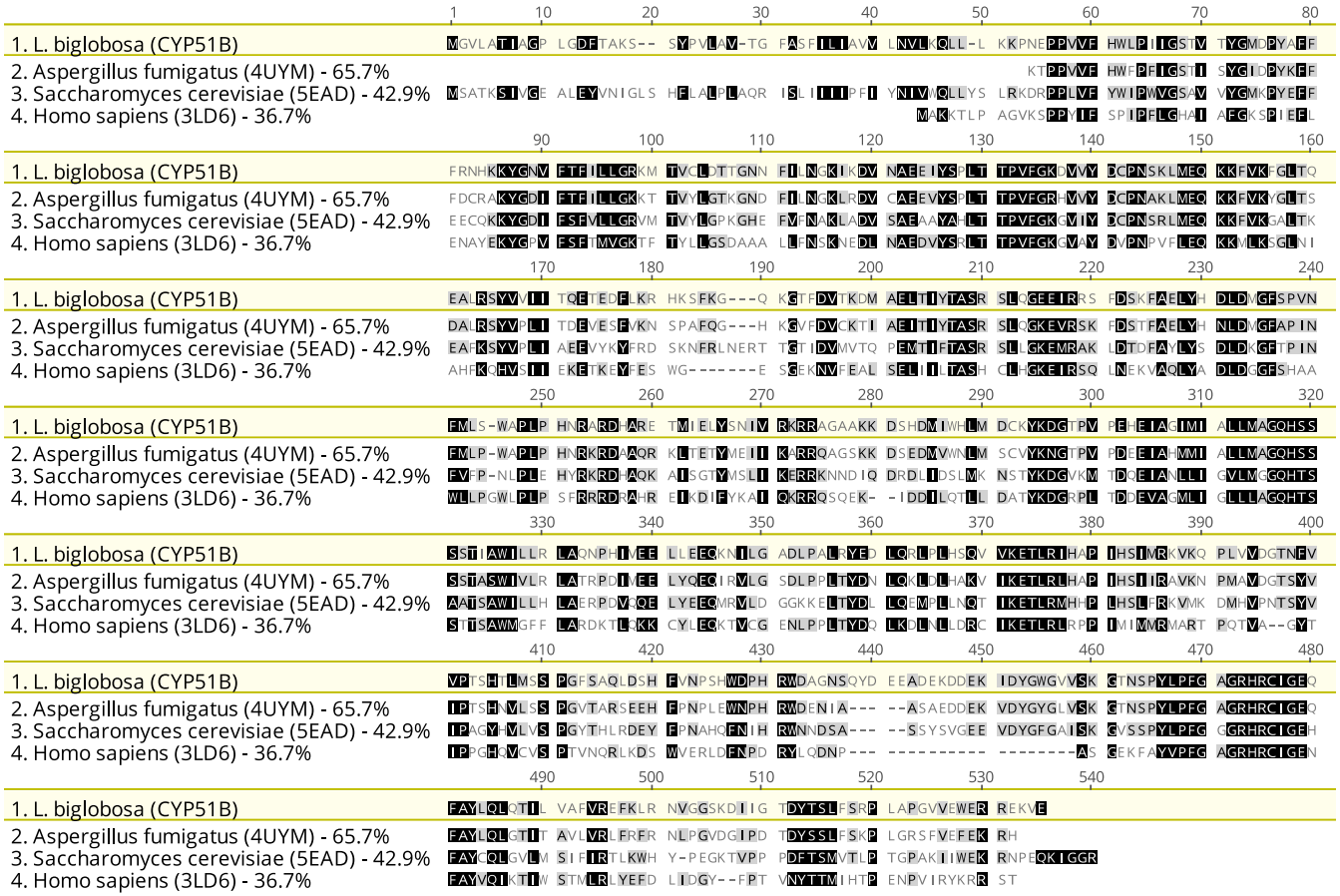



**Figure 5.2.** Protein sequence alignment (Clustal Omega) of the three most homologous CYP51B protein sequences that have been structurally solved, together with that of LbCYP51B. A BLASTp search of the protein data bank database was done individually on LmCYP51B and LbCYP51B protein sequences. PDB code and percentage similarity (%) are given next to the species name.

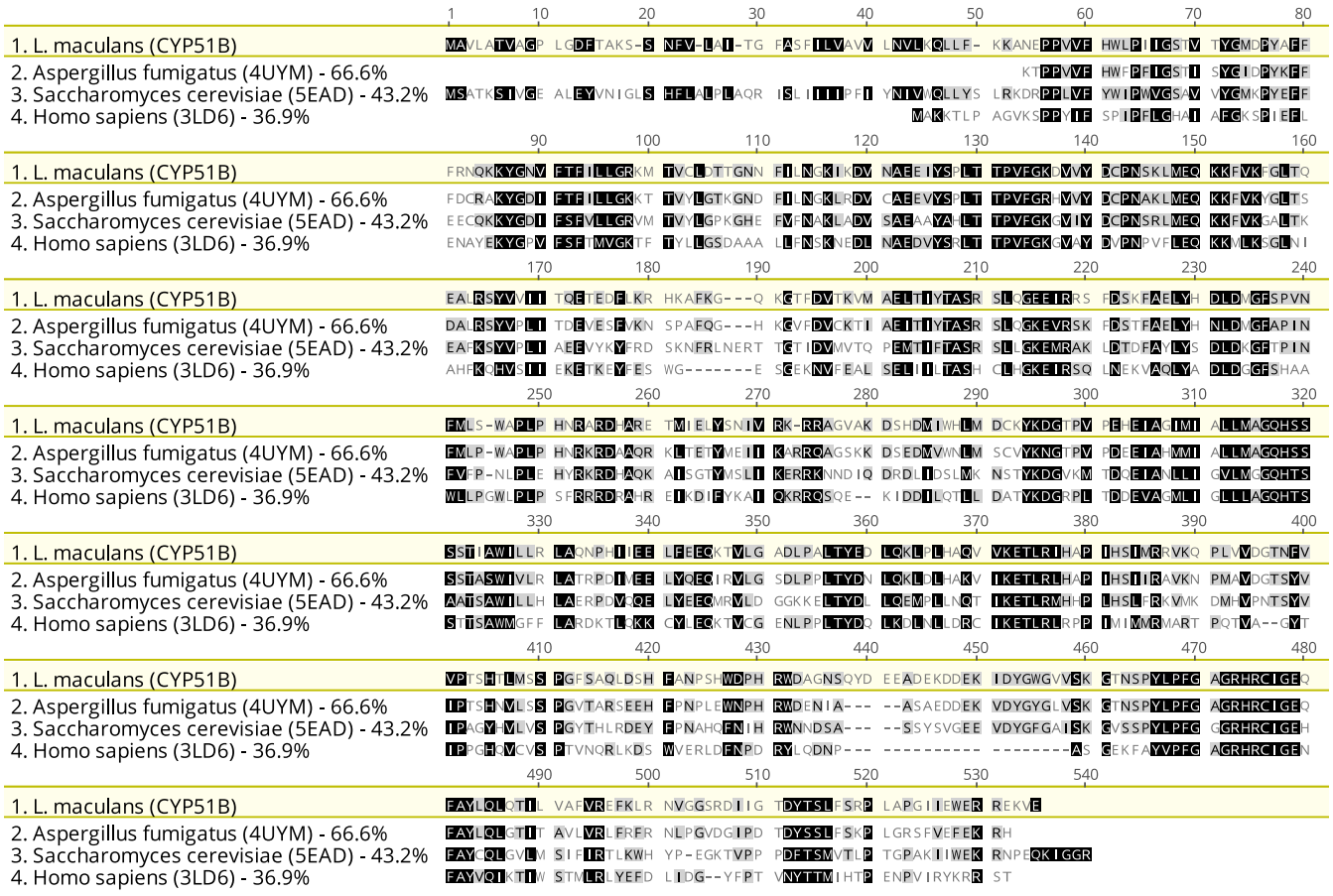



**Figure 5.3.** Protein sequence alignment (Clustal Omega) of the three most homologous CYP51B protein sequences that have been structurally solved, together with that of LmCYP51B. A BLASTp search of the protein data bank database was done individually on LmCYP51B and LbCYP51B protein sequences. PDB code and percentage similarity (%) are given next to the species name.

Finally, LigPlot+, a ligand-protein analysis tool, was used to compare prothioconazole-CYP51B interactions within the binding site of the predicted protein structure (Wallace et al. 1995).

### 5.2.3 Heterologous expression of LmCYP51B and LbCYP51B in *S. cerevisiae*

#### 5.2.3.1 Total RNA extraction

Total RNA was extracted from one *L. maculans* isolate (Hrox 12-2-1) and one *L. biglobosa* ($F_2$ dm 11-5) isolate using the Qiagen RNeasy Plant Mini Kit (Qiagen). Mycelium of each species was grown in saboroud dextrose broth for 10 days, harvested through centrifugation, flash-frozen in liquid nitrogen, freeze-dried for 24 hours and then ground into a powder. RNA extraction was done exactly to the manufacturer's instructions. A total of 50 µg of powdered mycelium was transferred into a liquid-nitrogen cooled 1.5 ml Eppendorf tube and 450 µl of buffer RLT was added before vortexing vigorously. The lysate was transferred to a QIAshredder spin column and centrifuged at maximum speed for 2 min. The supernatant from the flow-through was carefully transferred into a new 1.5 ml Eppendorf tube and mixed with 0.5 volumes of absolute ethanol. The sample was then transferred to an RNeasy spin column and centrifuged at 8,000x g for 15 sec; discarding the flow-through upon completion. The column was then washed once with 700 µl of buffer RW1 and two times with 500 µl of buffer RPE, respectively. Each wash step was centrifuged at 8,000x g for 15 sec with the flow-through discarded at each step; the column was dried by centrifugation (maximum speed for 1 min). Finally, the column-bound RNA was eluted using 50 $^{O}$C nuclease-free water and a centrifugation speed of 13,500 rpm.

#### 5.2.3.2 Synthesis of complementary DNA

Complementary DNA (cDNA) was synthesised from 1 µg of total RNA using the SuperScript® IV First-Strand Synthesis System (SSIV) according to manufacturer's guidelines (Invitrogen, CA, USA). Briefly, 1 µl of Oligo $d(T)_{20}$ primer was mixed with 1 µl dNTP mix (10mM), 1 µg total RNA and DEPC-treated water to a final volume of 13 µl; the sample was incubated at 65 $^{O}$C for 5 min to anneal primer to the template RNA.

Reverse transcription was initialised by the addition of 4 µl 5x SSIV buffer, 1 µl DTT

(100mM), 1 µl RNase inhibitor and 1 µl SSIV reverse transcriptase. The reaction was incubated at 50 $^{O}$C for 15 min and deactivated at 80 $^{O}$C for 10 min. The final cDNA was then diluted by 1/10.

#### 5.2.3.3 Restriction cloning into pYES2/CT yeast vector

The complete LmCYP51B and LbCYP51B coding sequences were amplified using primer pairs LmCYP51res (forward/reverse) and LbCYP51res (forward/reverse) (Table 2.2). Restriction primers introduced a non-specific *HindIII* restriction site to the 5" end and a *NotI* restriction site to the 3" of the amplified CYP51B sequences.

A touchdown-PCR (Akagi et al. 2011) was completed using EasyA High Fidelity PCR Cloning Enzyme (Agilent). Amplification conditions for LmCYP51B were one cycle at 94°C for 2 min; seven cycles at 94 °C for 30 sec, 59 to 53°C for 1 min (1 °C decrease per cycle), and 72 °C for 2 min; 28 cycles at 94 °C for 30 sec, 52°C for 1 min, and 72°C for 1 min; followed by a final extension at 72 °C for 7 min. Amplification conditions for LbCYP51B were one cycle at 94 °C for 2 min; seven cycles at 94 °C for 30 sec, 58 to 52 °C for 1 min (1 °C decrease per cycle), and 72 °C for 2 min; 28 cycles at 94 °C for 30 sec, 56 °C for 1 min, and 72 °C for 1 min; followed by a final extension at 72 °C for 7 min.

PCR products were purified using Qiagen PCR purification kit (Qiagen), cloned into the pGEM-T easy vector (Promega) (Appendix D.1) and transformed into high efficiency JM109 competent cells (Promega) all according to the manufacturer's specification. Ligation reactions combined 5µl 2x ligation buffer, 1µl T4 ligase, 50 ng/µl template DNA and nuclease-free water to a final volume of 10 µl. Control DNA (supplied) was used as positive control. Ligation reactions were incubated for 1 hour at 25 $^{O}$C or 16 hours (overnight) at 4 $^{O}$C.

Transformation of ligated DNA into high efficiency JM109 competent *E. coli* cells was completed according to the manufacturer's protocol (Promega). Briefly, 2 µl of ligated DNA was transferred into 50 µl of JM109 competent cells and incubated on ice for 20 min. The mixture was heat-shocked for 45 sec at 42 $^{O}$C and replaced on ice for 5 min. Super optimal broth (SOB) was equilibrated to room temperature and 950 µl was added to the transformed cells. After an incubation period of 1.5 h at 37 $^{O}$C with shaking, samples of 150, 100 and 50 µl, respectively, were spread on Luria-Bertani (LB) agar plates (Appendix A.1) amended with 40 µg/ml X-gal, 40 µg/ml IPTG and 50 µg/ml ampicillin. Inoculated agar plates were incubated at 30 $^{O}$C for 16 hours

(overnight).

Blue/white screening was used to identify potential transformants. Colony PCR, using LmCYP51res (forward/reverse) and LbCYP51res (forward/reverse) primers, was done on white *E. coli* colonies to determine successful ligation and transformation of LmCYP51B or LbCYP51B. A single *E. coli* colony was transferred into 50 µl of sterile distilled water, of which 1 µl was added to 19 µl of routine PCR master mix (Chapter 2.9). Colony PCR amplification parameters for LmCYP51B were 1 cycle at 95°C for 3 min; 30 cycles at 95 °C for 30 sec, 52 °C for 40 sec and 72 °C for 2 min; followed by a final extension at 72 °C for 7 min. Amplification parameters for LbCYP51B were 1 cycle at 95°C for 3 min; 30 cycles at 95 °C for 30 sec, 51 °C for 40 sec and 72 °C for 2 min; followed by a final extension at 72 °C for 7 min.

Plasmid DNA (pDNA) extraction was done using QIAprep Spin Miniprep Kit according to manufacturer's guidelines. Briefly, bacterial transformants confirmed to have either pGEM-T-LmCYP51B or pGEM-T-LbCYP51B constructs were grown for 16 hours (overnight) in 5 ml LB broth and centrifuged at 6800x g for 3 min. Cells were suspended in buffer P1 and buffer P2 was added subsequently. After inverting six times, buffer N3 was added and the sample was centrifuged at 13,500 rpm for 10 min. The supernatant was then applied to a QIAprep spin column and centrifuged at 13,500 rpm for 30 sec. The column was then washed with buffer PB and PE, respectively. Both steps were centrifuged at 13,500 rpm for 30 sec. The extracted pDNA was eluted from the column using 50 $^{O}$C nuclease-free water and centrifugation at 13,500 for 1 min.

Restriction digestions of pGEM-T-LmCYP51B and pGEM-T-LbCYP51B constructs were prepared using *HindIII* and *NotI* enzymes (New England Biolabs, USA) according to manufacturer's double-digestion specification. The reaction was incubated for 16 hours (overnight) and contained 5 µl of 10X CutSmart buffer, 1 µl of *HindIII* (10 units), 1µl *NotI* (10 units) and 1 µg of pDNA, with nuclease free water to a final volume of 50 µl. The yeast vector, pYES2/CT (Thermo Fisher) (Appendix D.2) was digested under the same conditions.

The 50 µl digestion samples for pYES2/CT, LmCYP51B and LbCYP51B were purified by gel electrophoresis (0.7 % agarose). Purified samples were then ligated and transformed into high efficiency JM109 competent *E. coli* cells, as previously described. Colony PCR was used to confirm which *E. coli* colonies had the correct size insert. Positive transformants underwent plasmid extraction and the purified pDNA was used for transformation of *S. cerevisiae* strain *YUG37:erg11 (MATa ura3-*

*52 trp1-63 LEU2::tTA tetO- CYC1::erg11)* (Revankar et al. 2004) using the S.c EasyComp transformation kit (Invitrogen). *YUG37:*LmCYP51B and *YUG37:*LbCYP51B transformants were plated out onto synthetic dropout minimal medium (SD) containing 4 g/l yeast nitrogen base without amino acids (Sigma), 3.92 g/l dropout medium supplement without uracil (Sigma), 2 % galactose and 2 % raffinose (SD GAL+RAF, Appendix A.1).

#### 5.2.3.4 Complementation evaluation of *YUG37:*LmCYP51B and *YUG37:*LmCYP51B transformants

*YUG37:*LmCYP51B, *YUG37:*LbCYP51B and *YUG37:pYES2/CT* (negative control) transformants were grown in 10 ml liquid SD medium for 24 hours at 30 $^{O}$C with shaking (250 RPM). Cell suspensions were diluted to a concentration of 1 x $10^6$ cells/ml and inoculated onto SD GAL+RAF agar plates with ($Dox^+$) or without ($Dox^-$) 3 µg/ml doxycycline (5 µl of five 5-fold dilutions). Plates were incubated at 30 $^{O}$C and photographed after 5 days.

#### 5.2.3.5 Fungicide-sensitivity on *YUG37:*LmCYP51B and *YUG37:*LmCYP51B transformants

Fungicide-sensitivity of *YUG37:*LmCYP51B and *YUG37:*LbCYP51B transformants was tested using fluconazole, tebuconazole, prothioconazole and prothioconazole-desthio fungicides (method similar to Chapter 4.2.2). A single transformant colony was grown in 10 ml liquid SD GAL+RAF for 24 hours at 30 $^{O}$C with shaking. The resultant cell suspensions were diluted to a concentration of 1 x $10^6$ cells/ml and 100 µl aliquots were added to a flat-bottomed 96-well microtitre plate. The microtitre plate was subsequently populated with 100 µl of SD GAL+ RAF media with doxycycline (6 µg/ml), which had been amended with decreasing concentration of fungicide (5, 1.7, 0.56, 0.19, 0.062, 0.021, 0.0069, 0.0023, 0.00076, 0.00025, 8.47 x $10^{-5}$, 2.82 x $10^{-5}$, 0 µg/ml). Plates were incubated at 30 $^{O}$C for 6 days. Fungal growth was measured by absorbance using a FLUOstar OPTIMA microplate reader (BMG Labtech, Offenburg, Germany) set to 630 nm. Fungicide sensitivities for each isolate were calculated as 50% effective concentrations (EC50s) using a dose-response relationship curve generated by the FLUOstar OPTIMA microplate software.

## 5.3 Results

### 5.3.1 Characterisation of *Lmerg11*/CYP51B and *Lberg11*/CYP51B

All 11 *L. maculans* isolate sequence assemblies encompassed *Lmerg11* (GenBank accession numbers KY500978 - KY500989), which was 1635 bp in length and consisted of two exons and one 54 bp intron; nucleotide sequence similarity between all isolates was 100% (Figure 5.4a). LmCYP51B was identical in all 11 isolates but differed by 1 amino acid (K337N) when compared with the published LmCYP51B sequence (AAN28927.1) from Australia. *Lberg11* was encompassed within all *L. biglobosa* sequence assemblies (GenBank accession numbers KY500970 - KY500977), was 1642 bp in length and consisted of two exons and one 58 bp intron (Figure 5.4b). Of the eight isolates sequenced, six had an identical *erg11* gene, while two isolates ($F_2$ dm 11-5 and H Dr 12 12) contained the same single nucleotide polymorphism (SNP) C1467T. Nonetheless, the SNP proved synonymous and all LbCYP51B sequences were 100 % similar. Neither species contained amino acid alterations previously recognised as conferring reduced sensitivity to azoles in other plant pathogenic fungi (Price et al. 2015).

A pair-wise alignment between LmCYP51B and LbCYP51B identified 24 amino acid alterations, one amino acid deletion and a pair-wise percentage identity of 97.5 % (Score matrix = BLOSUM80, threshold = 1) (Figure 5.4). Of the 24 alterations, 12 were considered a change to amino acids with similar physical-chemical properties and 12 were considered a change to amino acids with dissimilar physical-chemical properties (Score matrix = BLOSUM80, threshold = 1).

Phylogenetic analysis by maximum likelihood predicted that CYP51B from *L. maculans* and *L. biglobosa* share a similar evolutionary trajectory, distinct from other asomycete plant pathogen fungi, such as *Pyrenophora tritici-repentis* and *Alternaria alternata* (Figure 5.5).

### 5.3.2 Comparative homology modelling

For both species, all five CYP51B models that were predicted by Modeller had a very high structural fold confidence ($P$ <0.001) and a global model quality score of 0.89 (0 to 1 scale). The best models for each species, Lm_cyp51_model_final_5.pdb (Figure 5.6a) and Lb_cyp51_model_final_4.pdb (Figure 5.6b), were selected for further analysis.

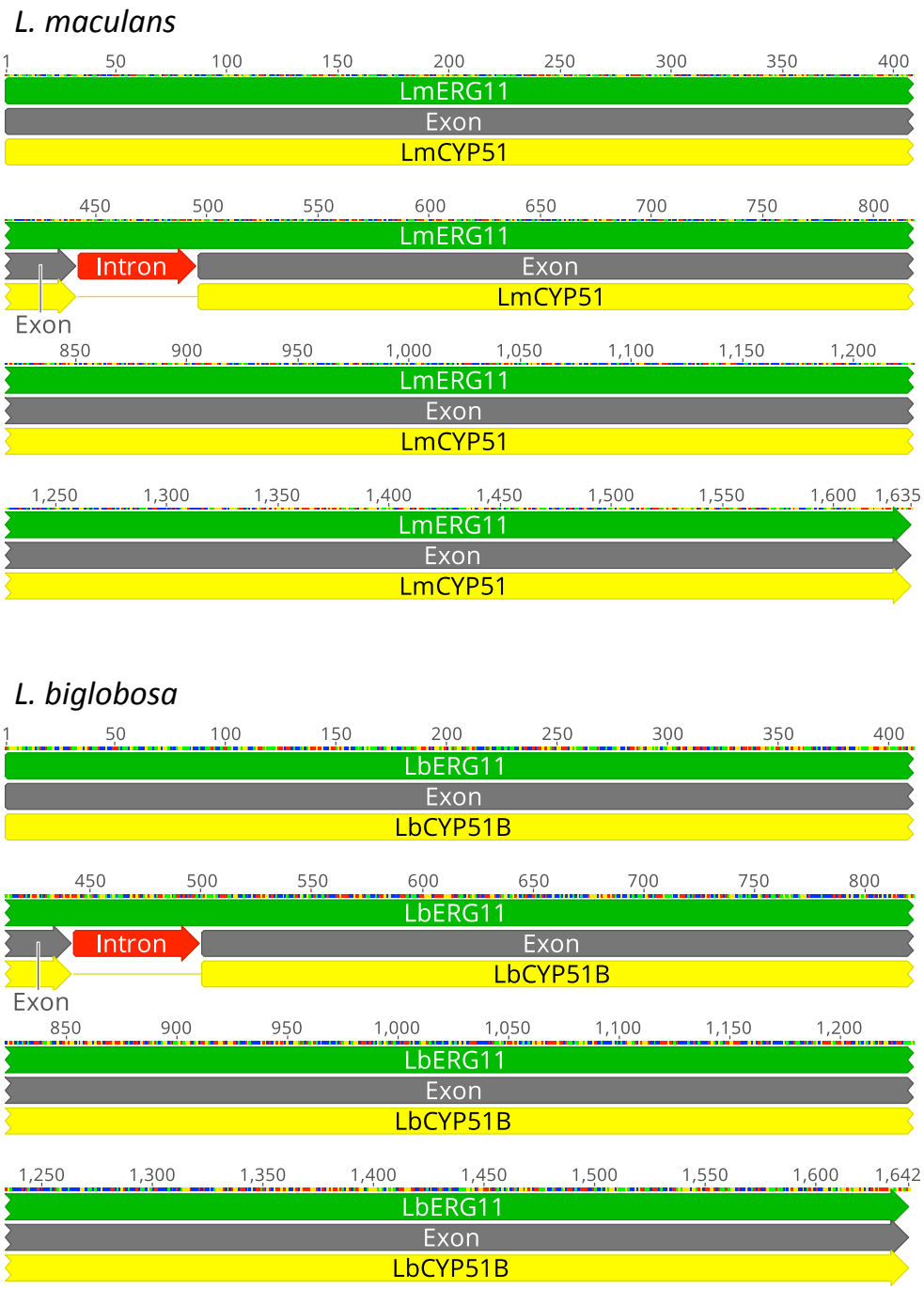



**Figure 5.4.** Characterisation of *erg11* in *L. maculans (Lmerg11)* and *L. biglobosa (Lberg11)*. To determine wild type *erg11* sequence in 11 *L. maculans* and eight *L. biglobosa* isolates, DNA extraction, purification and Sanger sequencing was done. Contigs were constructed using Cap3. Geneious was used for sequence translation, annotation and imaging. Cloning of both *Lmerg11* and *Lberg11* later confirmed sequence characterisation (Chapter 5.2.4.3).

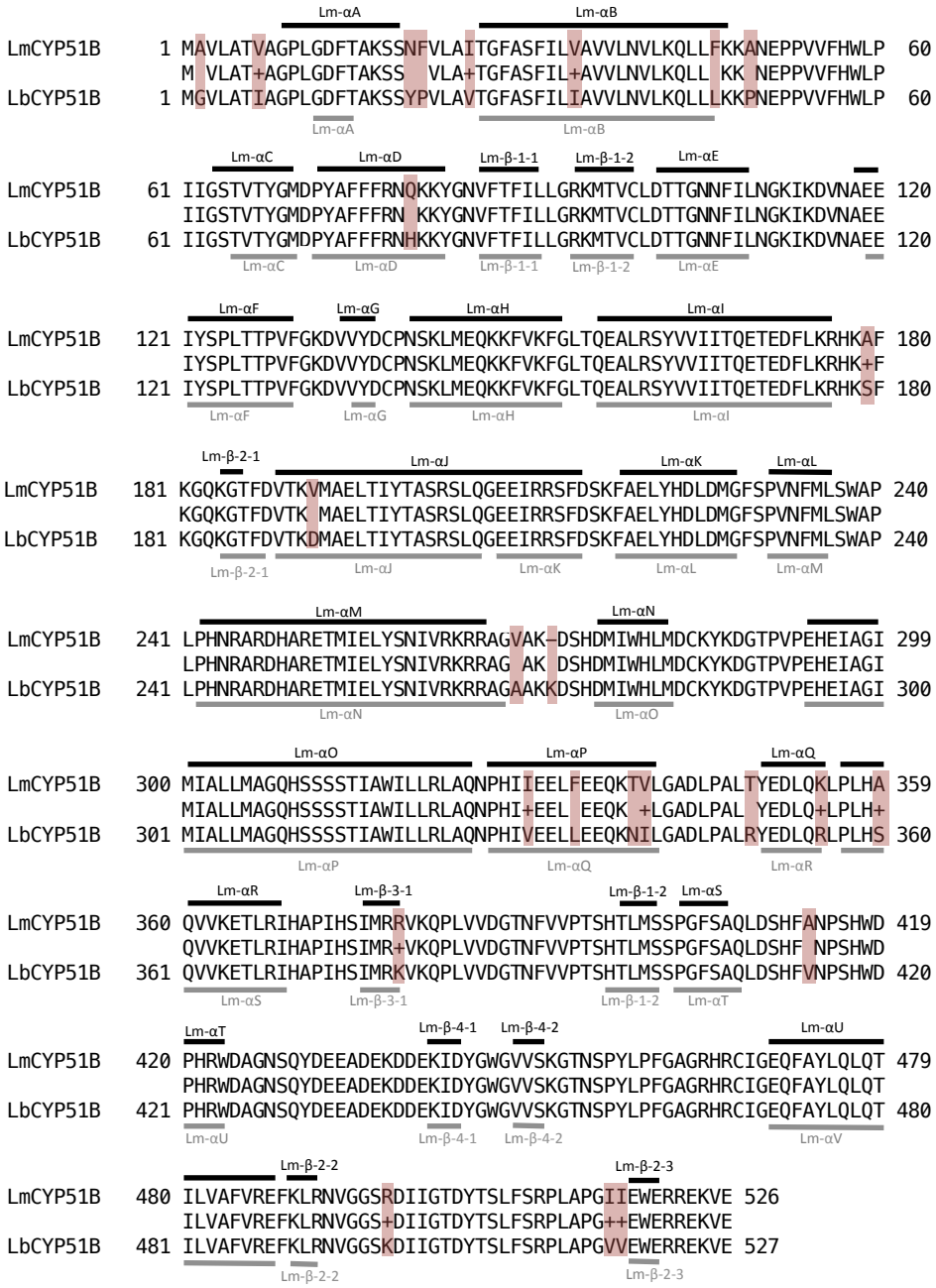



**Figure 5.5.** Wild type LmCYP51B and LbCYP51B pair-wise amino acid sequence alignment (Clustal Omega). CYP51B sequence was determined in 11 *L. maculans* isolates and eight *L. biglobosa* isolates. There were no alterations within species and 25 alterations between species (highlighted in red). Secondary structure annotation was determined using Chimera.

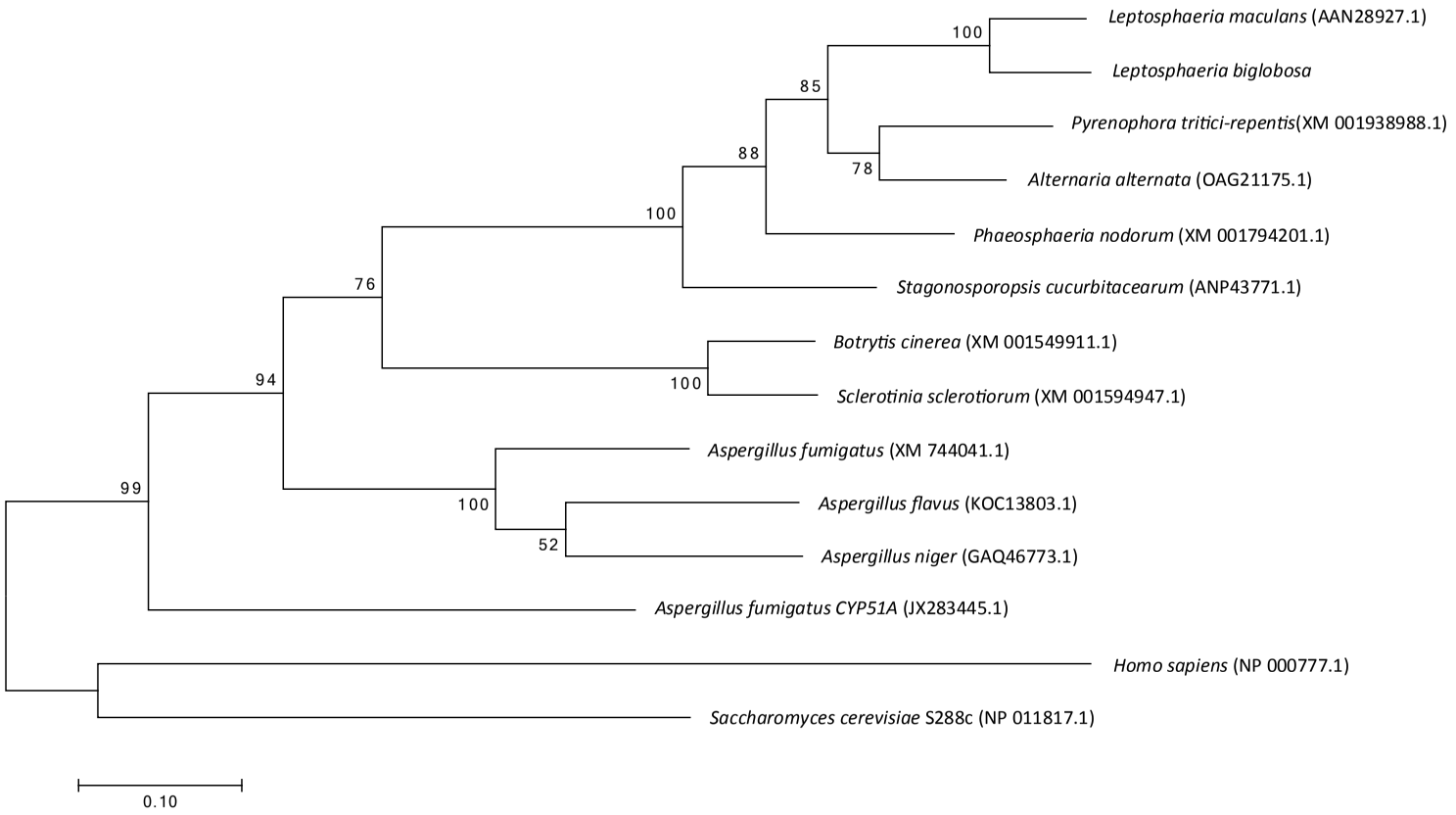



**Figure 5.6.** Molecular phylogenetic analysis of CYP51B sequences by Maximum Likelihood with 1000 bootstrap. The evolutionary history was inferred by using the maximum likelihood method based on the Tamura-Nei model (Tamura and Nei 1993). The percentage of trees in which the associated taxa clustered together is shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Evolutionary analyses were conducted in MEGA7.

The chosen predicted structures of LmCYP51B and LbCYP51B were very similar (TM-score = 0.90). The root-mean-square deviation (RMSD) of the common residues was 2.76 Å on a 526 amino acid alignment length (Figure 5.7). The globular active sites of the proteins appear to have the highest structural conservation. Noteworthy differences in the predicted structures were distinguishable only in the trans-membrane tail although some amino acid substitutions did slightly alter the predicted fold of the globular structure. The heme cofactor and subsequently bound prothioconazole-desthio ligand were shown to be located inside the binding pocket of the proteins. The surrounding amino acids, which form this pocket, were all highly conserved (Figure 5.8). Moreover, important residues involved in the development of a water-mediated hydrogen bond network that facilitates CYP51B/azole interaction were conserved structurally; specifically Tyr126, Tyr140 and Ser382 (amino acid locations from *S. cerevisiae* CYP51B).

Detailed two-dimensional schematics of protein-ligand interactions identified further similarities between the predicted structures of LmCYP51B (Figure 5.9a) and LbCYP51B (Figure 5.9b). A water-mediated hydrogen bond network was maintained in both structures, specifically facilitated by the presence of Tyr136, a water molecule and the relative location of the heme cofactor. Furthermore, five other residues, Tyr122, Ala306 (Ala307 in LbCYP51B), Ile372 (Ile373 in LbCYP51B), Ser374 (Ser375 in LbCYP51B) and Phe508 (Phe509 in LbCYP51B), which were interacting with the bound ligand (prothioconazole-desthio), were correspondingly located in the binding pocket. The predicted structure of LbCYP51B had two extra residues, Ser311 and Leu508, both located sufficiently close to interact with the ligand.

### 5.3.3 Heterologous expression of LmCYP51B and LbCYP51B in *S. cerevisiae*

The vector-only transformed strain *YUG37:pYES2/CT*, which lacked a complementary CYP51B protein, was unable to grow on the doxycycline amended SD GAL+RAF medium due to the repression of native CYP51B expression in *S. cerevisiae*. Complementation of native CYP51B was achieved in *YUG37:*LmCYP51B and *YUG37:*LbCYP51B *S. cerevisiae* transformants, which both showed robust growth across five 5-fold dilutions on SD GAL+RAF medium amended with doxycycline; demonstrating that expression of the predicted wild-type CYP51B protein from *L. maculans* or *L. biglobosa* can support ergosterol synthesis in *S. cerevisiae* (Figure 5.10)

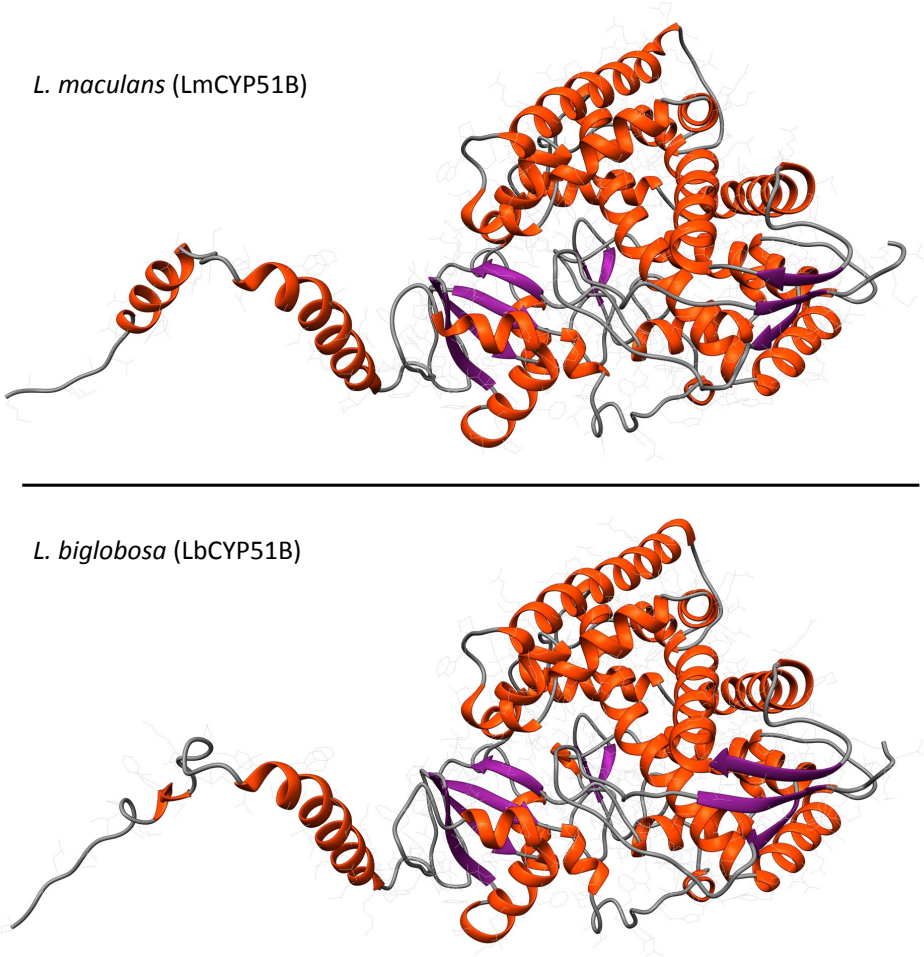



**Figure 5.7.** Three-dimensional representation of LmCYP51B and LbCYP51B homology models. Structural predictions were inferred through homology modelling (Modeller v9.14) of solved CYP51B structures from *Aspergillus nidulans*, *Saccharomyces cerevisiae* and *Homo sapiens*. Secondary structures are illustrated in colour: α-helices (orange), β-sheets (purple) and coils (grey). Images were generated in Chimera.

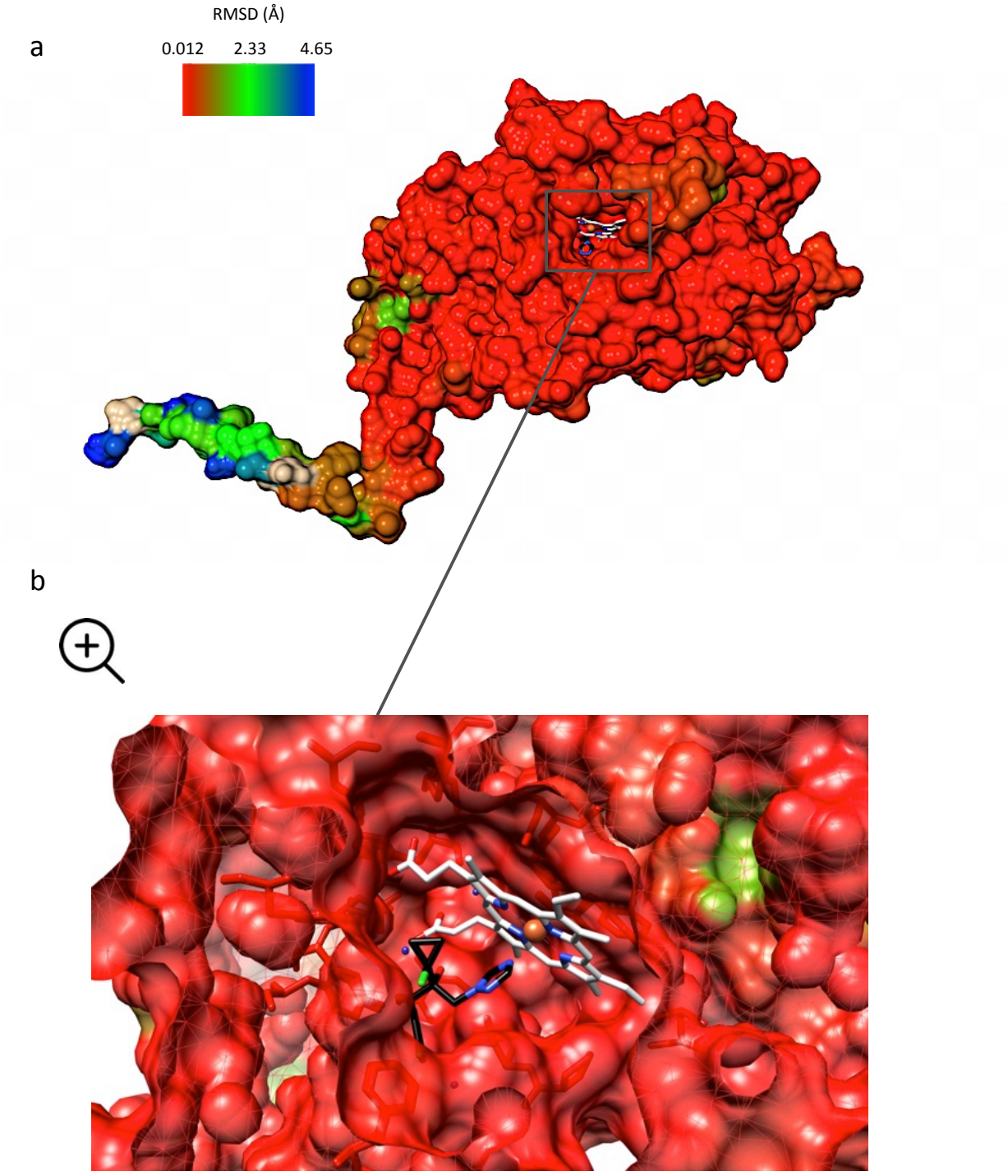



**Figure 5.8.** Structural pair-wise alignment (Match > Align; Chimera) on the predicted homology models of LmCYP51B and LbCYP51B. Difference in colour represents distance (Å) between aligned residues, with red representing residues close in structural locality and blue representing residues that are further apart. Zoomed image is a cross section of the binding pocket of aligned proteins. Images were generated in Chimera.

.

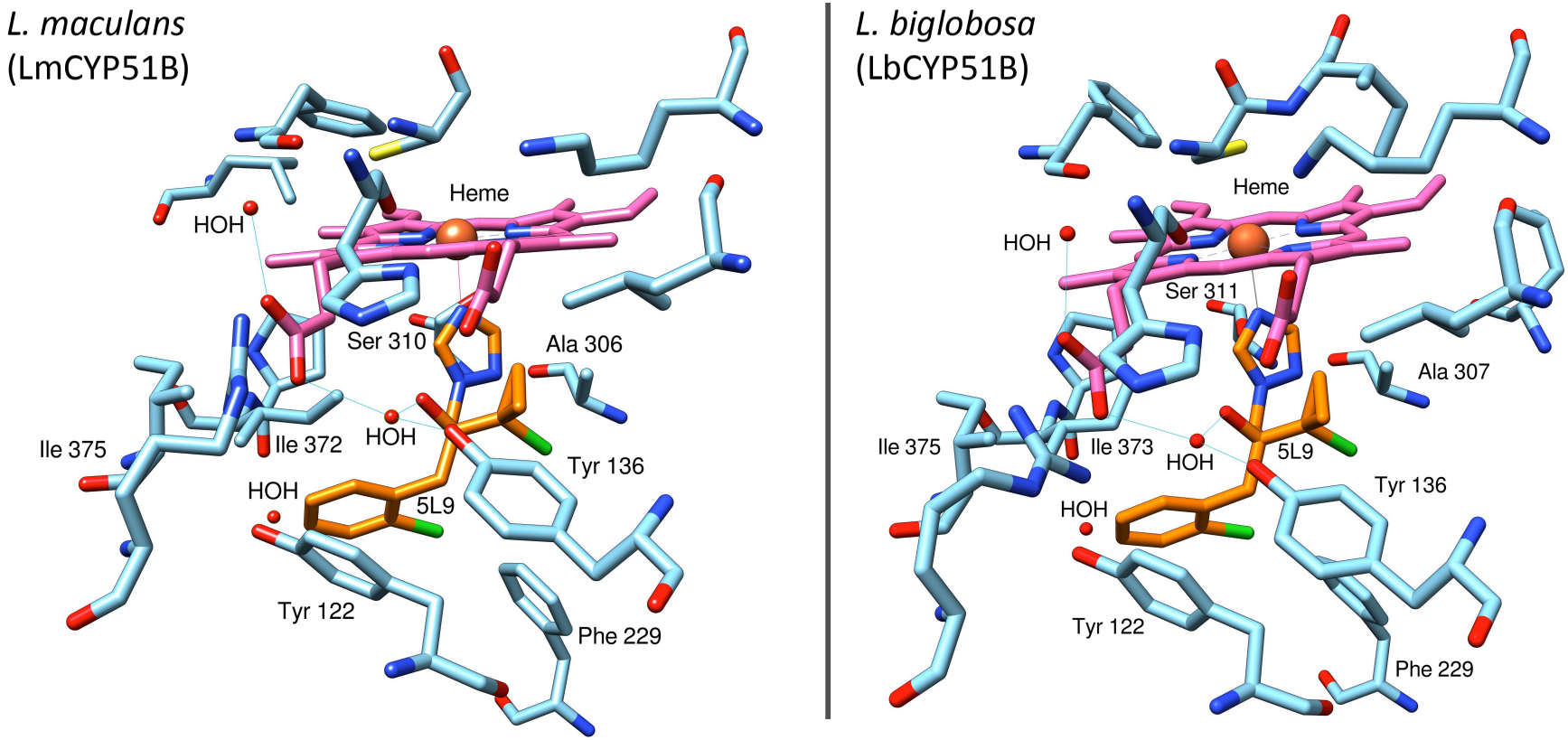



**Figure 5.9.** Azole binding site in the LmCYP51B (a) and LbCYP51B (b) proteins inferred by homology. Superimposed prothioconazole-desthio (5L9) is highlighted in orange with residues < 5 Å from ligand (5L9) labelled. The heme cofactor is highlighted in pink and the metal-ligand bond in purple. Hydrogen bonds are highlighted in blue and water molecules (HOH) in red. Images were generated in Chimera.

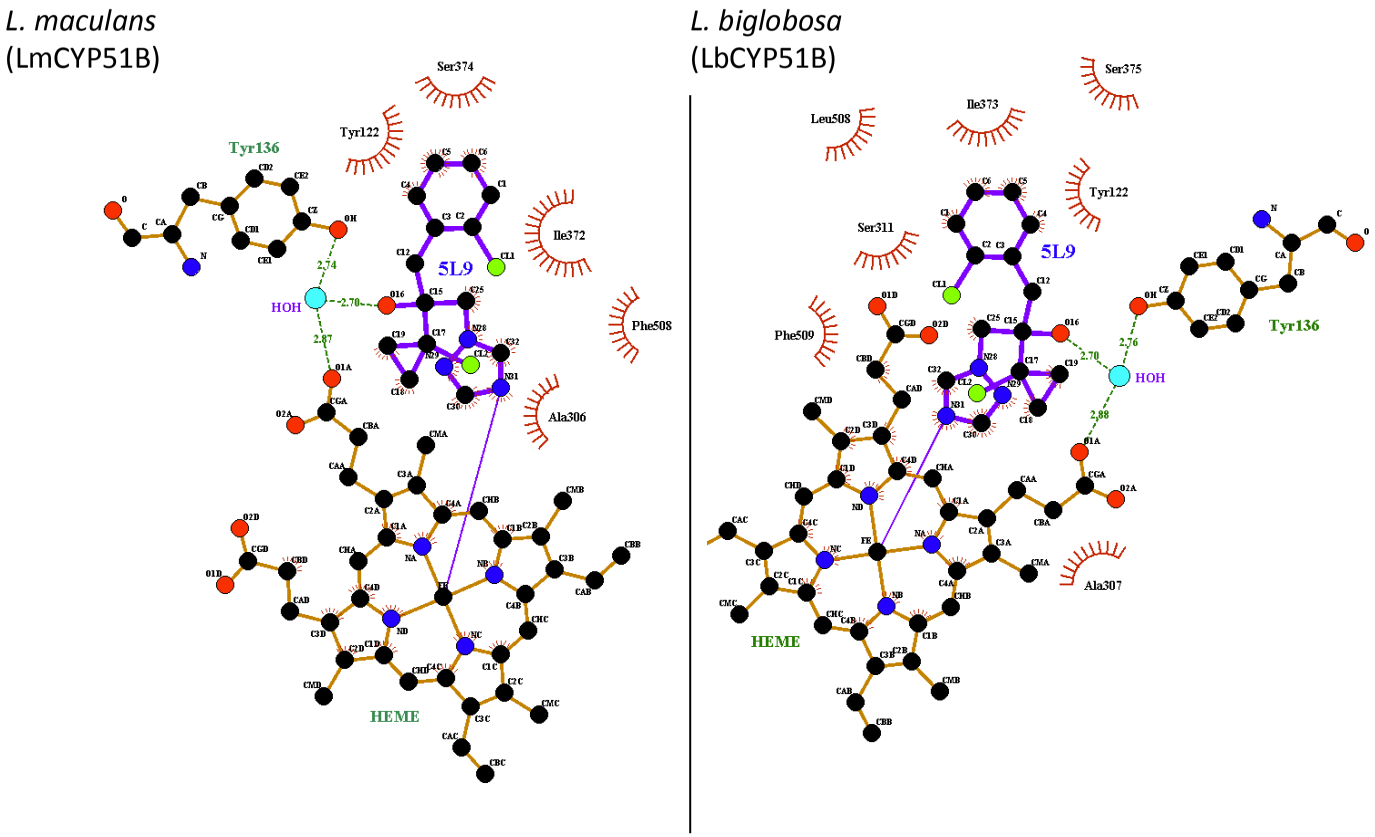



**Figure 5.10.** Two-dimensional protein-ligand interaction schematic (LigPlot$^{+}$) demonstrating LmCYP51B (a) and LbCYP51B (b) in association with fungicide prothioconazole-desthio (5L9). Prothioconazole-desthio, CYP51B side chain (Tyr136) and heme cofactor are shown in ball-and-stick representation. Ligand-metal bonds coloured in purple, hydrogen bonds are shown as green dotted lines, while the red-spoked arcs represent protein residues involved in hydrophobic interactions with the ligand. Water molecules (HOH) are displayed in light blue.

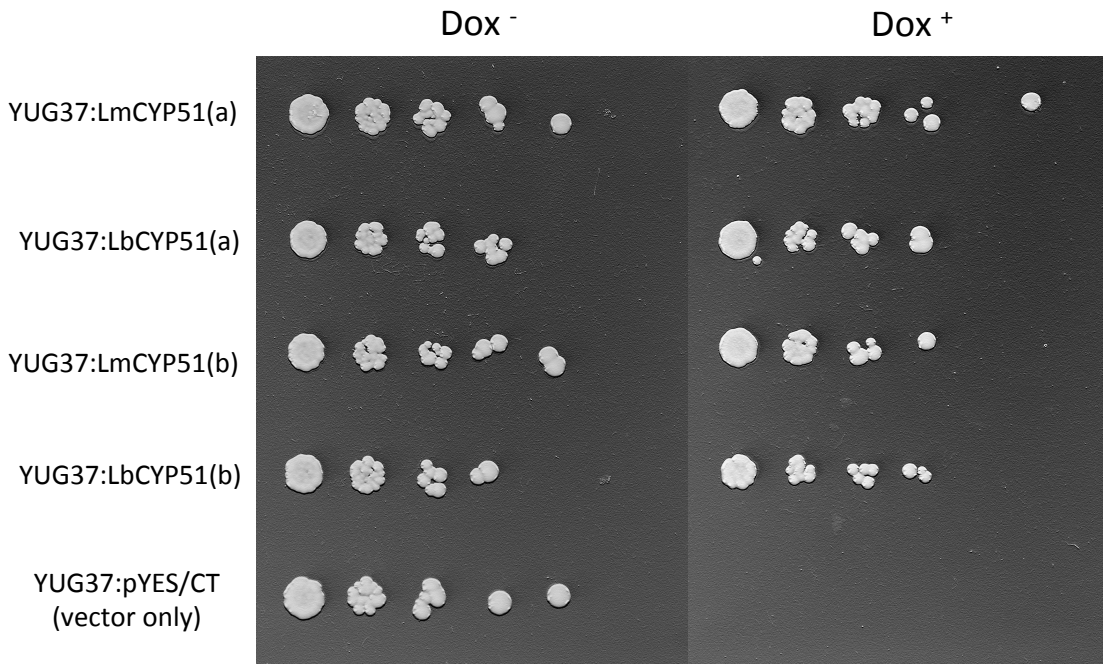



**Figure 5.11.** Complementation of *S. cerevisiae* strain *YUG37:erg11* with LmCYP51B and LbCYP51B. Yeast transformants grown for 16 hours (overnight) in SD + GAL + RAF media were inoculated onto SD + GAL + RAF agar without ($DOX^-$) or with ($DOX^+$) doxycycline, which represses the native *S. cerevisiae* CYP51B. The *YUG37:pYES2/CT* vector-only strain was used as a control. Lettering (a,b) refers to biological replicates. Inoculated plates were incubated at 30 $^{O}$C for 5 days and photographed. Images were visually enhanced using ImageJ editing software.

All *YUG37* transformants expressing LmCYP51B or LbCYP51B were very sensitive to tebuconazole, flusilazole, prothioconazole and prothioconazole-desthio (Table 5.1). There was a significant difference the $EC_{50}$ in values between *YUG37:LmCYP51* and YUG37:LbCYP51 transformants (1 df, $P$ <0.05). On each occasion, *YUG37:LmCYP51* was marginally less sensitive to the fungicides than *YUG37:LbCYP51*.

## 5.4 Discussion

In this present study, the protein sequence and predicted three-dimensional structure of lanosterol 14 alpha demthylase (CYP51B), an important enzyme in the synthesis of ergosterol in fungi, were found to be highly similar in coexisting winter oilseed rape pathogens *L. maculans* (LmCYP51B) and *L. biglobosa* (LbCYP51B).

Furthermore, the role of CYP51B in the difference between azole fungicide-sensitivity, previously described by Eckert et al. (2010) and Huang et al. (2011), appears to be unrelated to sequence alterations within the wild type LmCYP51B and wild type LbCYP51B proteins.

For the isolates analysed, both LmCYP51B and LbCYP51B contained no previously identified CYP51B mutations that confer azole resistance in other plant and human pathogenic fungi (Flowers et al. 2015; Howard et al. 2009; Lucas et al. 2015; Oliver and Hewitt 2014). Therefore, there was no evidence that evolution of resistance to azole fungicides, which has been strongly linked to the gradual development of missense mutations in the CYP51B amino acid sequence (Carter et al. 2014; Cools et al. 2010; Cools and Fraaije 2008; Fraaije et al. 2007; Hawkins et al. 2014; Mullins et al. 2011), had occurred in either the *L. maculans* or the *L. biglobosa* populations sampled. These data are supported by previous findings, which suggested that both species are sensitive to tebuconazole, flusilazole and prothioconazole-desthio (Chapter 4).

Although there were no missense mutations that either a) conferred reduced sensitivity or b) had previously been identified in other plant pathogenic fungi treated with similar antifungal azoles, there were many amino acid differences between LmCYP51B and LbCYP51B that could potentially affect the secondary structure of the proteins and thus the azole-binding potential.

**Table 5.1.** Fungicide sensitivity (EC50 µg ml-1) of YUG37::LmCYP51B and YUG37::LbCYP51B Sacharomyces cerevisiae transformants. A 96-well plate was populated with decreasing concentrations (5, 1.7, 0.56, 0.19, 0.062, 0.021, 0.0069, 0.0023, 0.00076, 0.00025, 8.47 x 10-5, 2.82 x 10-5, 0 µg ml-1) of fungicide-amended SD + GAL + RAF media with doxycycline and cell suspension (106 cells ml -1). Established assays were incubated at 28 OC for 6 days and absorbance was recorded by well-scanning (12 locations per well; 630 nm). EC50 values were calculated automatically. Standard deviations are given in brackets and were calculated from two biological replicates. Asterisks denote significance when compared with the control (* P < 0.05, ** P < 0.01 *** P < 0.001).

| Transformant | Fungicide sensitivity ($EC_{50}$ µg $ml^{-1}$) | | | | | |
|---|---|---|---|---|---|---|
| | Tebuconazole | Fold difference | Flusilazole | Fold difference | Prothioconazole-desthio | Fold difference |
| YUG37::LmCYP51B | 0.0084 (0.0017) | | 0.036 (0.014) | | 0.0007 (0.0006) | |
| YUG37::LbCYP51B | 0.0062 (0.0005) | **1.35 *** | 0.032 (0.0056) | **1.13** | 0.0004 (0.0002) | **1.75** |

Changes to the structure of CYP51B have previously been proposed to alter the affinity of azoles, such as tebuconazole or triadimenol; this was determined through predictive homology modelling of wild type (*MgCYP51B*) and mutant CYP51B proteins found in insensitive *Mycosphaerella graminicola* populations (Mullins et al. 2011).

It was hypothesised that the gradual development of polymorphisms and a deletion (ΔY459/G460) increased the size of the binding pocket, which encompasses both the heme cofactor and ligand azole. By increasing the size of the binding pocket, interacting residues moved away from the heme-bound azole (>4.5 Å), thus reducing the affinity of the fungicides to CYP51B.

Additionally, this particular study identified important substitutions at locations directly interacting with the azole, an example being the amino acid substitution Y136F (in *MgCYP51B*), which was shown to change the angle of the side chain into a region that would have been occupied by triadimenol in the wild-type protein.

More recently though, analysis of a high resolution *S. cerevisiae* CYP51B structure (PDB:4WMZ), co-crystallised with the medical azole fluconazole, identified two water molecules within the active site of the proteins that form a network of hydrogen bonds adhering the azole to the protein (Sagatova et al. 2015). The presence of Y136F/H (*ScCYP51B*) breaks one part of this water-mediated hydrogen bond network due to the loss of the hydroxyl group incurred by the tyrosine to phenylalanine or histidine substitution. This reduces the binding capacity of the proteins, acknowledged through spectral characterisation of fluconazole binding with the wildtype *ScCYP51B* protein or Y140F/H mutants (Sagatova et al. 2016).

Here, the predicted structures of LmCYP51B and LbCYP51B have been compared through the structural alignment of homology models. The agricultural azole prothioconazole-desthio was superimposed using its confirmation in co-crystallised *ScCYP51B* structure PDB:5EAD. Analysis of the alignment noticeably illustrates that structural conservation is maintained throughout the globular cysteine pocket. Only in the transmembrane tail was there any noticeable distance between the aligned residues.

Moreover, locality of residues Y136, K147 and *G461/G462 (*LmCYP51B/LbCYP51B, respectively), which correspond to substitutions Y132F, K143R and G464S in resistant *Candida albicans* isolates (Y140F, K151R and G464S in *ScCYP51B*) (Flowers et al. 2015), was conserved in both *L. maculans* and *L.*

*biglobosa*. In addition to this, Y122 (Y126 in *ScCYP51B*) and *S374/S375 (S383 in *ScCYP51B*), which form a water-mediated hydrogen bond network with another water molecule when fluconazole is bound, were also structurally conserved. In fact, according to the multiple sequence alignment with other plant and human pathogenic fungi, many residues interacting with the azole, heme cofactor and/or the water-mediated hydrogen bond network were uniformly conserved, along with that of the *S. cerevisiae* and the *H. sapiens* orthologs.

Consequentially using these data, it can be postulated that the wild-type LmCYP51B and LbCYP51B proteins would interact with azole fungicides similarly due to the likeness of key structural locations and the preservation of a water-mediated hydrogen bond network in the example shown.

Testing of this hypothesis was done using heterologous yeast expression and subsequent fungicide-sensitivity assays, to investigate the efficacy of various azole fungicides on LmCYP51B and LbCYP51B within an isogenic background. Repression of the native *S. cerevisiae erg11* gene allowed this comparison. Both YUG37:LmCYP51B and YUG37:LbCYP51B yeast transformants responded similarly to azole treatment, although there was a minor difference in sensitivity between the two strains, which could explain the phenotypic difference in azole-sensitivity between *L. maculans and L. biglobosa* (Chapter 4).

Previous studies have used this method to investigate the effects of CYP51B polymorphisms on fungicide efficacy, and have shown that the presence of these alterations confers decreased sensitivity (Carter et al. 2014; Cools et al. 2010; Hawkins et al. 2014). This is not the case in our example, which supports the structural similarities observed in the predicated models of LmCYP51B and LbCYP51B.

Ultimately, these conclusions do not fully explain the minor differences in sensitivity described in Chapter 4 and previously (Eckert et al. 2010; Huang et al. 2011), and further work investigating the two other known mechanisms of resistance to azoles (target site over-expression and increased fungicide efflux) would provide a more complete understanding of why there is a minor difference in sensitivity to azole fungicides between *L. maculans* and *L. biglobosa*.

# Chapter 6 General discussion

The overall aim of this project was to further understand the role of fungicides in controlling phoma stem canker, by investigating their efficacy against *Leptosphaeria maculans* and *L. biglobosa* in crops, *in vitro* and *in planta*. This chapter will discuss main findings of the project and propose future research.

## 6.1 Azole fungicides are still very effective for control of phoma stem canker.

This is the first study to highlight the effects of penthiopyrad plus picoxystrobin at reducing *L. maculans* and *L. biglobosa* type symptoms on UK winter oilseed rape crops (Sewell et al. 2016). Confidence in the efficacy of the mixture has been further increased by indication that the fungicide decreases potential of the pathogens to colonise winter oilseed rape cotyledons under controlled conditions and reduces the growth of both pathogens on nutrient-rich medium (Chapter 4). Comparisons with azole fungicide prothioconazole (and its active antifungal metabolite prothioconazole-desthio) suggest that both products offer a similar level of control in winter oilseed rape crops, *in planta* and *in vitro*. Dissemination of these findings will provide growers with an opportunity to improve selection of fungicides from different fungicide classes.

In recent years, reliance on azole fungicides has increased. Since 2000, the foliar application of triazole fungicides on all crops has increased by almost six times (Garthwaite et al. 2014). Diversifying fungicides classes, through the application of non-azole based fungicides such as penthiopyrad plus picoxystrobin, will decrease selection pressure generated through the continued application of single-site fungicides of the same class (Lucas et al. 2015).

Despite this continued reliance on them, results presented here suggest that current azole fungicides are still effective at reducing phoma stem canker symptoms in winter oilseed rape crops and that both *Leptosphaeria* spp. have yet to evolve resistance to that particular class of fungicide. This supports previous findings that described effective control of phoma stem canker symptoms in field experiments using the azole fungicide flusilazole (Huang et al. 2011), and showed that resistance had yet to appear in the isolates of *Leptosphaeria* spp. tested *in vitro* with flusilazole and tebuconazole (Eckert et al. 2010; Huang et al. 2011).

Nevertheless, azole fungicides are classed as at a medium to high risk for evolution of resistance (Oliver and Hewitt 2014) and stewardship of active ingredients with the same mode of action is essential (Fungicide Resistance Action Committee 2014, www.frac.info, last accessed September 19, 2016). It has frequently been suggested that alternation or mixtures of fungicides decreases the risk of resistance evolution (Hollomon and Kendall 1997), a finding that has been further reinforced by mathematical modelling (Hobbelen et al. 2011). Through the alteration or mixing of fungicides with different modes of action, selected pathogen populations generated by one class of fungicide will be killed by the other class of fungicide. Use of penthiopyrad plus picoxystrobin will not only decrease selection generated from over-reliance on azole fungicides but also protect against the development of SDHI and QoI resistance, which is imperative considering that both are considered high-risk to develop resistance (Oliver and Hewitt 2014; Fungicide Resistance Action Committee 2014, www.frac.info).

Continued monitoring of fungicide efficacy is vital to ensure that growers are still provided with cost-effective benefits from applying fungicides to their crops. Future work should continue to investigate the effects of penthiopyrad plus picoxystrobin at reducing phoma stem canker symptoms and improving oilseed rape yield. Azole fungicides should also be monitored to ensure that efficacy in crops has not decreased. Isolate collections should be maintained, updated and tested for fungicide-sensitivity both *in vitro* and *in planta*. Generally, insensitive populations are at a low level, and alleles conferring fungicide-resistance are at a low frequency when compared to the wild type (Brent et al. 2007). Extensive collection and testing of *L. maculans* and *L. biglobosa* isolates from fungicide-treated plots is essential.

## 6.2 Difference in sensitivity to azole fungicides

Importantly, what has been elucidated in this present study is that differences in the wild-type amino acid sequence between the two co-existing species do not have a substantial effect on the secondary structure of lanosterol 14α-demethylase, signifying that the CYP51B protein is very similar in *L. maculans* and *L. biglobosa.* However, despite structural similarities, heterologous expression of LmCYP51B and LbCYP51B in yeast does suggest that CYP51B could have a role in minor azole sensitivity differences between the two species, although more work investigating other mechanisms of resistance is required for conclusive evidence.

Before this study, two publications had suggested that *L. maculans* and *L. biglobosa* differed in their sensitivity to azole fungicides. The first study proposed that the growth of *L. maculans* and *L. biglobosa* mycelium was affected differently by azole fungicides flusilazole and tebuconazole; this was determined through the calculation of $EC_{50}$ on fungicide-amended media (Eckert et al. 2010). The second study identified a difference between the two species whilst colonising winter oilseed rape plants under controlled conditions (Huang et al. 2011). The plants were inoculated with either *L. maculans* or *L. biglobosa* and sprayed with a flusilazole-based fungicide. Lesion development was measured and relative amounts of DNA were quantified using qPCR. In both cases, *L. biglobosa* was found to be less sensitive to azole fungicides than *L. maculans*.

These results can only be partially supported by these findings. Indeed, *in vitro* there was a difference in sensitivity to flusilazole but not to tebuconazole. Additionally, *in planta* results suggested that both *L. maculans* and *L. biglobosa* were similarly affected by application of prothioconazole. Although *L. maculans* was less sensitive to prothioconazole-deshio treatment *in vitro*, this was not the case during the *in planta* experiment. One explanation could be the extent of differences in $EC_{50}$ between the two species. Studies investigating resistance in other plant pathogens, where polymorphisms confer azole-resistance, have reported an $EC_{50}$-resistance factor ratio of >100 between sensitive and insensitive populations (Cools et al. 2011; Mullins et al. 2011).

The difference in $EC_{50}$ between *L. maculans* and *L. biglobosa* described in this study and in Eckert et al. (2010) and Huang et al. (2011) is a ratio of < 5, which would be considered small had this been a sensitivity shift in populations of the same species; nonetheless, it is difficult to compare the two species in this way because of their vast genetic differences (Grandaubert et al. 2014a; Grandaubert et al. 2014b). Furthermore, a small number of differences in $EC_{50}$ does not explain why Huang et al. (2011) found flusilazole to be less effective at reducing *L. biglobosa* colonisation of winter oilseed rape true leaves compared to *L. maculans*.

A lack of consistency between experiments could be the explanation for the different results. Both this study and Huang et al. (2011) analysed just one *L. maculans* and one *L. biglobosa* isolate during *in planta* experiments. Furthermore, growth stages of the plant and fungicide concentration were also different as was the azole fungicide used for experimentation. Consequently, the evidence is still not clear about whether there is actually a difference in sensitivity between *L. maculans* and *L. biglobosa.*

Considering that a sensitivity difference in coexisting eyespot pathogens *Tapesia yallundae* and *T. acuformis* has been shown to result in population shifts (Bierman et al. 2002), it is vital that a more detailed analysis of azole-sensitivity alterations in *L. maculans* and *L. biglobosa* is undertaken. A large-scale investigation, both *in planta* and *in vitro,* using a large set of isolates collected across the UK would provide more conclusive evidence on the existence of a difference in azole sensitivity between *Leptosphaeria* spp.

## 6.3 *L. maculans* is still the dominant phoma stem canker pathogen in the UK

In all four winter oilseed rape cropping seasons (2010/11, 2011/12, 2012/13 and 2014/15), *L. maculans* was the predominant causal agent of phoma leaf spotting, suggesting that the phoma stem canker epidemic was dominated by *L. maculans*. Furthermore, in the 2013/14 cropping season, in which there was a severe phoma stem canker epidemic, there was very little evidence of *L. biglobosa* in either upper stem lesions or basal stem cankers sampled.

In the UK, it was suggested that *L. maculans* was the main causative agent of phoma stem canker in 2000, and that the incidence of *L. biglobosa* was small (West et al. 2002). More recently, this finding was supported by a similar study that again demonstrated that epidemics in several cropping seasons were dominated by *L. maculans*, based on greater amounts of *L. maculans* DNA in stem canker samples. Our data support these studies and improves our understanding of phoma stem canker epidemics in the south-east region of the UK. However, they do not explain recent preliminary findings that have shown large amounts of *L. biglobosa* DNA in stem base cankers and upper stem lesions; intriguingly in cultivars harbouring good resistance against *L. maculans* (Huang et al. 2014a).

Additionally, observations in regions of Poland where *L. biglobosa* is considered the dominant of the two phoma stem canker pathogens have highlighted symptoms with yield loss potential (Brachaczek et al. 2010). Controlled environment experiments presented show that *L. biglobosa* colonises oilseed rape cotyledons faster than *L. maculans*, thus displaying its potential as a pathogen of susceptible winter oilseed rape cultivars (e.g. cv. Catana). Furthermore, growth *in vitro* suggests that *L. biglobosa* is a better saprophyte, utilising both V8 agar and PDA more efficiently than *L. maculans*. A large-scale multi-site field experiment across the entire UK focusing on *L. biglobosa* would contribute to a better understanding of where and when this

pathogen causes phoma stem canker and whether its symptoms result in decreased oilseed rape yield.

## 6.4 Improved understanding of co-existing fungal plant pathogens and their reaction to fungicide treatment

The molecular characterisation of lanosteol 14α-demethylase (CYP51B) in *L. maculans* and *L. biglobosa* will provide a better understanding of coexisting plant pathogenic fungi and the strategies used to control them. These data have shown that two coexisting species, which appear to be closely related, have a similar response to azole fungicide application, possibly due to the homologous structure of CYP51B.

The emergence of fungal plant pathogens is a continual threat to global food security (Fisher et al. 2012). Species complexes, such as the Sigatoka complex consisting of *Mycosphaerella fijiensis*, *M. musae* and *M. eumusae,* have the potential to cause worldwide suffering through loss of staple crop yields (Churchill 2011; Marín et al. 2003). Genetic diversity of banana is lacking due to the propagation methods used; almost a single cultivar (Cavendish) is used across the globe for banana production (Heslop-Harrison and Schwarzacher 2007). A lack of genetic diversity can lead to increased vulnerability to environmental fluctuations and pathogens. To counter susceptibility to pathogens, banana crops are subjected to weekly fungicide treatments, controlling diseases caused by *Mycosphaerella* spp. and *Fusarium* spp. (Friesen 2016).

Using *L. maculans* and *L. biglobosa* as an example, it is reasonable to suggest that wild-type CYP51B proteins from other species complexes are likely to be structurally conserved and therefore the species are likely to interact with azoles similarly, unless evolved resistance has occurred.

Due to the synthetic origin of azole fungicides and the proposal that no known natural analogs exist (Hawkins et al. 2014), it is unlikely that an large intrinsic difference in azole sensitivity would exist between two or more species sharing a similar environmental niche. This is because any phenotypic differences in fungicide sensitivity between sibling pathogens would have had to evolve after the first application of fungicides targeting CYP51B and therefore cannot be considered intrinsic. Intrinsic sensitivity is generally ancient and has developed through long-term contact with naturally forming antimicrobial chemicals (D'Costa et al. 2011; Davies and Davies 2010; Perron et al. 2015).

A more ominous feature of species complexes is that they share a host and cause similar disease symptoms; sometimes they coexist and therefore share similar human control strategies; and yet ultimately they have different evolutionary pathways. With this considered, emphasis on whether species complexes differ in their potential evolution of resistance would be more valuable than any further investigation into what is a very minor difference in fungicide-sensitivity. For example, a difference in the rate of resistance evolution would have a far greater effect on fungicide-mediated selection. Through the generation of mutant laboratory strains, developed by UV-mutagenesis, it may be possible to investigate the evolutionary potential for resistance of species complexes that are controlled using agricultural fungicides.

## 6.5 Conclusions

To summarise, fungicides are still an effective method for reducing phoma stem canker symptoms caused by UK *L. maculans* and *L. biglobosa* populations and, although reliance on azole fungicides has increased, resistance has not materialised in populations of *Leptosphaeria* spp. Nonetheless, diversification of fungicides, by applying novel mixtures such as penthiopyrad plus picoxystrobin, should increase the longevity of other active ingredients used for the protection of oilseed rape crops. These steps will help to safeguard the future of sustainable oilseed rape production in the UK.

## References


Agrios, G. (2005). *Plant Pathology. 5th ed*. Generic, New York, USA: Academic Press.

AHDB Cereals & Oilseeds. (2015). Oilseed rape guide. *Agriculture and Horticulture Development Board*. Warwickshire

Akagi, A., Dandekar, A. M., & Stotz, H. U. (2011). Resistance of *Malus domestica* fruit to *Botrytis cinerea* depends on endogenous ethylene biosynthesis. *Phytopathology*, *101*, 1311–1321.

Anke, T., Oberwinkler, F., Steglich, W., & Schramm, G. (1977). The strobilurins - New antifungal antibiotics from the basidiomycete *Strobilurus tenacellus*. *The Journal of Antibiotics*, *30*, 806–810.

Arzanlou, M., Groenewald, J. Z., Fullerton, R. A., Abeln, E. C. A., Carlier, J., Zapater, M.-F., et al. (2008). Multiple gene genealogies and phenotypic characters differentiate several novel species of *Mycosphaerella* and related anamorphs on banana. *Persoonia: Molecular Phylogeny and Evolution of Fungi*, *20*, 19–37.

Attard, A., Gout, L., Ross, S., Parlange, F., Cattolico, L., Balesdent, M.-H., & Rouxel, T. (2005). Truncated and RIP-degenerated copies of the LTR retrotransposon Pholy are clustered in a pericentromeric region of the *Leptosphaeria maculans* genome. *Fungal Genetics and Biology*, *42*, 30–41.

Aubertot, J. N., West, J. S., Bousset-Vaslin, L., Salam, M. U., Barbetti, M. J., & Diggle, A. J. (2006). Improved resistance management for durable disease control: a case study of phoma stem canker of oilseed rape (*Brassica napus*). *European Journal of Plant Pathology*, *114*, 91–106.

Avenot, H. F., & Michailides, T. (2009). Monitoring the sensitivity to boscalid of *Alternaria alternata* populations from California pistachio orchards. *Annual Meeting of the American Phytopathology Society, 99, 6.*

Avenot, H. F., & Michailides, T. J. (2010). Progress in understanding molecular mechanisms and evolution of resistance to succinate dehydrogenase inhibiting (SDHI) fungicides in phytopathogenic fungi. *Crop Protection*, *29*, 643–651.

Barbetti, M. J. (1975). Late blackleg infections in rape are important. *Australian Plant Pathology Society Newsletter*, *4*, 3–4.

Bartlett, D. W., Clough, J. M., Godwin, J. R., Hall, A. A., Hamer, M., & Parr-Dobrzanski, B. (2002). The strobilurin fungicides. *Pest Management Science*, *58*, 649–662.

Bateman, G. L., Landau, S., & Welham, S. J. (1995). Sensitivity to prochloraz in populations of the eyespot fungus, *Pseudocercosporella herpotrichoides*, in

relation to fungicide treatments and their efficacy in continuous winter wheat. *Annals of Applied Biology*, *126*, 235–247.

Bengtsson, S. B. K., Vasaitis, R., Kirisits, T., Solheim, H., & Stenlid, J. (2012). Population structure of *Hymenoscyphus pseudoalbidus* and its genetic relationship to *Hymenoscyphus albidus*. *Fungal Ecology*, *5*, 147–153.

Bierman, S. M., Fitt, B. D. L., Van den Bosch, F., Bateman, G. L., Jenkyn, J. F., & Welham, S. J. (2002). Changes in populations of the eyespot fungi *Tapesia yallundae* and *T. acuformis* under different fungicide regimes in successive crops of winter wheat, 1984–2000. *Plant Pathology*, *51*, 191–201.

Bowen, J. K., Mesarich, C. H., Bus, V. G. M., Beresford, R. M., Plummer, K. M., & Templeton, M. D. (2011). *Venturia inaequalis*: the causal agent of apple scab. *Molecular Plant Pathology*, *12*, 105–122.

Brachaczek, A., Kaczmarek, J., & Jedryczka, M. (2010). Economic value of oilseed rape cultivars under production conditions with full fungicide protection technology using the SPEC forecasting system. *Rośliny Oleiste*, *31*, 67–83.

Brent, K. J., & Hollomon, D. W. (2007). *Fungicide resistance: the assessment of risk*. FRAC Monograph No.1. 2nd (revised) edn. Fungicide Resistance Action Committee, Brussels, Belgium, 1-52.

Brun, H., Chèvre, A., Fitt, B. D. L., Powers, S., Besnard, A., Ermel, M., et al. (2010). Quantitative resistance increases the durability of qualitative resistance to *Leptosphaeria maculans* in *Brassica napus*. *New Phytologist*, *185*, 285–299.

Cañas-Gutiérrez, G. P., Angarita-Velásquez, M. J., Restrepo-Flórez, J. M., Rodríguez, P., Moreno, C. X., & Arango, R. (2009). Analysis of the CYP51 gene and encoded protein in propiconazole-resistant isolates of *Mycosphaerella fijiensis*. *Pest Management Science*, *65*, 892–899.

Carlier, J., Lebrun, M. H., Zapater, M. F., Dubois, C., & Mourichon, X. (1996). Genetic structure of the global population of banana black leaf streak fungus, *Mycosphaerella fijiensis*. *Molecular Ecology*, *5*, 499–510.

Carlier, J., Zapater, M.-F., Lapeyre, F., Jones, D. R., & Mourichon, X. (2000). Septoria leaf spot of banana: a newly discovered disease caused by *Mycosphaerella eumusae* (anamorph *Septoria eumusae*). *Phytopathology*, *90*, 884–890.

Carter, H. E., Fraaije, B. A., West, J. S., Kelly, S. L., Mehl, A., Shaw, M. W., & Cools, H. J. (2014). Alterations in the predicted regulatory and coding regions of the sterol 14α-demethylase gene (CYP51) confer decreased azole sensitivity in the oilseed rape pathogen *Pyrenopeziza brassicae*. *Molecular Plant Pathology*, 15, 513-522.

Chen, X. M. (2005). Epidemiology and control of stripe rust *Puccinia striiformis* f. sp. *tritici* on wheat. *Canadian Journal of Plant Pathology*, *27*, 314–337.

Chen, X., Penman, L., Wan, A., & Cheng, P. (2010). Virulent races of *Puccinia striiformis* f. sp. *tritici* in 2006 and 2007 and development of wheat stripe rust and distributions, dynamics, and evolutionary relationships of races from 2000 to 2007 in the United States. *Canadian Journal of Plant Pathology*, *32*, 315–333.

Chisholm, S. T., Coaker, G., Day, B., & Staskawicz, B. J. (2006). Host-microbe interactions: shaping the evolution of the plant immune response. *Cell*, *124*, 803–814.

Churchill, A. C. L. (2011). *Mycosphaerella fijiensis*, the black leaf streak pathogen of banana: progress towards understanding pathogen biology and detection, disease development, and the challenges of control. *Molecular Plant Pathology*, *12*, 307–328.

Cools, H. J., & Fraaije, B. A. (2008). Are azole fungicides losing ground against Septoria wheat disease? Resistance mechanisms in *Mycosphaerella graminicola*. *Pest Management Science*, *64*, 681–684.

Cools, H. J., & Fraaije, B. A. (2013). Update on mechanisms of azole resistance in *Mycosphaerella graminicola* and implications for future control. *Pest Management Science*, *69*, 150–155.

Cools, H. J., Hawkins, N. J., & Fraaije, B. A. (2013). Constraints on the evolution of azole resistance in plant pathogenic fungi. *Plant Pathology*, *62*, 36–42.

Cools, H. J., Mullins, J. G. L., Fraaije, B. A., Parker, J. E., Kelly, D. E., Lucas, J. A., & Kelly, S. L. (2011). Impact of recently emerged sterol 14α-demethylase (CYP51) variants of Mycosphaerella graminicola on azole fungicide sensitivity. *Applied and Environmental Microbiology*, *77*, 3830–3837.

Cools, H. J., Parker, J. E., Kelly, D. E., Lucas, J. A., Fraaije, B. A., & Kelly, S. L. (2010). Heterologous expression of mutated eburicol 14α-demethylase (CYP51) proteins of *Mycosphaerella graminicola* to assess effects on azole fungicide sensitivity and intrinsic protein function. *Applied and Environmental Microbiology*, *76*, 2866–2872.

Couch, B. C., Fudal, I., Lebrun, M.-H., Tharreau, D., Valent, B., van Kim, P., et al. (2005). Origins of host-specific populations of the blast pathogen *Magnaporthe oryzae* in crop domestication with subsequent expansion of pandemic clones on rice and weeds of rice. *Genetics*, *170*, 613–630.

Coyne, J. A., & Orr, H. A. (2004). *Speciation*, Sinauer Associates, Sunderland, MA, USA.

D'Costa, V. M., King, C. E., Kalan, L., Morar, M., Sung, W. W. L., Schwarz, C., et al.

(2011). Antibiotic resistance is ancient. *Nature*, *477*, 457–461.

Daboussi, M.-J., & Capy, P. (2003). Transposable elements in filamentous fungi. *Annual Reviews in Microbiology*, *57*, 275–299.

Daboussi, M.-J., Langin, T., & Brygoo, Y. (1992). Fot1, a new family of fungal transposable elements. *Molecular and General Genetics*, *232*, 12–16.

Davies, J., & Davies, D. (2010). Origins and evolution of antibiotic resistance. *Microbiology and Molecular Biology Reviews*, *74*, 417–433.

De Backer, M. D., Raponi, M., & Arndt, G. M. (2002). RNA-mediated gene silencing in non-pathogenic and pathogenic fungi. *Current Opinion in Microbiology*, *5*, 323–329.

De Gruyter, J., Woudenberg, J. H. C., Aveskamp, M. M., Verkley, G. J. M., Groenewald, J. Z., & Crous, P. W. (2013). Redisposition of Phoma-like anamorphs in Pleosporales. *Studies in Mycology*, *75*, 1-36.

Dean, R., Van Kan, J. A. L., Pretorius, Z. A., Hammond-Kosack, K. E., Di Pietro, A., Spanu, P. D., et al. (2012). The top 10 fungal pathogens in molecular plant pathology. *Molecular Plant Pathology*, *13*, 414–430.

Delourme, R., Chevre, A. M., Brun, H., Rouxel, T., Balesdent, M. H., Dias, J. S., et al. (2006). Major gene and polygenic resistance to *Leptosphaeria maculans* in oilseed rape (*Brassica napus*). *European Journal of Plant Pathology*, *114*, 41–52.

Derbyshire, M. C., & Denton-Giles, M. (2016). The control of sclerotinia stem rot on oilseed rape (*Brassica napus*): current practices and future opportunities. *Plant Pathology*, *65*, 859–877.

Dethlefsen, L., McFall-Ngai, M., & Relman, D. A. (2007). An ecological and evolutionary perspective on human–microbe mutualism and disease. *Nature*, *449*, 811–818.

Dilmaghani, A., Balesdent, M. H., Didier, J. P., Wu, C., Davey, J., Barbetti, M. J., et al. (2009). The *Leptosphaeria maculans–Leptosphaeria biglobosa* species complex in the American continent. *Plant Pathology*, *58*, 1044–1058

Dilmaghani, A., Gladieux, P., Gout, L., Giraud, T., Brunner, P. C., Stachowiak, A., et al. (2012). Migration patterns and changes in population biology associated with the worldwide spread of the oilseed rape pathogen *Leptosphaeria maculans*. *Molecular Ecology*, *21*, 2519–2533.

Dobzhansky, T. (1973). Nothing in biology makes sense except in the light of evolution. *The American Biology Teacher, 75,* 87-91.

Dodd, D. M. B. (1989). Reproductive isolation as a consequence of adaptive divergence in *Drosophila pseudoobscura*. *Evolution*, 1308–1311.

Dooley, H., Shaw, M. W., Mehenni-Ciz, J., Spink, J., & Kildea, S. (2016). Detection of *Zymoseptoria tritici* SDHI-insensitive field isolates carrying the SdhC-H152R and SdhD-R47W substitutions. *Pest Management Science*. doi: 10.1002/ps.4269.

Eckert, M. R. (2005). *Investigating populations of* Leptosphaeria maculans *and* L. biglobosa *on winter oilseed rape in England* (PhD thesis). Nottingham University, Nottingham.

Eckert, M. R., Rossall, S., Selley, A., & Fitt, B. D. L. (2010). Effects of fungicides on *in vitro* spore germination and mycelial growth of the phytopathogens *Leptosphaeria maculans* and *L. biglobosa* (phoma stem canker of oilseed rape). *Pest Management Science*, *66*, 396–405.

European Commission. (2007). Review report for the active substance flusilazole. *EC Review Reports 6850/VI/97 final*. Brussels.

Evans, N., Baierl, A., Semenov, M. A., Gladders, P., & Fitt, B. D. L. (2008). Range and severity of a plant disease increased by global warming. *Journal of the Royal Society Interface*, *5*, 525–531.

Faugeron, G. (2000). Diversity of homology-dependent gene silencing strategies in fungi. *Current Opinion in Microbiology*, *3*, 144–148.

Fisher, M. C., Henk, D. A., Briggs, C. J., Brownstein, J. S., Madoff, L. C., McCraw, S. L., & Gurr, S. J. (2012). Emerging fungal threats to animal, plant and ecosystem health. *Nature*, *484*, 186–194.

Fisher, N., & Meunier, B. (2008). Molecular basis of resistance to cytochrome bc1 inhibitors. *FEMS Yeast Research*, *8*, 183–192.

Fitt, B. D. L., Brun, H., Barbetti, M. J., & Rimmer, S. R. (2006a). World-wide importance of phoma stem canker (*Leptosphaeria maculans* and *L. biglobosa*) on oilseed rape (*Brassica napus*). *European Journal of Plant Pathology, 114*, 3–15.

Fitt, B. D. L., Huang, Y.-J., Van Den Bosch, F., & West, J. S. (2006b). Coexistence of related pathogen species on arable crops in space and time. *Annual Review of Phytopathology, 44*, 163–82.

Flor, H. H. (1956). The complementary genic systems in flax and flax rust. *Advances in Genetics*, *8*, 29–54.

Flor, H. H. (1971). Current status of the gene-for-gene concept. *Annual Review of Phytopathology*, *9*, 275–296.

Flowers, S. A., Colón, B., Whaley, S. G., Schuler, M. A., & Rogers, P. D. (2015). Contribution of clinically derived mutations in *erg11* to azole resistance in Candida albicans. *Antimicrobial Agents and Chemotherapy*, *59*, 450–460.

Fones, H., & Gurr, S. (2015). The impact of *Septoria tritici* blotch disease on wheat:

an EU perspective. *Fungal Genetics and Biology*, *79*, 3–7.

Fraaije, B. A., Bayon, C., Atkins, S., Cools, H. J., Lucas, J. A., & Fraaije, M. W. (2012). Risk assessment studies on succinate dehydrogenase inhibitors, the new weapons in the battle to control Septoria leaf blotch in wheat. *Molecular Plant Pathology*, *13*, 263–275.

Fraaije, B. A., Cools, H. J., KIM, S., Motteram, J., Clark, W. S., & Lucas, J. A. (2007). A novel substitution I381V in the sterol 14α-demethylase (CYP51) of *Mycosphaerella graminicola* is differentially selected by azole fungicides. *Molecular Plant Pathology*, *8*, 245–254.

Friesen, T. L. (2016). Combating the Sigatoka disease complex on banana. *PLoS Genetics*, *12*, e1006234.

Friesen, T. L., Stukenbrock, E. H., Liu, Z., Meinhardt, S., Ling, H., Faris, J. D., et al. (2006). Emergence of a new disease as a result of interspecific virulence gene transfer. *Nature Genetics*, 38, 953–956.

Garthwaite, D. G., Hudson, S., Barker, I., Parrish, G., Smith, L., & Pietravalle, S. (2014). Pesticide Usage Survery Report. *Arable Crops in the United Kingdom 2014*, Fera, 263, 1-87. York.

Georgopoulos, S. G., & Ziogas, B. N. (1977). A new class of carboxin-resistant mutants of *Ustilago maydis*. *Netherlands Journal of Plant Pathology*, *83*, 235–242.

Giraud, T., Gladieux, P., & Gavrilets, S. (2010). Linking the emergence of fungal plant diseases with ecological speciation. *Trends in Ecology & Evolution*, *25*, 387–395.

Giraud, T., Refrégier, G., Le Gac, M., de Vienne, D. M., & Hood, M. E. (2008). Speciation in fungi. *Fungal Genetics and Biology*, *45*, 791–802.

Giraud, T., Villaréal, L. M. M. A., Austerlitz, F., Le Gac, M., & Lavigne, C. (2006). Importance of the life cycle in sympatric host race formation and speciation of pathogens. *Phytopathology*, *96*, 280–287.

Gisi, U., Sierotzki, H., Cook, A., & McCaffery, A. (2002). Mechanisms influencing the evolution of resistance to Qo inhibitor fungicides. *Pest Management Science*, *58*, 859–867.

Gladieux, P., Guérin, F., Giraud, T., Caffier, V., Lemaire, C., Parisi, L., et al. (2011). Emergence of novel fungal pathogens by ecological speciation: importance of the reduced viability of immigrants. *Molecular Ecology*, *20*, 4521–4532.

Godfray, H. C. J., Beddington, J. R., Crute, I. R., Haddad, L., Lawrence, D., Muir, J. F., et al. (2010). Food security: the challenge of feeding 9 billion people. *Science*, *327*, 812–818.

Grandaubert, J., Balesdent, M.-H., & Rouxel, T. (2014a). Evolutionary and adaptive role of transposable elements in fungal genomes. *Fungi*, *70*, 79–107.

Grandaubert, J., Lowe, R. G., Soyer, J. L., Schoch, C. L., Van de Wouw, A. P., Fudal, I., et al. (2014b). Transposable element-assisted evolution and adaptation to host plant within the *Leptosphaeria maculans-Leptosphaeria biglobosa* species complex of fungal pathogens. *BMC Genomics*, *15*, 1-27.

Grasso, V., Palermo, S., Sierotzki, H., Garibaldi, A., & Gisi, U. (2006). Cytochrome b gene structure and consequences for resistance to Qo inhibitor fungicides in plant pathogens. *Pest Management Science*, *62*, 465–472.

Griffiths, K. M., Bacic, A., & Howlett, B. J. (2003). Sterol composition of mycelia of the plant pathogenic ascomycete *Leptosphaeria maculans*. *Phytochemistry*, *62*, 147–153.

Gudelj, I., Fitt, B. D. L., & Van den Bosch, F. (2004). Evolution of sibling fungal plant pathogens in relation to host specialization. *Phytopathology*, *94*, 789–795.

Guérin, F., & Le Cam, B. (2004). Breakdown of the scab resistance gene *Vf* in apple leads to a founder effect in populations of the fungal pathogen *Venturia inaequalis*. *Phytopathology*, *94*, 364–369.

Halmschlager, E., & Kirisits, T. (2008). First report of the ash dieback pathogen *Chalara fraxinea* on *Fraxinus excelsior* in Austria. *Plant Pathology*, *57*, 1177.

Hamamoto, H., Hasegawa, K., Nakaune, R., Lee, Y. J., Makizumi, Y., Akutsu, K., & Hibi, T. (2000). Tandem repeat of a transcriptional enhancer upstream of the sterol 14α-demethylase gene (CYP51) in *Penicillium digitatum*. *Applied and Environmental Microbiology*, *66*, 3421–3426.

Hammond-Kosack, K. E., & Jones, J. D. G. (1997). Plant disease resistance genes. *Annual Review of Plant Biology*, *48*, 575–607.

Hammond, K. E., & Lewis, B. G. (1987). Variation in stem infections caused by aggressive and non-aggressive isolates of *Leptosphaeria maculans* on *Brassica napus* var. *oleifera*. *Plant Pathology*, *36*, 53–65.

Hassell, M. P., & Comins, H. N. (1976). Discrete time models for two-species competition. *Theoretical Population Biology*, *9*, 202–221.

Hawkins, N. J., Cools, H. J., Sierotzki, H., Shaw, M. W., Knogge, W., Kelly, S. L., et al. (2014). Paralog re-emergence: a novel, historically contingent mechanism in the evolution of antimicrobial resistance. *Molecular Biology and Evolution*, *31*, 1793–1802.

Hawksworth, D. L. (1991). The fungal dimension of biodiversity: magnitude, significance, and conservation. *Mycological Research*, *95*, 641–655.

Hawksworth, D. L. (2001). The magnitude of fungal diversity: the 1.5 million species

estimate revisited. *Mycological Research*, *105*, 1422–1432.

Hayashi, K., Schoonbeek, H., & De Waard, M. A. (2002). Expression of the ABC transporter BcatrD from *Botrytis cinerea* reduces sensitivity to sterol demethylation inhibitor fungicides. *Pesticide Biochemistry and Physiology*, *73*, 110–121.

Hayden, H. L., Carlier, J., & Aitken, E. A. B. (2003). Population differentiation in the banana leaf spot pathogen *Mycosphaerella musicola*, examined at a global scale. *Plant Pathology*, *52*, 713–719.

Henderson, M. P. (1918). *The blackleg disease of cabbage caused by Phoma lingam* (Tode) Desmaz (PhD Thesis), University of Wisconsin-Madison, USA. 431pp.

Heslop-Harrison, J. S., & Schwarzacher, T. (2007). Domestication, genomics and the future for banana. *Annals of Botany*, *100*, 1073–1084.

Hobbelen, P. H. F., Paveley, N. D., & Van Den Bosch, F. (2011). Delaying selection for fungicide insensitivity by mixing fungicides at a low and high risk of resistance development: a modeling analysis. *Phytopathology*, *101*, 1224–1233.

Hollomon, D. W., & Kendall, S. J. (1997). Mixtures of DMI fungicides in cereal disease control. *International Journal of Nutraceuticals, Functional Foods and Healthy Ingredients*, *8*, 44–46.

Horsefield, R., Yankovskaya, V., Sexton, G., Whittingham, W., Shiomi, K., Ōmura, S., et al. (2006). Structural and computational analysis of the quinone-binding site of complex II (succinate-ubiquinone oxidoreductase) a mechanism of electron transfer and proton conduction during ubiquinone reduction. *Journal of Biological Chemistry*, *281*, 7309–7316.

Howard, S. J., Cerar, D., Anderson, M. J., Albarrag, A., Fisher, M. C., Pasqualotto, A. C., et al. (2009). Frequency and evolution of azole resistance in *Aspergillus fumigatus* associated with treatment failure. *Emerging Infectious Diseases*, *15*, 1068–1076.

Howlett, B. J., Idnurm, A., & Pedras, M. S. (2001). *Leptosphaeria maculans*, the causal agent of blackleg disease of *Brassicas*. *Fungal Genetics and Biology*, *33*, 1–14.

Huang, X., & Madan, A. (1999). CAP3: A DNA sequence assembly program. *Genome Research*, *9*, 868–877.

Huang, Y.-J., Fitt, B. D. L., & Hall, A. M. (2003). Survival of A-group and B-group *Leptosphaeria maculans* (phoma stem canker) ascospores in air and mycelium on oilseed rape stem debris. *Annals of Applied Biology*, *143*, 359–369.

Huang, Y.-J., Fitt, B. D. L., Jedryczka, M., Dakowska, S., West, J. S., Gladders, P., et al. (2005). Patterns of ascospore release in relation to phoma stem canker

epidemiology in England (*Leptosphaeria maculans*) and Poland (*Leptosphaeria biglobosa*). *European Journal of Plant Pathology*, *111*, 263–277.

Huang, Y.-J., Hood, J. R., Eckert, M. R., Stonard, J. F., Cools, H. J., King, G. J., et al. (2011). Effects of fungicide on growth of *Leptosphaeria maculans* and *L. biglobosa* in relation to development of phoma stem canker on oilseed rape (*Brassica napus*). *Plant Pathology*, *60*, 607–620.

Huang, Y.-J., Karandeni-Dewage CS, & Fitt, B. D. L. (2014a). Importance of *Leptosphaeria biglobosa* as a cause of phoma stem canker on winter oilseed rape in the UK. *Aspects of Applied Biology*, *127*, 117–122.

Huang, Y.-J., Li, Z.-Q., Evans, N., Rouxel, T., Fitt, B. D. L., & Balesdent, M.-H. (2006). Fitness cost associated with loss of the AvrLm4 avirulence function in *Leptosphaeria maculans* (phoma stem canker of oilseed rape). *European Journal of Plant Pathology*, *114*, 77–89.

Huang, Y.-J., Pirie, E. J., Evans, N., Delourme, R., King, G. J., & Fitt, B. D. L. (2009). Quantitative resistance to symptomless growth of *Leptosphaeria maculans* (phoma stem canker) in *Brassica napus* (oilseed rape). *Plant Pathology*, *58*, 314–323.

Huang, Y.-J., Qi, A., King, G. J., & Fitt, B. D. L. (2014b). Assessing quantitative resistance against *Leptosphaeria maculans* (phoma stem canker) in *Brassica napus* (oilseed rape) in young plants. *PloS One*, *9*, e84924.

Huang, Y.-J., Toscano-Underwood, C., Fitt, B. D. L., Todd, A. D., West, J. S., Koopmann, B., & Balesdent, M.-H. (2001). Effects of temperature on germination and hyphal growth from ascospores of A-group and B-group *Leptosphaeria maculans* (phoma stem canker of oilseed rape). *Annals of Applied Biology*, *139*, 193–207.

Joosten, M. H. A. J., Cozijnsen, T. J., & De Wit, P. J. G. M. (1994). Host resistance to a fungal tomato pathogen lost by a single base-pair change in an avirulence gene. *Nature*, *367*, 384–386.

Kang, S., Lebrun, M. H., Farrall, L., & Valent, B. (2001). Gain of virulence caused by insertion of a Pot3 transposon in a *Magnaporthe grisea* avirulence gene. *Molecular Plant-Microbe Interactions*, *14*, 671–674.

Kelly, S. L., Lamb, D. C., Corran, A. J., Baldwin, B. C., & Kelly, D. E. (1995). Mode of action and resistance to azole antifungals associated with the formation of 14α-methylergosta-8, 24 (28)-dien-3β, 6α-diol. *Biochemical and Biophysical Research Communications*, *207*, 910–915.

Kidwell, M. G., & Lisch, D. (1997). Transposable elements as sources of variation in animals and plants. *Proceedings of the National Academy of Sciences*, *94*,

7704–7711.

Kim, Y.-S., Dixon, E. W., Vincelli, P., & Farman, M. L. (2003). Field resistance to strobilurin (QoI) fungicides in *Pyricularia grisea* caused by mutations in the mitochondrial cytochrome b gene. *Phytopathology*, *93*, 891–900.

Kimber, D. S., & McGregor, D. I. (1995). *Brassica oilseeds: production and utilization. CAB International,* Wallingford*,* UK.

Kinross, J. M., Darzi, A. W., & Nicholson, J. K. (2011). Gut microbiome-host interactions in health and disease. *Genome Medicine*, *3*, 1-12.

Kohn, L. M. (2005). Mechanisms of fungal speciation. *Annual Review of Phytopathology*, *43*, 279–308.

Kowalski, T. (2006). *Chalara fraxinea* sp. nov. associated with dieback of ash (*Fraxinus excelsior*) in Poland. *Forest Pathology*, *36*, 264–270.

Kowalski, T., & Holdenrieder, O. (2009). The teleomorph of *Chalara fraxinea*, the causal agent of ash dieback. *Forest Pathology*, *39*, 304–308.

Kumar, S., Stecher, G., & Tamura, K. (2016). MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. *Molecular Biology and Evolution*, msw054.

Lacey, M. E., & West, J. S. (2006). *The air spora: a manual for catching and identifying airborne biological particles*, Springer Netherlands,1-156.

Larkan, N. J., Lydiate, D. J., Parkin, I. A. P., Nelson, M. N., Epp, D. J., Cowling, W. A., et al. (2013). The *Brassica napus* blackleg resistance gene *LepR3* encodes a receptor-like protein triggered by the *Leptosphaeria maculans* effector AVRLM1. *New Phytologist*, *197*, 595–605.

Leach, R. (1946). Banana leaf spot (*Mycosphaerella musicola*) on the Gros Michel variety in Jamaica. *Investigations on the Aetiology of the Disease and the Principles of Control by Spraying*, 1-118.

Leach, R. (1964). A new form of Banana leaf spot in Fiji. Black leaf streak. *World Crops*, *16*, 60–64.

Lepesheva, G. I., & Waterman, M. R. (2007). Sterol 14α-demethylase cytochrome P450 (CYP51), a P450 in all biological kingdoms. *Biochimica et Biophysica Acta (BBA)-General Subjects*, *1770*, 467–477.

Li, H., Sivasithamparam, K., & Barbetti, M. J. (2003). Breakdown of a *Brassica rapa* subsp. *sylvestris* single dominant blackleg resistance gene in *B. napus* rapeseed by *Leptosphaeria maculans* field isolates in Australia. *Plant Disease*, *87*, 752.

Line, R. F. (2002). Stripe rust of wheat and barley in North America: a retrospective historical review. *Annual Review Phytopathology, 40*, 75–118.

Liu, S. Y., Liu, Z., Fitt, B. D. L., Evans, N., Foster, S. J., Huang, Y.-J., et al. (2006). Resistance to *Leptosphaeria maculans* (phoma stem canker) in *Brassica napus* (oilseed rape) induced by *L. biglobosa* and chemical defence activators in field and controlled environments. *Plant Pathology*, *55*, 401–412.

Liu, X., Yu, F., Schnabel, G., Wu, J., Wang, Z., & Ma, Z. (2011). Paralogous CYP51 genes in *Fusarium graminearum* mediate differential sensitivity to sterol demethylation inhibitors. *Fungal Genetics and Biology*, *48*, 113–123.

Lô-Pelzer, E., Aubertot, J. N., Bousset, L., Pinochet, X., & Jeuffroy, M. H. (2009). Phoma stem canker (*Leptosphaeria maculans*/*L. biglobosa*) of oilseed rape (*Brassica napus*): is the G2 Disease Index a good indicator of the distribution of observed canker severities? *European Journal of Plant Pathology*, *125*, 515–522.

Lucas, J. A., Hawkins, N. J., & Fraaije, B. A. (2015). The evolution of fungicide resistance. *Advances in Applied Microbiology*, *90*, 29–92.

Ma, L.-J., Van Der Does, H. C., Borkovich, K. A., Coleman, J. J., Daboussi, M.-J., Di Pietro, A., et al. (2010). Comparative genomics reveals mobile pathogenicity chromosomes in *Fusarium*. *Nature*, *464*, 367–373.

Marichal, P., Gorrens, J., Laurijssens, L., Vermuyten, K., Van Hove, C., Le Jeune, L., et al. (1999). Accumulation of 3-ketosteroids induced by itraconazole in azole-resistant clinical Candida albicans isolates. *Antimicrobial Agents and Chemotherapy*, *43*, 2663–2670.

Marín, D. H., Romero, R. A., Guzmán, M., & Sutton, T. B. (2003). Black Sigatoka: an increasing threat to banana cultivation. *Plant Disease*, *87*, 208–222.

Marx-Stoelting, P., Niemann, L., Ritz, V., Ulbrich, B., Gall, A., Hirsch-Ernst, K. I., et al. (2014). Assessment of three approaches for regulatory decision making on pesticides with endocrine disrupting properties. *Regulatory Toxicology and Pharmacology*, *70*, 590–604.

Mayr, E. (1942). *Systematics and the origin of species, from the viewpoint of a zoologist*, Harvard University Press, USA, 334pp.

Mayr, E. (1963). Animal species and evolution. *Animal species and their evolution.* Harvard University Press, USA, 811pp.

Mayr, E. (1970). *Populations, species, and evolution: an abridgment of animal species and evolution*., Harvard University Press, USA, 453pp.

Mayr, E. (1996). What is a species, and what is not? *Philosophy of Science*, 63, 262–277.

McClintock, B. (1951). Chromosome organization and genic expression. In *Cold Spring Harbor Symposia on Quantitative Biology*. Cold Spring Harbor

Laboratory Press, 16, 13–47.

McDonald, B. A., & Linde, C. (2002). Pathogen population genetics, evolutionary potential, and durable resistance. *Annual Review of Phytopathology*, *40*, 349–379.

McGuffin, L. J., Buenavista, M. T., & Roche, D. B. (2013). The ModFOLD4 server for the quality assessment of 3D protein models. *Nucleic Acids Research*, gkt294.

McKinney, L. V., Thomsen, I. M., Kjær, E. D., Bengtsson, S. B. K., & Nielsen, L. R. (2012). Rapid invasion by an aggressive pathogenic fungus (*Hymenoscyphus pseudoalbidus*) replaces a native decomposer (*Hymenoscyphus albidus*): a case of local cryptic extinction? *Fungal Ecology*, *5*, 663–669.

Mendes-Pereira, E., Balesdent, M.-H., Brun, H., & Rouxel, T. (2003). Molecular phylogeny of the *Leptosphaeria maculans-L. biglobosa* species complex. *Mycological Research*, *107*, 1287–1304.

Meredith, D. S., & Lawrence, J. S. (1969). Black leaf streak disease of bananas (*Mycosphaerella fijiensis*): Symptoms of disease in Hawaii, and notes on the conidial state of the causal fungus. *Transactions of the British Mycological Society*, *52*, 459-476.

Mooney, H. A., & Cleland, E. E. (2001). The evolutionary impact of invasive species. *Proceedings of the National Academy of Sciences*, *98*, 5446–5451.

Mullins, J. G. L., Parker, J. E., Cools, H. J., Togawa, R. C., Lucas, J. A., Fraaije, B. A., et al. (2011). Molecular modelling of the emergence of azole resistance in *Mycosphaerella graminicola*. *PLoS One*, 6, e20973.

Murray, G. M., & Brennan, J. P. (2009). Estimating disease losses to the Australian wheat industry. *Australasian Plant Pathology*, *38*, 558–570.

Muszewska, A., Hoffman-Sommer, M., & Grynberg, M. (2011). LTR retrotransposons in fungi. *PLoS One*, *6*, e29425.

Nakaune, R., Adachi, K., Nawata, O., Tomiyama, M., Akutsu, K., & Hibi, T. (1998). A novel ATP-Binding cassette transporter Involved in multidrug resistance in the phytopathogenic fungus *Penicillium digitatum*. *Applied and Environmental Microbiology*, *64*, 3983–3988.

NCBI, R. C. (2016). Database resources of the National Center for Biotechnology Information. *Nucleic Acids Research*, *44*, 7-19.

Niks, R. E., Parlevliet, J. E., Lindhout, P., & Bai, Y. (2011). *Breeding crops with resistance to diseases and pests*. Wageningen Academic Publishers, The Netherlands, 198pp.

Odds, F. C., Brown, A. J. P., & Gow, N. A. R. (2003). Antifungal agents: mechanisms of action. *Trends in Microbiology*, *11*, 272–279.

Oliver, R. P., & Hewitt, H. G. (2014). *Fungicides in crop protection*. 2nd edition (revised), CABI, Oxfordshire, UK, 200pp.

Oliver, R. P., & Ipcho, S. V. S. (2004). Arabidopsis pathology breathes new life into the necrotrophs-vs.-biotrophs classification of fungal pathogens. *Molecular Plant Pathology*, *5*, 347–352.

Parker, J. E., Warrilow, A. G. S., Cools, H. J., Fraaije, B. A., Lucas, J. A., Rigdova, K., et al. (2013). Prothioconazole and prothioconazole-desthio activities against *Candida albicans* sterol 14-α-demethylase. *Applied and Environmental Microbiology*, *79*, 1639–1645.

Parker, J. E., Warrilow, A. G. S., Cools, H. J., Martel, C. M., Nes, W. D., Fraaije, B. A., et al. (2011). Mechanism of binding of prothioconazole to *Mycosphaerella graminicola* CYP51 differs from that of other azole antifungals. *Applied and Environmental Microbiology*, *77*, 1460–1465.

Parker, J. E., Warrilow, A. G. S., Price, C. L., Mullins, J. G. L., Kelly, D. E., & Kelly, S. L. (2014). Resistance to antifungals that target CYP51. *Journal of Chemical Biology*, *7*, 143–161.

Perron, G. G., Whyte, L., Turnbaugh, P. J., Goordial, J., Hanage, W. P., Dantas, G., & Desai, M. M. (2015). Functional characterization of bacteria isolated from ancient arctic soil exposes diverse resistance mechanisms to modern antibiotics. *PLoS One*, *10*, e0069533.

Price, C. L., Parker, J. E., Warrilow, A. G. S., Kelly, D. E., & Kelly, S. L. (2015). Azole fungicides–understanding resistance mechanisms in agricultural fungal pathogens. *Pest Management Science*, 8, 1054-1058.

Prins, T. W., Tudzynski, P., von Tiedemann, A., Tudzynski, B., Ten Have, A., Hansen, M. E., et al. (2000). Infection strategies of *Botrytis cinerea* and related necrotrophic pathogens. *Fungal Pathology*, Springer Netherlands, 33–64.

R Development Core Team. (2011). R: A Language and Environment for Statistical Computing. *R Foundation for Statistical Computing*. Vienna, Austria.

Revankar, S. G., Fu, J., Rinaldi, M. G., Kelly, S. L., Kelly, D. E., Lamb, D. C., et al. (2004). Cloning and characterization of the lanosterol 14α-demethylase (*erg11*) gene in *Cryptococcus neoformans*. *Biochemical and Biophysical Research Communications*, *324*, 719–728.

Rhodes, P. L. (1964). A new Banana disease in Fiji. *Commonwealth Phytopathological News*, *10*, 38–41.

Ridley, M. (2004). *Evolution*. Blackwell, UK, 784pp

Rosewich, U. L., & Kistler, H. C. (2000). Role of horizontal gene transfer in the evolution of fungi. *Annual Review of Phytopathology*, *38*, 325–363.

Rouxel, T., & Balesdent, M. H. (2005). The stem canker (blackleg) fungus, *Leptosphaeria maculans*, enters the genomic era. *Molecular Plant Pathology*, *6*, 225–241.

Rouxel, T., Grandaubert, J., Hane, J. K., Hoede, C., van de Wouw, A. P., Couloux, A., et al. (2011). Effector diversification within compartments of the *Leptosphaeria maculans* genome affected by Repeat-Induced Point mutations. *Nature Communications*, 202, 1-10.

Rundle, H. D., & Nosil, P. (2005). Ecological speciation. *Ecology Letters*, *8*, 336–352.

Sagatova, A. A., Keniya, M. V, Wilson, R. K., Monk, B. C., & Tyndall, J. D. A. (2015). Structural insights into binding of the antifungal drug fluconazole to *Saccharomyces cerevisiae* lanosterol 14α-demethylase. *Antimicrobial Agents and Chemotherapy*, *59*, 4982–4989.

Sagatova, A. A., Keniya, M. V, Wilson, R. K., Sabherwal, M., Tyndall, J. D. A., & Monk, B. C. (2016). Triazole resistance mediated by mutations of a conserved active site tyrosine in fungal lanosterol 14α-demethylase. *Scientific Reports*, *6*. doi: 10.1038/srep26213.

Šali, A., & Blundell, T. L. (1993). Comparative protein modelling by satisfaction of spatial restraints. *Journal of Molecular Biology*, *234*, 779–815.

Selker, E. U. (1997). Epigenetic phenomena in filamentous fungi: useful paradigms or repeat-induced confusion? *Trends in Genetics*, *13*, 296–301.

Servedio, M. R., Doorn, G., Kopp, M., Frame, A. M., & Nosil, P. (2011). Magic traits in speciation: “magic” but not rare? *Trends in Ecology & Evolution*, *26*, 389–397.

Sewell, T. R., Moloney, S., Ashworth, M., Ritchie, F., Mashanova, A., Huang, Y. J., et al. (2016). Effects of a penthiopyrad and picoxystrobin fungicide mixture on phoma stem canker (*Leptosphaeria* spp.) on UK winter oilseed rape. *European Journal of Plant Pathology*, *145*, 675-685.

Shaw, M. W., Bearchell, S. J., Fitt, B. D. L., & Fraaije, B. A. (2008). Long-term relationships between environment and abundance in wheat of *Phaeosphaeria nodorum* and *Mycosphaerella graminicola*. *New Phytologist*, *177*, 229–238.

Shoemaker, R. A., & Brun, H. (2001). The teleomorph of the weakly aggressive segregate of *Leptosphaeria maculans*. *Canadian Journal of Botany*, *79*, 412–419.

Sierotzki, H., Frey, R., Wullschleger, J., Palermo, S., Karlin, S., Godwin, J., & Gisi, U. (2007). Cytochrome b gene sequence and structure of *Pyrenophora teres* and *P. tritici-repentis* and implications for QoI resistance. *Pest Management Science*, *63*, 225–233.

Sierotzki, H., & Scalliet, G. (2013). A review of current knowledge of resistance

aspects for the next-generation succinate dehydrogenase inhibitor fungicides. *Phytopathology*, *103*, 880–887.

Sievers, F., Wilm, A., Dineen, D., Gibson, T. J., Karplus, K., Li, W., et al. (2011). Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. *Molecular Systems Biology*, *7*, 539-544

Sleper, D. A., & Poehlman, J. M. (2006). *Breeding field crops*, 5th edition. Blackwell, UK, 424pp.

Stammler, G., Brix, H. D., Glättli, A., Semar, M., & Schoefl, U. (2007). Biological properties of the carboxamide boscalid including recent studies on its mode of action. *Proceedings 16th International Plant Protection Congress, Glasgow*, 40–45.

Stammler, G., & Speakman, J. (2006). Microtiter method to test the sensitivity of *Botrytis cinerea* to boscalid. *Journal of Phytopathology*, *154*, 508–510.

Steed, J. M., Baierl, A., & Fitt, B. D. L. (2007). Relating plant and pathogen development to optimise fungicide control of phoma stem canker (*Leptosphaeria maculans*) on winter oilseed rape (*Brassica napus*). *European Journal of Plant Pathology*, *118*, 359–373.

Steyskal, G. C. (1972). The meaning of the term “sibling species.” *Systematic Biology*, *21*, 446.

Stonard, J. F., Latunde-Dada, A. O., Huang, Y.-J., West, J. S., Evans, N., & Fitt, B. D. L. (2010). Geographic variation in severity of phoma stem canker and *Leptosphaeria maculans/L. biglobosa* populations on UK winter oilseed rape (*Brassica napus*). *European Journal of Plant Pathology*, *126*, 97–109.

Stotz, H. U., Mitrousia, G. K., de Wit, P. J. G. M., & Fitt, B. D. L. (2014). Effector-triggered defence against apoplastic fungal pathogens. *Trends in Plant Science*, 19, 491-500.

Stukenbrock, E. H., & McDonald, B. A. (2008). The origins of plant pathogens in agro-ecosystems. *Annual Review of Phytopathology*, *46*, 75–100.

Sylvester-Bradley, R., & Makepeace, R. J. (1984). Code for stages of development in oilseed rape (*Brassica napus* L.). *Aspects of Applied Biology*.

Sylvester-Bradley, R. (1985). Revision of a code for stages of development in oilseed rape (*Brassica napus* L.). *Aspects of Applied Biology*.

Tamura, K., & Nei, M. (1993). Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Molecular Biology and Evolution*, *10*, 512–526.

Taylor, J. W., Jacobson, D. J., & Fisher, M. C. (1999). The evolution of asexual fungi: reproduction, speciation and classification. *Annual Review of Phytopathology*,

*37*, 197–246.

Timmermann, V., Børja, I., Hietala, A. M., Kirisits, T., & Solheim, H. (2011). Ash dieback: pathogen spread and diurnal patterns of ascospore dispersal, with special emphasis on Norway. *EPPO Bulletin*, *41*, 14–20.

Toscano-Underwood, C., Huang, Y.-J., Fitt, B. D. L., Hall, A. M., Toscano-Underwood, C., Huang, Y.-J., et al. (2003). Effects of temperature on maturation of pseudothecia of *Leptosphaeria maculans* and *L. biglobosa* on oilseed rape stem debris. *Plant Pathology*, *52*, 726–736.

Toscano-Underwood, C., West, J. S., Fitt, B. D. L., Todd, A. D., & Jedryczka, M. (2001). Development of phoma lesions on oilseed rape leaves inoculated with ascospores of A-group or B-group *Leptosphaeria maculans* (stem canker) at different temperatures and wetness durations. *Plant Pathology*, *50*, 28–41.

Untergasser, A., Cutcutache, I., Koressaar, T., Ye, J., Faircloth, B. C., Remm, M., & Rozen, S. G. (2012). Primer3—new capabilities and interfaces. *Nucleic Acids Research*, *40*, 115–115.

Van den Bosch, F., Oliver, R., Van den Berg, F., & Paveley, N. (2014). Governing principles can guide fungicide-resistance management tactics. *Annual Review of Phytopathology*, *52*, 175–195.

Vincenot, L., Balesdent, M. H., Li, H., Barbetti, M. J., Sivasithamparam, K., Gout, L., & Rouxel, T. (2008). Occurrence of a new subclade of *Leptosphaeria biglobosa* in Western Australia. *Phytopathology*, *98*, 321–329.

Wallace, A. C., Laskowski, R. A., & Thornton, J. M. (1995). LIGPLOT: a program to generate schematic diagrams of protein-ligand interactions. *Protein Engineering*, *8*, 127–134.

West, J. S., Balesdent, M., Rouxel, T., Narcy, J. P., Huang, Y.-J., Roux, J., et al. (2002). Colonization of winter oilseed rape tissues by A/Tox$^{+}$ and B/Tox$^{0}$ *Leptosphaeria maculans* (phoma stem canker) in France and England. *Plant Pathology*, *51*, 311–321.

West, J. S., Biddulph, J. E., Fitt, B. D. L., & Gladders, P. (1999). Epidemiology of *Leptosphaeria maculans* in relation to forecasting stem canker severity on winter oilseed rape in the UK. *Annals of Applied Biology*, *135*, 535–546.

West, J. S., Fitt, B. D. L., Leech, P. K., Biddulph, J. E., Huang, Y., & Balesdent, M. (2002). Effects of timing of *Leptosphaeria maculans* ascospore release and fungicide regime on phoma leaf spot and phoma stem canker development on winter oilseed rape (*Brassica napus*) in southern England. *Plant Pathology*, *51*, 454–463.

West, J. S., Kharbanda, P. D., Barbetti, M. J., & Fitt, B. D. L. (2001). Epidemiology

and management of *Leptosphaeria maculans* (phoma stem canker) on oilseed rape in Australia, Canada and Europe. *Plant Pathology*, *50*, 10–27.

Williamson, B., Tudzynski, B., Tudzynski, P., & van Kan, J. A. L. (2007). *Botrytis cinerea*: the cause of grey mould disease. *Molecular Plant Pathology*, *8*, 561–580.

Wood, P. M., & Hollomon, D. W. (2003). A critical evaluation of the role of alternative oxidase in the performance of strobilurin and related fungicides acting at the Qo site of complex III. *Pest Management Science*, *59*, 499–511.

Wyand, R. A., & Brown, J. K. M. (2005). Sequence variation in the CYP51 gene of *Blumeria graminis* associated with resistance to sterol demethylase inhibiting fungicides. *Fungal Genetics and Biology*, *42*, 726–735.

Yan, L., Yang, Q., Zhou, Y., Duan, X., & Ma, Z. (2009). A real-time PCR assay for quantification of the Y136F allele in the CYP51 gene associated with *Blumeria graminis* f. sp. *tritici* resistance to sterol demethylase inhibitors. *Crop Protection*, *28*, 376–380.

Yang, Z., Lasker, K., Schneidman-Duhovny, D., Webb, B., Huang, C. C., Pettersen, E. F., et al. (2012). UCSF Chimera, MODELLER, and IMP: an integrated modeling system. *Journal of Structural Biology*, *179*, 269–278.

Young, N. D. (1996). QTL mapping and quantitative disease resistance in plants. *Annual Review of Phytopathology*, *34*, 479–501.

Zandjanakou-Tachin, M., Vroh-Bi, I., Ojiambo, P. S., Tenkouano, A., Gumedzoe, Y. M., & Bandyopadhyay, R. (2009). Identification and genetic diversity of Mycosphaerella species on banana and plantain in Nigeria. *Plant Pathology*, *58*, 536–546.

Zeigler, R. S., Leong, S. A., & Teng, P. S. (1994). *Rice blast disease*, International. Rice Research Institute, CAB International, UK. 626pp.

Zhang, X., White, R. P., Demir, E., Jedryczka, M., Lange, R. M., Islam, M., et al. (2014). *Leptosphaeria* spp., phoma stem canker and potential spread of *L. maculans* on oilseed rape crops in China. *Plant Pathology*, *63*, 598–612.

Zhang, Y., Crous, P. W., Schoch, C. L., & Hyde, K. D. (2012). Pleosporales. *Fungal Diversity*, *53*, 1–221.

Zhang, Y., Schoch, C. L., Fournier, J., Crous, P. W., de Gruyter, J., Woudenberg, J. H. C., et al. (2009). Multi-locus phylogeny of Pleosporales: a taxonomic, ecological and evolutionary re-evaluation. *Studies in Mycology*, *64*, 85-102

Zhang, Y., & Skolnick, J. (2005). TM-align: a protein structure alignment algorithm based on the TM-score. *Nucleic Acids Research*, *33*, 2302–2309.

Zhou, Y., Fitt, B. D. L., Welham, S. J., Gladders, P., Sansford, C. E., & West, J. S.

(1999). Effects of severity and timing of stem canker (*Leptosphaeria maculans*) symptoms on yield of winter oilseed rape (*Brassica napus*) in the UK. *European Journal of Plant Pathology*, *105*, 715–728.

# Appendices

## A. Growth media and antibiotic preparation

All *in vitro* growth media were autoclaved (121 $^{O}$C, 15 min, 1.52 kg/cm$^2$) and antibiotic added when cooled to approximately 55$^{O}$C. Media was poured under a laminar flow hood into either 9cm Petri dishes or 12cm square plates.

### A.1 Growth media

Potato Dextrose Agar (PDA) – 400 ml
15.6g – potato dextrose agar
400 ml – Distilled water

V8 Agar (V8) – 400 ml
80 ml – V8 vegetable juice
0.8 g – $CaCO_3$
8g – Agar
320 ml – Distilled water

LB media – 500 ml
5 g NaCl
5 g Tryptone
2.5 g Yeast Extract
8 g Agar (omitted if broth)
500 ml distilled water

Sabouraud dextrose broth – 500 ml
5 g Mycological peptone
10 g Dextrose
(double weight for 2x concentration)

Yeast peptone dextrose media – 500 ml
5 g – Yeast extract
10 g – Peptone
10 g – Dextrose

8 g – Agar
500 ml distilled water

Synthetic dropout media (SD + GAL + RAF) – 500 ml
1g - Yeast nitrogen base w/o amino acids
0.96g - Yeast synthetic drop-out medium supplement without uracil
400 ml – Distilled water
8g – Agar (omitted for broth)

Following autoclave process

50 ml – Raffinose (20 mg/ml filter sterilised)
50 ml – Galactose (20 mg/ml filter sterilised)
100 µl – Doxycycline (30 mg/ml filter sterilised)

### A.2 Antibiotic supplements

- Streptomycin sulphate: stock was prepared at a concentration of 50 mg/ml using sterile distilled water.
- Penicillin G sodium: stock was prepared at a concentration of 50 mg/ml using sterile distilled water.
- Tetracycline: stock was prepared at a concentration of 10 mg/ml using 50% ethanol 50% distilled water.

### A.3 Compost and soil

Compost mixture consisted of 50% Miracle-Gro all purpose compost and 50 % John Innes number three compost.

## B. Consumables

| B.1. Antibiotics | Supplier | Cat. No. |
|---|---|---|
| Penicillin G sodium | Sigma | P3032 |
| Streptomycin sulphate | Sigma | S9137 |
| Tetracycline hydrochloride | Sigma | 87128 |

| | | |
|---|---|---|
| Doxycycline hyclate | Sigma | D9891 |
| | | |
| **B.2. Fungicides (technical)** | | |
| Penthiopyrad | DuPont | DPX-LEM17-126 |
| Picoxystrobin | DuPont | MAR14CE65B |
| Prothioconazole | Sigma | 34232 |
| Prothioconazole-desthio | Sigma | 32429 |
| Tebuconazole | Sigma | 32013 |
| Flusilazole | Sigma | 45753 |
| | | |
| **B.3. Fungicides (commercial)** | | |
| Refinzar | DuPont | MAPP 16590 |
| Proline 275 | Bayer Crop Science | 79910681 |
| | | |
| **B.3. Chemicals** | | |
| Glycerol | Fisher | GP450/08 |
| 2-β-mercaptoethanol | Sigma | M6250 |
| Hexane | Sigma | 296090 |
| Water, PCR-grade | Sigma | W1754 |
| Trypan Blue | Sigma | T6146 |
| Tris HCl | Fisher | BP1756 |
| Ethanol, absolute | Fisher | BP28184 |

| | | |
|---|---|---|
| Phenol:Chloroform:Isoamyl Alcohol 25:24:1 | Sigma | P3803 |
| $CaCO_3$ | Sigma | C6763 |
| NaCl | Sigma | 31434 |
| Isoamyl alcohol | Fisher | A393-4 |
| Raffinose | Sigma | R0250 |
| Galactose | Sigma | G0750 |
| Acetone | Fisher | A18-4 |
| Industrial methylated spirits | Fisher | 11492874 |
| | | |
| **B.3. Commercial kits** | | |
| DNAMITE plant DNA extraction kit | Microzone | 2PLK1-100 |
| RNAeasy plant mini kit | Qiagen | 74904 |
| QIAquick PCR purification kit | Qiagen | 28104 |
| QIAprep Spin Miniprep Kit | Qiagen | 27104 |
| pGEM-T-easy cloning kit | Promega | A1380 |
| S.c. EasyComp yeast transformation | Invitrogen | K5050-01 |
| QIAquick Gel Extraction Kit | Qiagen | 28704 |
| Monarch® DNA Gel Extraction Kit | New England BioLabs | T1020G |
| Superscript IV 1ST Strand System | Thermo Sci | 18091050 |

| | | |
|---|---|---|
| **B.4. Restriction enzymes** | | |
| NoI-HF® | New England Biolabs | R3189S |
| HindIII-HF® | New England Biolabs | R3104S |
| | | |
| **B.5. Plasmids** | | |
| pGEM-T | Promega | Included in A1380 |
| pYES/CT | Invitrogen | V825120 |
| | | |
| **B.6. Gel electrophoresis** | | |
| Agarose, molecular biology grade | Sigma | A9539 |
| GelRed Nucleic acid stain | Biotium | 41003 |
| 1 KB DNA ladder | Sigma | D0428 |
| Direct Load 100 bp DNA ladder | Sigma | D3687 |
| Gel Pilot loading dye 5x | Qiagen | 239901 |
| | | |
| **B.7. TBE buffer 10x** | | |
| EDTA | Fisher | 10522965 |
| Tris borate | Alfa Aesar | 11332446 |
| | | |
| **B.8. Taq Polymerases** | | |
| REDTaq® ReadyMix™ | Sigma | R2523 |

| | | |
|---|---|---|
| PCR Reaction Mix | | |
| Brilliant III Ultra-Fast SYBR® Green Low ROX QPCR Master Mix | Agilent | 600892 |
| Easy-A High-Fidelity PCR Cloning Enzyme | Agilent | 600400 |
| | | |
| **B.9. Media** | | |
| Potato dextrose agar | Oxoid | CM0139 |
| V8 vegetable juice | Arnotts Biscuits | 1675650 |
| Agar Technical (Agar No. 3) | Oxoid | LP0013 |
| Yeast extract | Oxoid | LP0021 |
| Tryptone | Oxoid | LP0042 |
| Sabouraud dextrose broth | Sigma | 3306 |
| Yeast nitrogen base w/o amino acids | Sigma | Y0626 |
| Yeast synthetic drop-out medium supplement without uracil | Sigma | Y1501 |
| | | |
| **B.10. Miscellaneous Consumables** | | |
| Petri dish, 9 cm | Fisher | FB51506 |
| Petri dish, 120 mm, square | VWR | 391-0444 |
| Spreader, plastic | ISG | 153-532 |

| | | |
|---|---|---|
| Tube, free standing screw-top, 2 mL | Star Lab | E1420-2340 |
| Screw-top caps, standard | Star Lab | E1480-0100 |
| Parafilm | Bemis | PM992 |
| Miracloth | Millipore | 475855 |
| Reaction tube tube, attached cap, 1.5 mL | Greiner Bio-One Ltd. | 616-201 |
| Reaction tube tube, attached cap, 2 mL | Greiner Bio-One Ltd. | 623-201 |
| Glass beads, acid washed | Sigma | G-8772 |
| Filter Paper, no. 1 | Whatman | 1001 090 |
| Double sided, pressure sensitive, adhesive tape | 3M | 1322-18 |
| PCR plate, thermo-fast, 96 well | Sarstedt | 2018-11 |
| PCR plate ultra clear cap strips | Sarstedt | 2019-01 |
| PCR tube, attached flat cap, 0.2 mL | Star Lab | 11402-8100 |
| 0.2 ml 8-strip PCR tube, attached flat caps | Star Lab | A1402-3700 |
| Nunc™ MicroWell™ 96-Well Microplates | Thermo Scientific | 167008 |
| Tube, 15 mL, centrifuge tube | Greiner Bio-One Ltd. | 188271 |
| Tube, 50 mL, skirted centrifuge tube | Greiner Bio-One Ltd. | 210261 |
| 10 µl white pipette tips | Greiner Bio-One Ltd. | 771-290 |

| | | |
|---|---|---|
| 200 µl yellow pipette tips | Greiner Bio-One Ltd. | 739-290 |
| 1000 µl blue pipette tips | Greiner Bio-One Ltd. | 740-290 |
| 20 µl filter tips | Star Lab, TipOne | S1126-7810 |
| 10 µl filter tips | Star Lab, TipOne | S1121-3810 |
| 100 µl filter tips | Star Lab, TipOne | S1120-1840 |
| 1000 µl filter tips | Star Lab, TipOne | S1126-7810 |

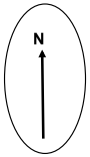
## C. Field experiments design and assessment

### C.1. 2011/12 cropping season field experiment information, treatment guidelines, randomised block design and spray timings.

| | |
|---|---|
| ADAS Code | XAC1710 |
| Trial title | University of Herts. Phoma |
| ADAS site | Boxworth |
| Host farmer/grower | J Witherow |
| Field | Pond field |
| Grid ref. | TL 33318 64660 |
| Year | 2011/2012 |
| Plot length (m) | 24.00 |
| Plot width (m) | 2.00 |

| | |
|---|---|
| Plan created by: | gfb |
| Plan created on: | 13/09/11 |
| Plan checked by: | PW |

| | |
|---|---|
| Host farmer/grower: | J Witherow |
| Contact number: | 07711096238 |




| | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| PLOT | DISCARD | DISCARD | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | DISCARD | DISCARD |
| BLOCK | | | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | | |
| TREATMENT | | | 6 | 12 | 8 | 13 | 10 | 14 | 1 | 9 | 4 | 7 | 5 | 3 | 11 | 2 | | |
| PLOT | DISCARD | DISCARD | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 | 26 | 27 | 28 | DISCARD | DISCARD |
| BLOCK | | | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | | |
| TREATMENT | | | 1 | 8 | 7 | 13 | 5 | 2 | 3 | 14 | 11 | 4 | 12 | 10 | 9 | 6 | | |
| PLOT | DISCARD | DISCARD | 29 | 30 | 31 | 32 | 33 | 34 | 35 | 36 | 37 | 38 | 39 | 40 | 41 | 42 | DISCARD | DISCARD |
| BLOCK | | | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | | |
| TREATMENT | | | 14 | 10 | 5 | 8 | 7 | 4 | 9 | 6 | 3 | 2 | 12 | 1 | 11 | 13 | | |

24.0m

2.5m

Treatment (l/ha)

| | T1 (first signs disease) | | T2 (T1 + 4 weeks) | | T3 (spring sclero. Spray) | |
|---|---|---|---|---|---|---|
| | Product | Rate | Product | Rate | Product | Rate |
| 1 | Untreated | - | Untreated | - | Untreated | - |
| 2 | Untreated | - | Untreated | - | Proline 275 | 0.63 |
| 3 | Sanction | 0.80 | Untreated | - | Proline 275 | 0.63 |
| 4 | LEM17 | 0.80 | Untreated | - | Proline 275 | 0.63 |
| 5 | Galileo | 0.32 | Untreated | - | Proline 275 | 0.63 |
| 6 | Q8Y78 | 1.00 | Untreated | - | Proline 275 | 0.63 |
| 7 | Untreated | - | Sanction | 0.80 | Proline 275 | 0.63 |
| 8 | Untreated | - | LEM17 | 0.80 | Proline 275 | 0.63 |
| 9 | Untreated | - | Galileo | 0.32 | Proline 275 | 0.63 |
| 10 | Untreated | - | Q8Y78 | 1.00 | Proline 275 | 0.63 |
| 11 | Sanction | 0.80 | Sanction | 0.80 | Proline 275 | 0.63 |
| 12 | LEM17 | 0.80 | LEM17 | 0.80 | Proline 275 | 0.63 |
| 13 | Galileo | 0.32 | Galileo | 0.32 | Proline 275 | 0.63 |
| 14 | Q8Y78 | 1.00 | Q8Y78 | 1.00 | Proline 275 | 0.63 |

Drilled on 22.08.11 with Catana @ 5.0 kgs/ha
T1 spray applied on 09.11.11
T2 spray applied on 09.01.12
T3 spray applied on 30.04.12

## C.2. 2012/13 cropping season field experiment information, treatment guidelines and randomised block design

| | |
|---|---|
| ADAS Code | XAC1710 |
| Trial title | Univercity of Herts. phoma |
| ADAS site | Boxworth |
| Host farmer/grower | Ian Lutey |
| Field | 102 |
| Grid ref. | TL 27783 57939 |
| Year | 2012/2013 |
| Plot length (m) | 29.00 |
| Plot width (m) | 2.00 |

| | |
|---|---|
| Plan created by: | gfb |
| Plan version date: | 19/10/12 |
| Plan checked by: | pw |

| | |
|---|---|
| Host farmer/grower: | Ian Lutey |
| Contact number: | 0 |

N

| | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| PLOT | DISCARD | DISCARD | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | DISCARD | DISCARD |
| BLOCK | DISCARD | DISCARD | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | DISCARD | DISCARD |
| TREATMENT | DISCARD | DISCARD | 12 | 14 | 13 | 10 | 11 | 2 | 1 | 5 | 9 | 8 | 4 | 6 | 7 | 3 | DISCARD | DISCARD |
| PLOT | DISCARD | DISCARD | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 | 26 | 27 | 28 | DISCARD | DISCARD |
| BLOCK | DISCARD | DISCARD | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | DISCARD | DISCARD |
| TREATMENT | DISCARD | DISCARD | 13 | 7 | 2 | 3 | 4 | 11 | 8 | 14 | 9 | 6 | 12 | 5 | 10 | 1 | DISCARD | DISCARD |
| PLOT | DISCARD | DISCARD | 29 | 30 | 31 | 32 | 33 | 34 | 35 | 36 | 37 | 38 | 39 | 40 | 41 | 42 | DISCARD | DISCARD |
| BLOCK | DISCARD | DISCARD | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | DISCARD | DISCARD |
| TREATMENT | DISCARD | DISCARD | 2 | 8 | 13 | 7 | 9 | 1 | 10 | 11 | 6 | 4 | 5 | 3 | 12 | 14 | DISCARD | DISCARD |

29.0m

2.5m

Treatment (l/ha)

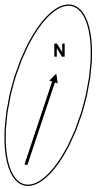
| | T1 (first signs disease) | | T2 (T1 + 4 weeks) | | T3 (spring sclero. Spray) | |
|---|---|---|---|---|---|---|
| | Product | Rate | Product | Rate | Product | Rate |
| 1 | Untreated | - | Untreated | - | Untreated | - |
| 2 | Untreated | - | Untreated | - | Proline 275 | 0.63 |
| 3 | Sanction | 0.80 | Untreated | - | Proline 275 | 0.63 |
| 4 | LEM17 | 0.80 | Untreated | - | Proline 275 | 0.63 |
| 5 | Galileo | 0.32 | Untreated | - | Proline 275 | 0.63 |
| 6 | Q8Y78 | 1.00 | Untreated | - | Proline 275 | 0.63 |
| 7 | Untreated | - | Sanction | 0.80 | Proline 275 | 0.63 |
| 8 | Untreated | - | LEM17 | 0.80 | Proline 275 | 0.63 |
| 9 | Untreated | - | Galileo | 0.32 | Proline 275 | 0.63 |
| 10 | Untreated | - | Q8Y78 | 1.00 | Proline 275 | 0.63 |
| 11 | Sanction | 0.80 | Sanction | 0.80 | Proline 275 | 0.63 |
| 12 | LEM17 | 0.80 | LEM17 | 0.80 | Proline 275 | 0.63 |
| 13 | Galileo | 0.32 | Galileo | 0.32 | Proline 275 | 0.63 |
| 14 | Q8Y78 | 1.00 | Q8Y78 | 1.00 | Proline 275 | 0.63 |

## C.3. 2013/2014 cropping season field experiment information, treatment guidelines, randomised block design and spray timings

| | |
|---|---|
| WRC: | XAC1710 |
| Trial title: | University Herts phoma |
| ADAS site | Boxworth |
| Host farmer/grower | John Witherow |
| Field | Brickyard Pond Field |
| Grid ref. | TL 34410 65722 |
| Year | 2013/2014 |
| Plot length (m) | 24.00 |
| Plot width (m) | 2.00 |

| | |
|---|---|
| Unique trial code: | BW14-025 |
| Plan created by: | gfb |
| Plan version date: | 09/09/13 |
| Plan checked by: | pw |

| | |
|---|---|
| Host farmer/grower: | John Witherow |
| Contact number: | 0 |




| | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| PLOT | DISCARD | DISCARD | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | DISCARD | DISCARD |
| BLOCK | | | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | | |
| TREATMENT | | | 10 | 3 | 8 | 5 | 13 | 7 | 11 | 2 | 6 | 1 | 9 | 4 | 14 | 12 | | |
| PLOT | DISCARD | DISCARD | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 | 26 | 27 | 28 | DISCARD | DISCARD |
| BLOCK | | | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | | |
| TREATMENT | | | 10 | 6 | 9 | 2 | 8 | 7 | 11 | 5 | 4 | 1 | 14 | 12 | 13 | 3 | | |
| PLOT | DISCARD | DISCARD | 29 | 30 | 31 | 32 | 33 | 34 | 35 | 36 | 37 | 38 | 39 | 40 | 41 | 42 | DISCARD | DISCARD |
| BLOCK | | | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | | |
| TREATMENT | | | 11 | 8 | 1 | 14 | 4 | 5 | 2 | 7 | 12 | 10 | 3 | 6 | 13 | 9 | | |

Marked out on 2.5m centres

| | T1 (first sign disease) | | T2 (T1 + 4 weeks) | | T3 (Early to mid-flower) | |
|---|---|---|---|---|---|---|
| | Chemical | Rate l/ha | Chemical | Rate l/ha | Chemical | Rate l/ha |
| 1 | Untreated | - | Untreated | - | Untreated | - |
| 2 | Untreated | - | Untreated | - | Galileo | 1.0 |
| 3 | Proline | 0.64 | Untreated | - | Galileo | 1.0 |
| 4 | LEM17 | 0.8 | Untreated | - | Galileo | 1.0 |
| 5 | Galileo | 0.32 | Untreated | - | Galileo | 1.0 |
| 6 | Q8Y78 | 1 | Untreated | - | Galileo | 1.0 |
| 7 | Untreated | - | Proline | 0.64 | Galileo | 1.0 |
| 8 | Untreated | - | LEM17 | 0.8 | Galileo | 1.0 |
| 9 | Untreated | - | Galileo | 0.32 | Galileo | 1.0 |
| 10 | Untreated | - | Q8Y78 | 1 | Galileo | 1.0 |
| 11 | Proline | 0.64 | Proline | 0.64 | Galileo | 1.0 |
| 12 | LEM17 | 0.8 | LEM17 | 0.8 | Galileo | 1.0 |
| 13 | Galileo | 0.32 | Galileo | 0.32 | Galileo | 1.0 |
| 14 | Q8Y78 | 1 | Q8Y78 | 1 | Galileo | 1.0 |

Drilled on 17.08.13

T1 applied on 24.10.13

T2 applied on 22.11.13

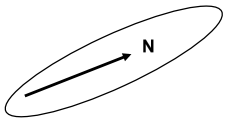
T3 applied on

## C.4. 2014/2015 cropping season field experiment information, treatment guidelines, randomised block design

| | |
|---|---|
| WRC: | XAC1841 |
| Trial title: | UoH wosr phoma |
| ADAS site | Boxworth |
| Host farmer/grower | John Witherow |
| Field | Brickyard Big Field |
| Grid ref. | TL 34580 65410 |
| Year | 2014/2015 |
| Plot length (m) | 24.00 |
| Plot width (m) | 2.00 |

| | |
|---|---|
| Unique trial code: | BW15-032 |
| Plan created by: | gfb |
| Plan version date: | 25/09/14 |
| Plan checked by: | PW |

| | |
|---|---|
| Host farmer/grower: | John Witherow |
| Contact number: | 0 |




| | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| PLOT | DISCARD | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | DISCARD |
| BLOCK | | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | |
| TREATMENT | | 4 | 10 | 2 | 13 | 6 | 14 | 1 | 5 | 11 | 9 | 3 | 12 | 8 | 7 | |
| PLOT | DISCARD | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 | 26 | 27 | 28 | DISCARD |
| BLOCK | | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | |
| TREATMENT | | 12 | 13 | 10 | 7 | 8 | 9 | 5 | 2 | 3 | 6 | 14 | 1 | 4 | 11 | |
| PLOT | DISCARD | 29 | 30 | 31 | 32 | 33 | 34 | 35 | 36 | 37 | 38 | 39 | 40 | 41 | 42 | DISCARD |
| BLOCK | | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | |
| TREATMENT | | 1 | 12 | 7 | 11 | 13 | 6 | 8 | 14 | 4 | 5 | 9 | 10 | 3 | 2 | |

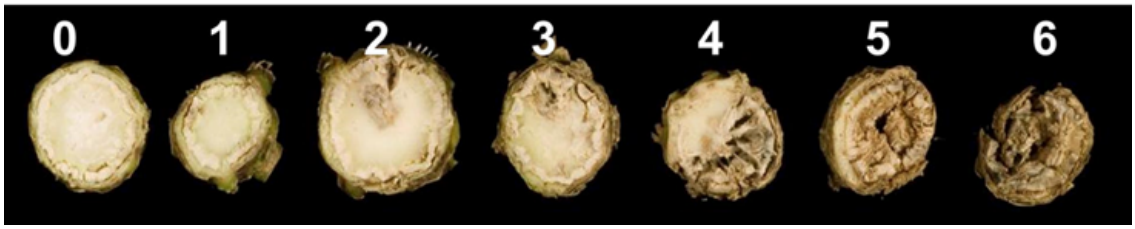
| | T1 (>20% plants affected) | | T2 (T1 + 4/6 weeks) | | T3 (Early to mid-flower) | |
|---|---|---|---|---|---|---|
| | Chemical | Rate l/ha | Chemical | Rate l/ha | Chemical | Rate l/ha |
| 1 | Untreated | - | Untreated | - | Untreated | - |
| 2 | Untreated | - | Untreated | - | Galileo | 1.0 |
| 3 | Proline | 0.64 | Untreated | - | Galileo | 1.0 |
| 4 | LEM17 | 0.80 | Untreated | - | Galileo | 1.0 |
| 5 | Galileo | 0.32 | Untreated | - | Galileo | 1.0 |
| 6 | Refinzar * | 1.00 | Untreated | - | Galileo | 1.0 |
| 7 | Untreated | - | Proline | 0.64 | Galileo | 1.0 |
| 8 | Untreated | - | LEM17 | 0.80 | Galileo | 1.0 |
| 9 | Untreated | - | Galileo | 0.32 | Galileo | 1.0 |
| 10 | Untreated | - | Refinzar * | 1.00 | Galileo | 1.0 |
| 11 | Proline | 0.64 | Proline | 0.64 | Galileo | 1.0 |
| 12 | LEM17 | 0.80 | LEM17 | 0.80 | Galileo | 1.0 |
| 13 | Galileo | 0.32 | Galileo | 0.32 | Galileo | 1.0 |
| 14 | Refinzar * | 1.00 | Refinzar * | 1.00 | Galileo | 1.0 |

* may be supplied as QY878 - but is same product

## C.5. Oilseed rape growth stages (Sylvester-Bradley and Makepeace, 1984; Sylvester-Bradley and Makepeace 1985)

| | |
|---|---|
| **0** - Germination and emergence | **5** - Pod development |
| **1** - Leaf production | **5,3** - 30% potential pods |
| **1,0** - Both cotyledons unfolded | **5,5** - 50% potential pods |
| **1,1** - First true leaf | **5,7** - 70% potential pods |
| **1,2** - Second true leaf | **5,9** - All potential pods |
| **1,5** - Fifth true leaf | **6** - Seed development |
| **1,10** - About tenth true leaf | **6,1** - Seeds expanding |
| **1,15** - About fifteenth true leaf | **6,2** - Most seeds translucent but full size |
| **2** - Stem extension | **6,3** - Most seeds green |
| **2,0** - No internodes ('rosette') | **6,4** - Most seeds green-brown mottled |
| **2,5** - About 5 internodes | **6,5** - Most seeds brown |
| **3** - Flower bud development | **6,6** - Most seeds dark brown |
| **3,0** - Only leaf buds present | **6,7** - Most seeds black but soft |
| **3,1** - Flower buds enclosed by leaves | **6,8** - Most seeds black and hard |
| **3,3** - Flower buds visible from above | **6,9** - All seeds black and hard |
| **3,5** - Flower buds raised above leaves | **7**- Leaf senescence |
| **3,6** - Flower stalks extending | **8** - Stem senescence |
| **3,7** - First flower buds yellow ('yellowbud') | **8,1** - Most stem green |
| **4** - Flowering | **8,5** - Half stem green |
| **4,0** - First flower opened | **8,9** - Little stem green |
| **4,1** - 10% all buds opened | **9** - Pod senescence |
| **4,3** - 30% all buds opened | **9,1** - Most pods green |
| **4,5** - 50% all buds opened | **9,5** - Half pods green |
| **4,7** - 70% all buds opened | **9,9** - Few pods green |
| **4,9** - 90 % all buds opened | |

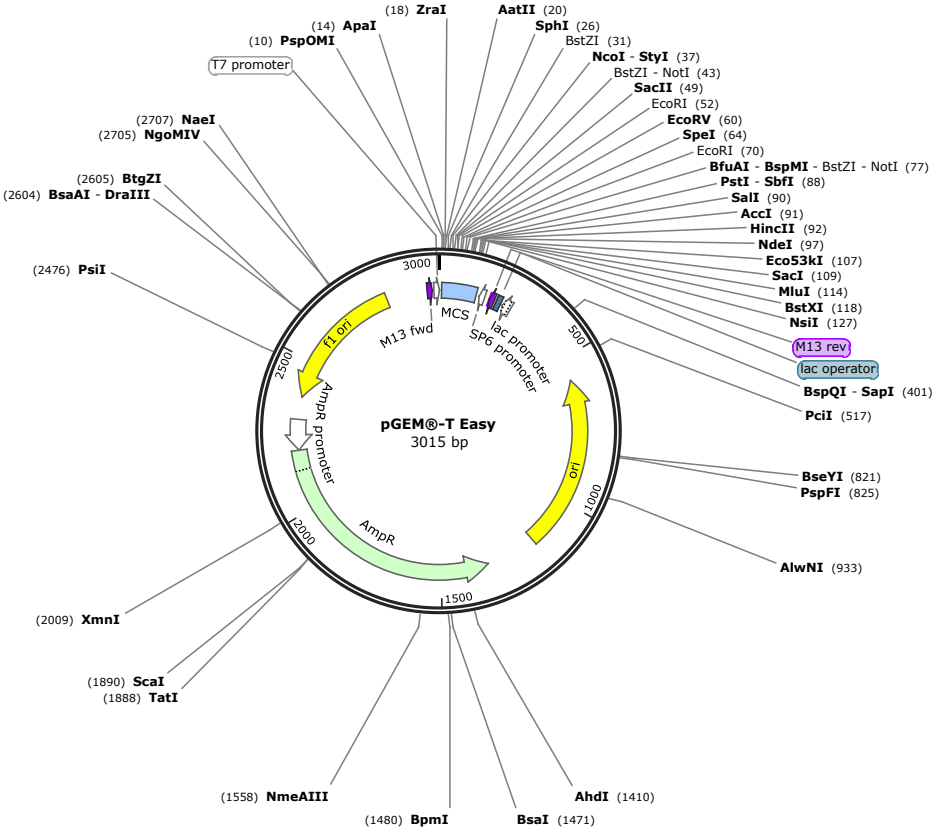
**C.6. Phoma stem canker disease severity scoring**



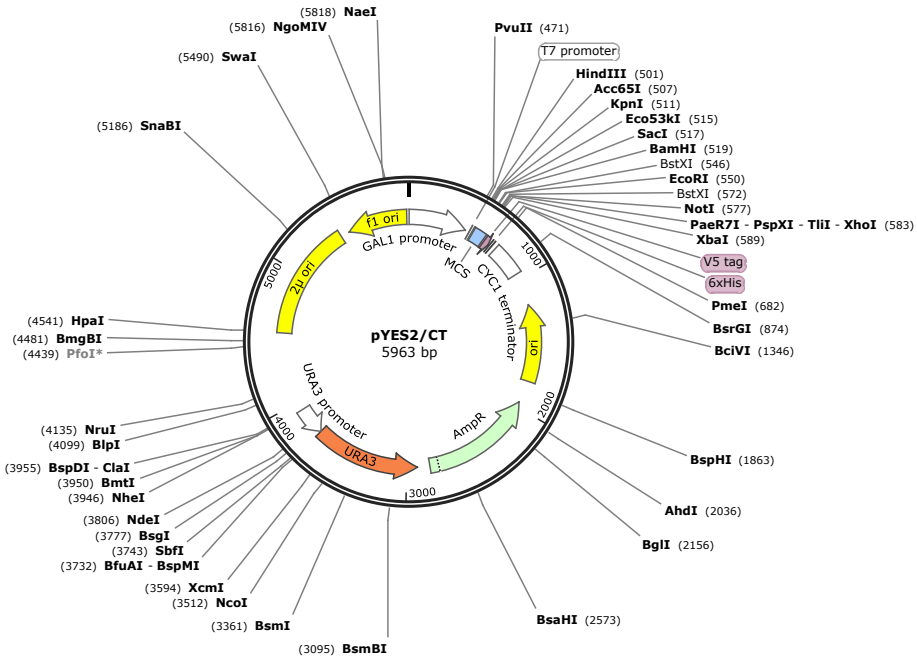

0 = healthy; 1 < 5% stem cross-section necrotic; 2 < 25% necrotic; 3 < 50% necrotic; 4 < 75% necrotic; 5 < 100% necrotic, stem firm; 6 = 100% necrotic, stem weak; 7 = 100% necrotic, stem broken or dead plant. Taken from Lô-Pelzer et al. (2009).

## D. Plasmid vector maps

### D.1. pGEM-T – *E. coli* cloning vector

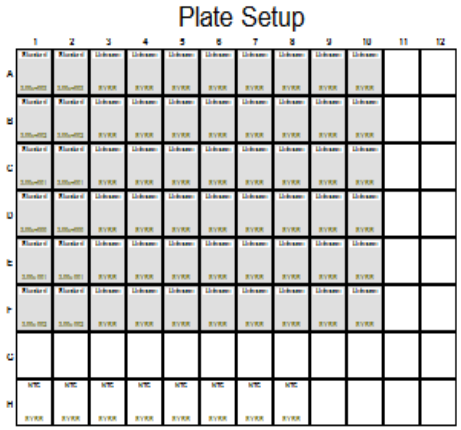


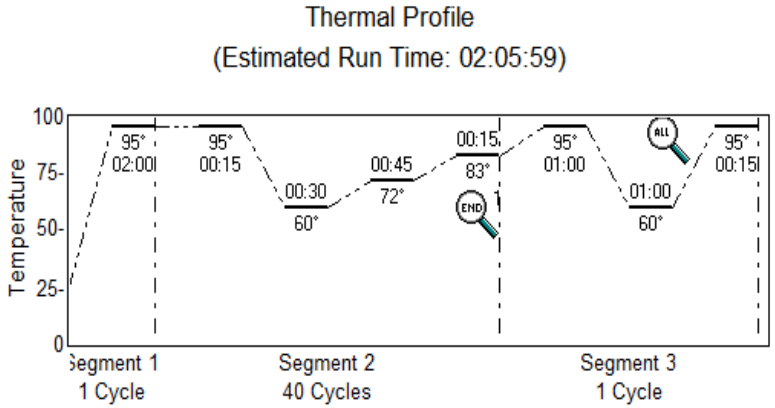

### D.2. pYES2/CT – *S. cerevisiae* expression vector

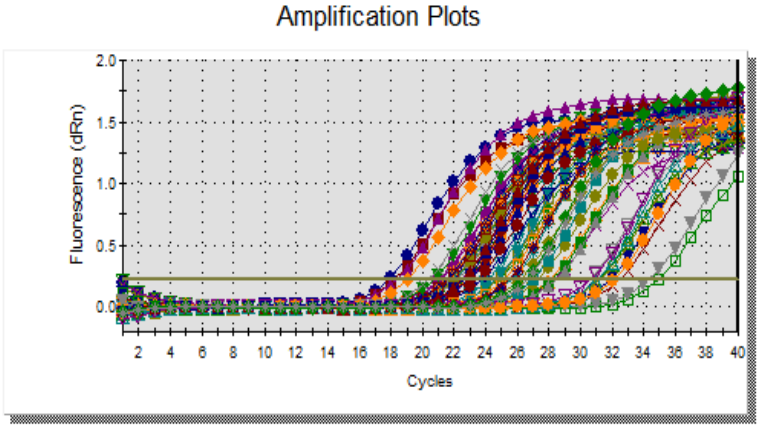


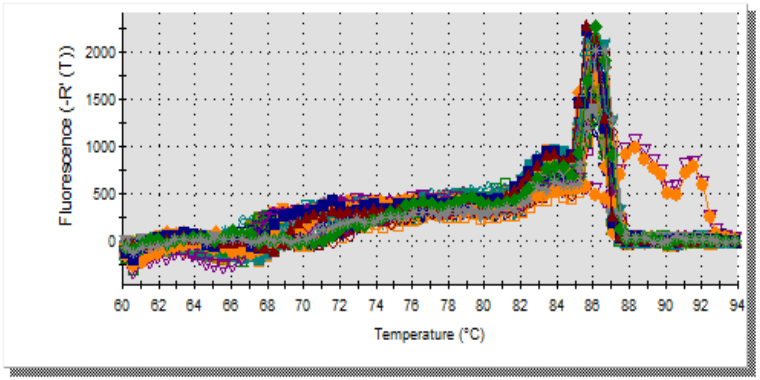
## E. Quantitative polymerase chain reaction reports and standard curves

E.1. *L. maculans* DNA quantification *in planta*

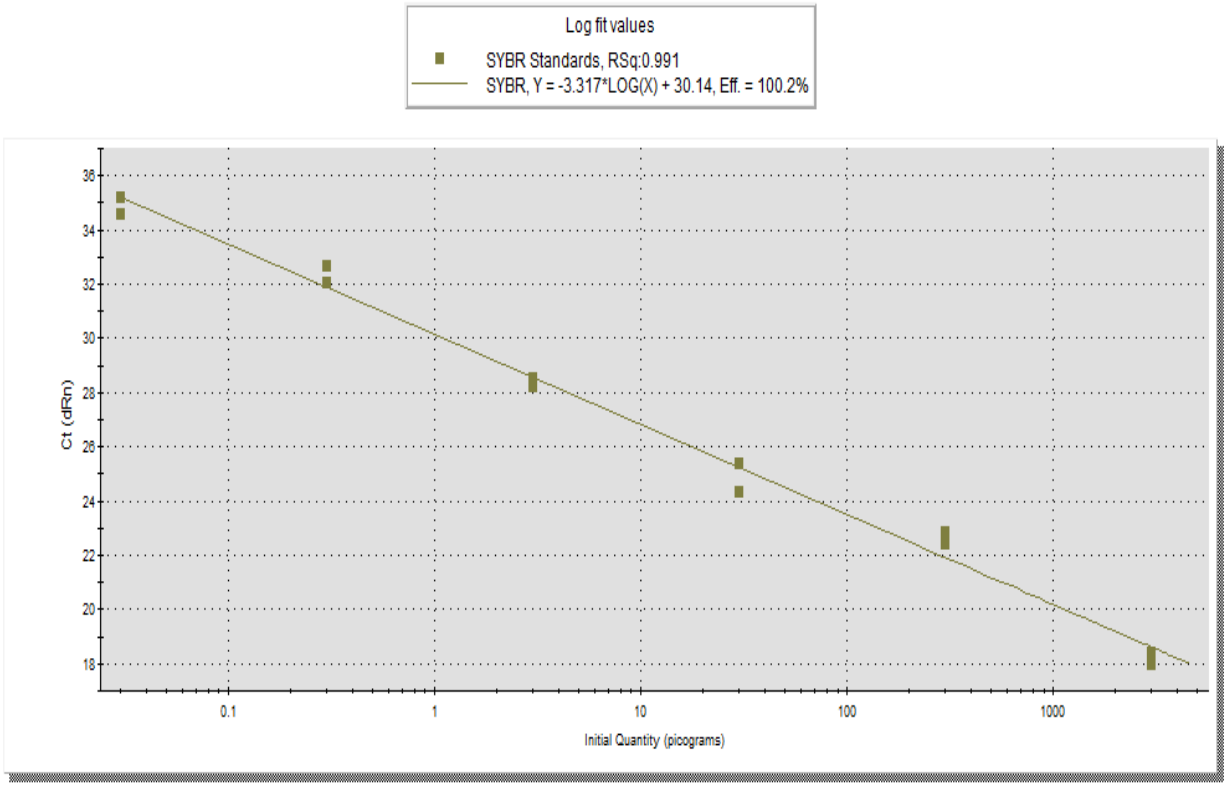


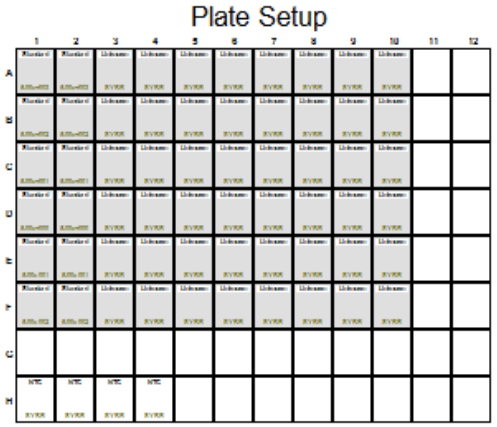



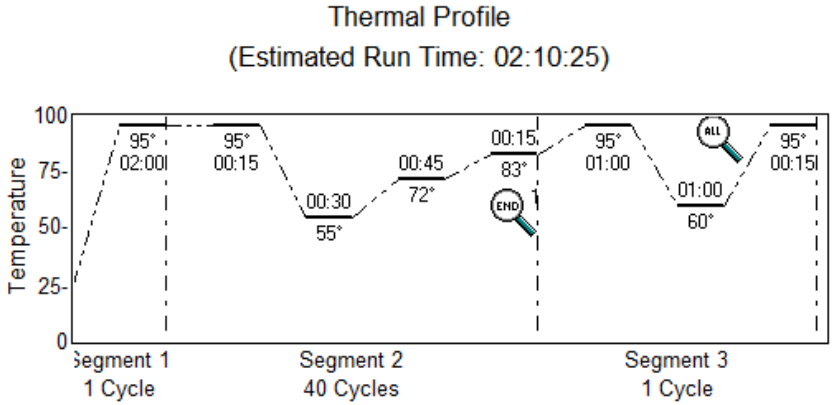



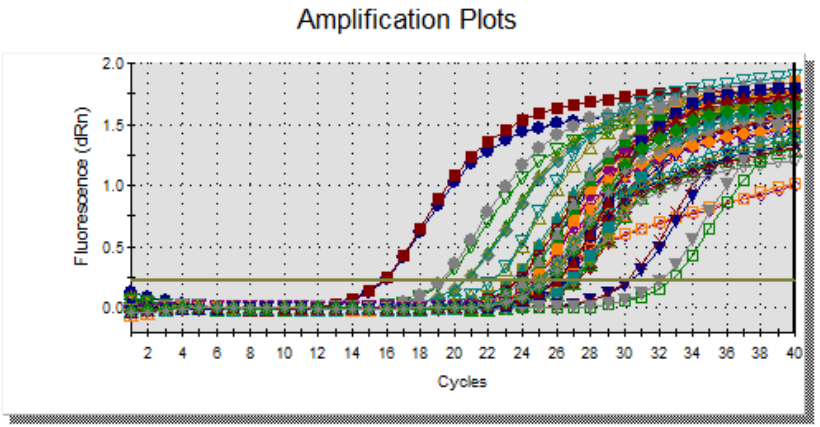
## Standard Curve

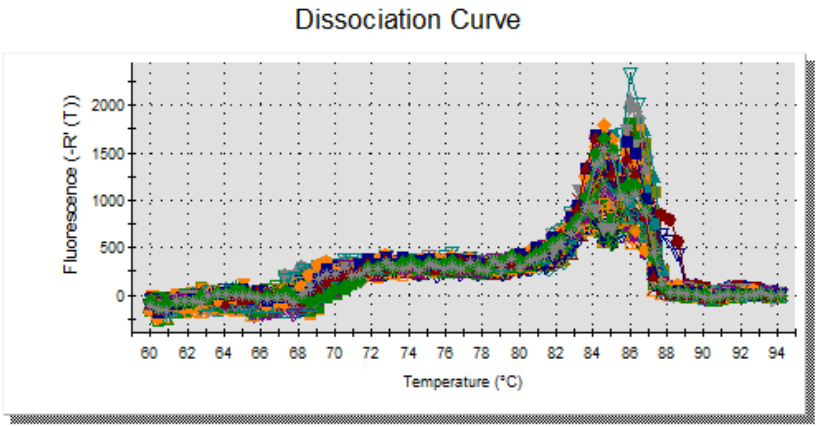


E.1. *L. biglobosa* DNA quantification *in planta*

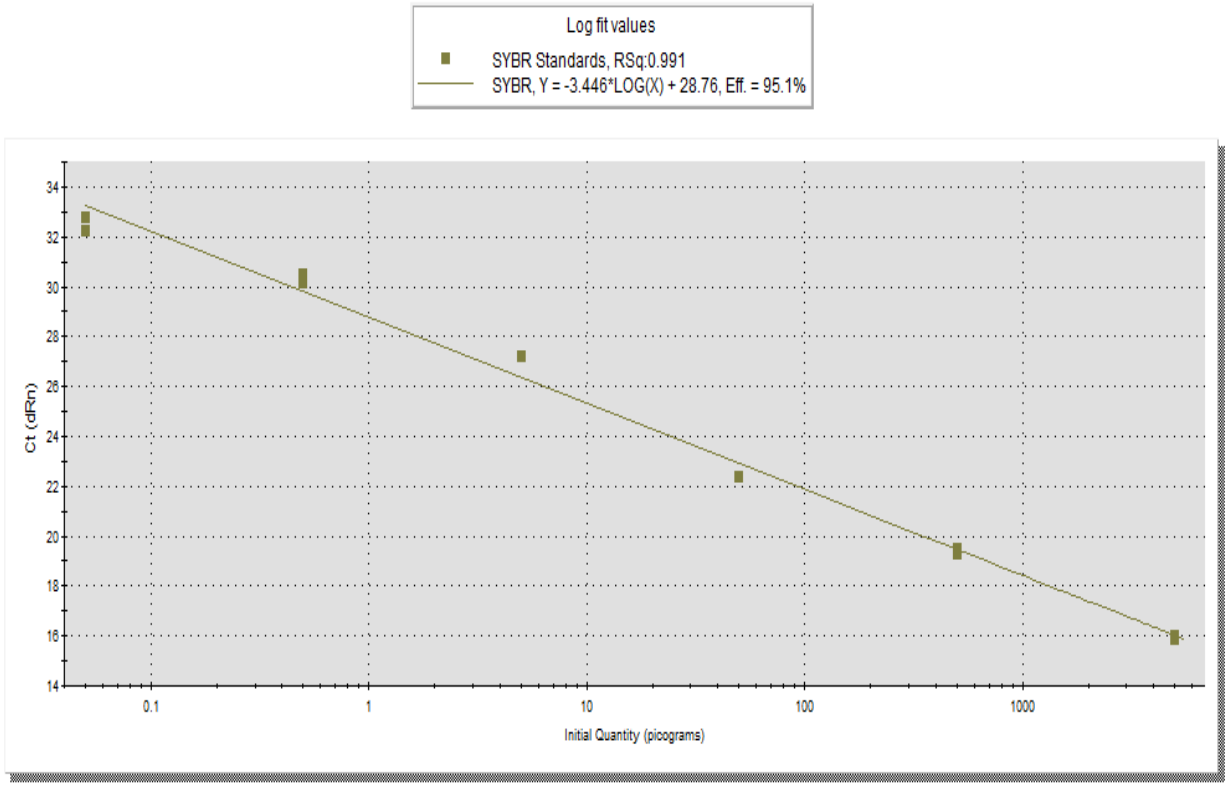


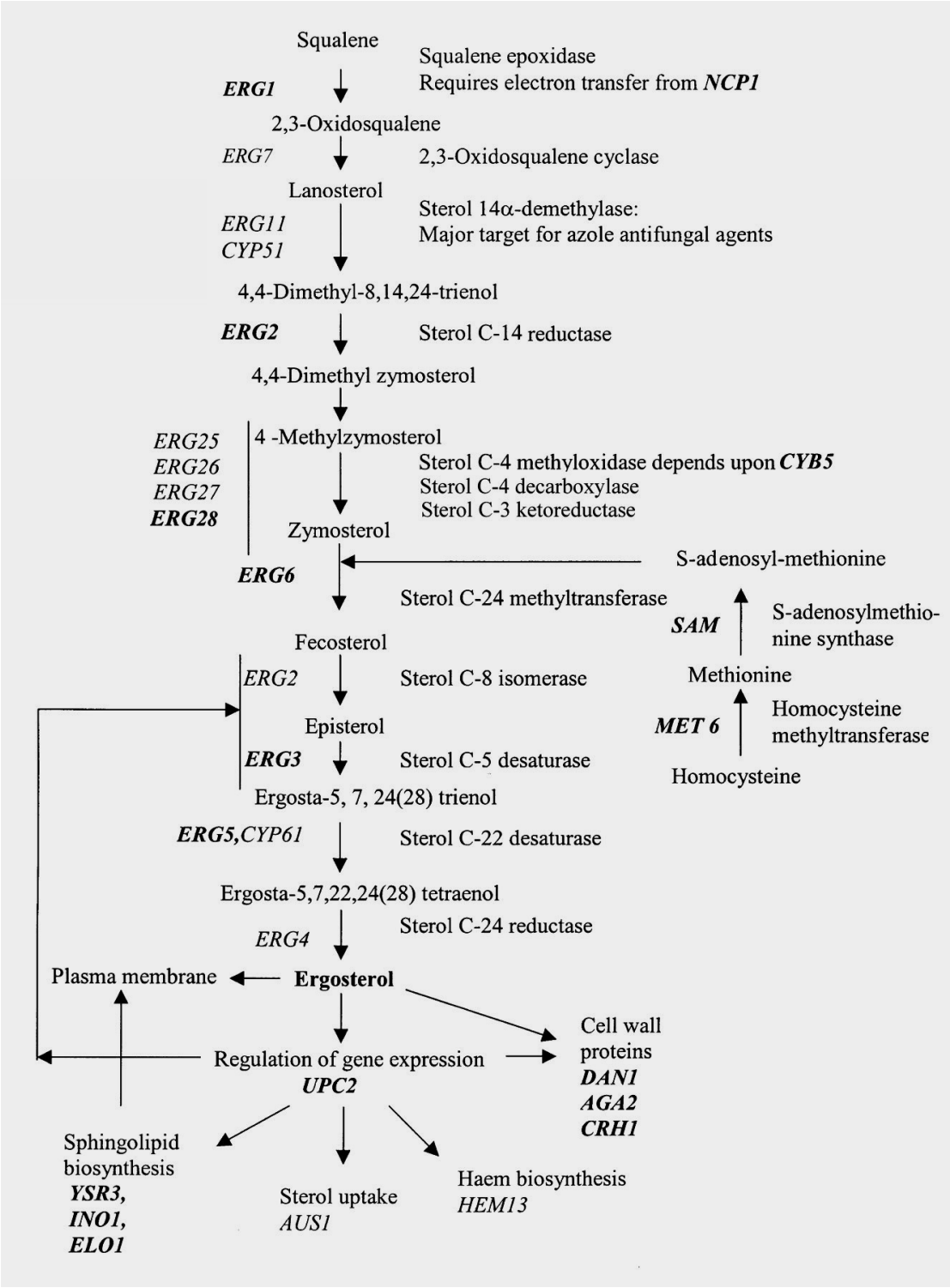



## Standard Curve

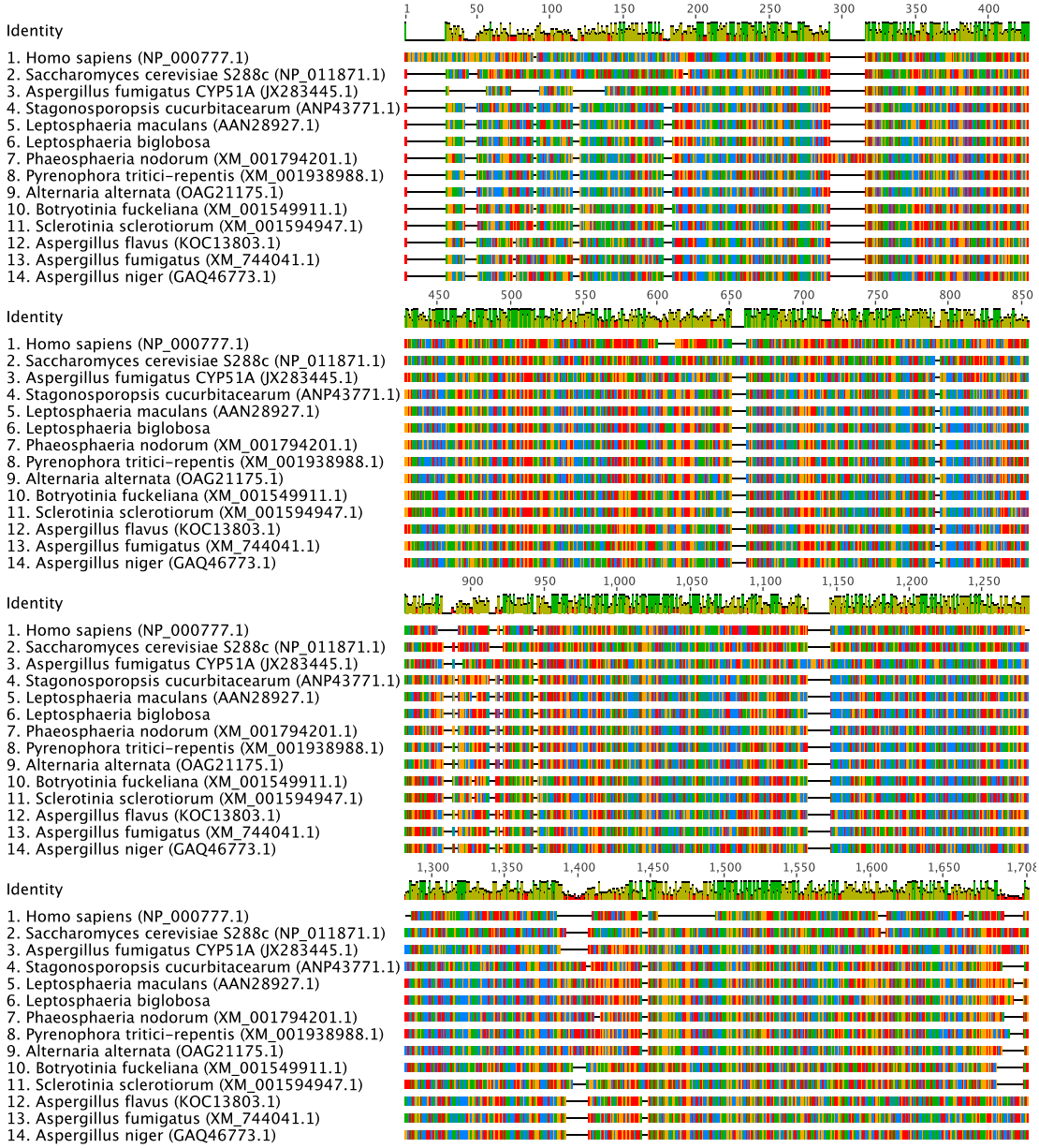


## F. Ergosterol synthetic pathway

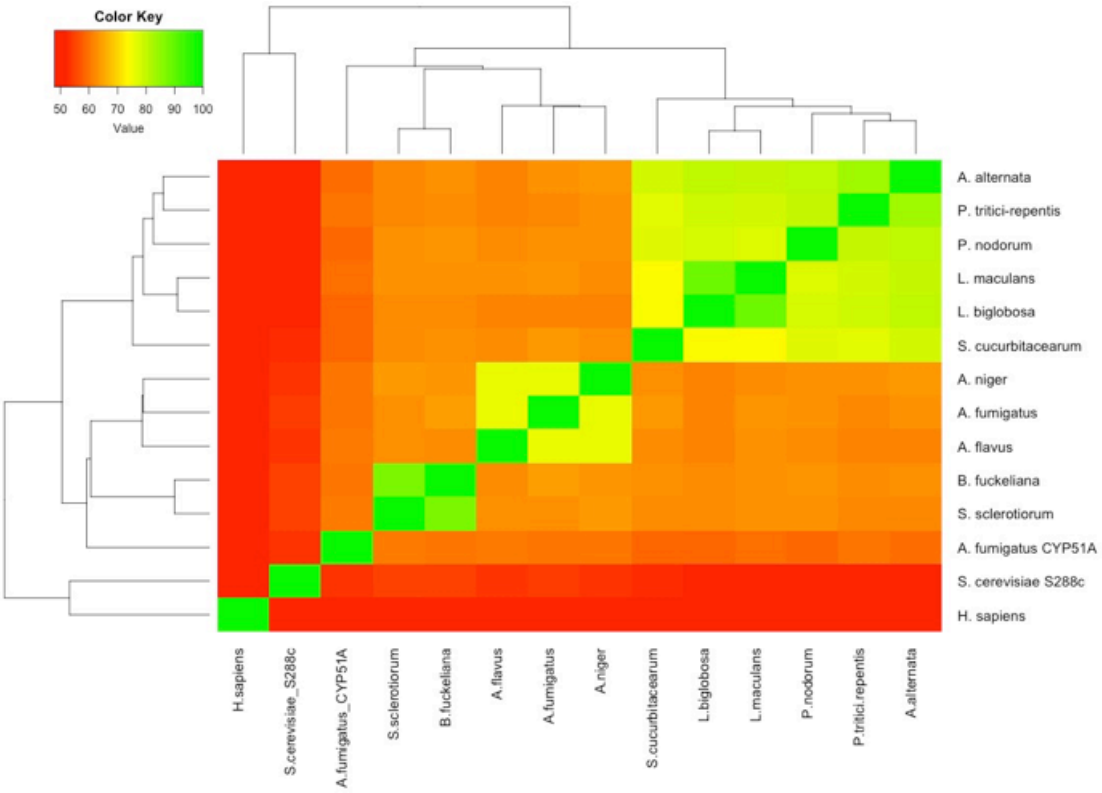


## G. Phylogenetic Tree input (MEGA7)

## H.1 CYP51 sequences alignment



## H.2. Pair-wise identity heat cap of aligned CYP51 sequences



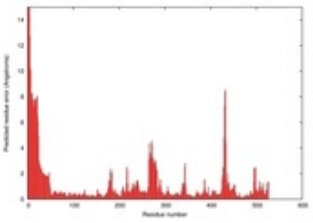
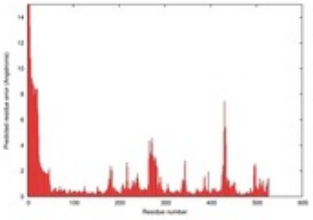
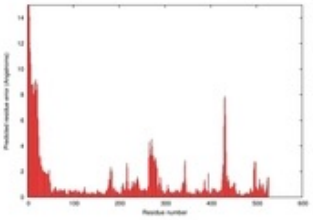
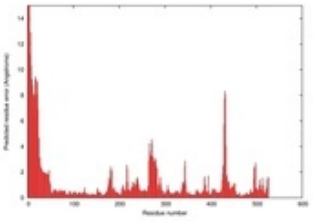
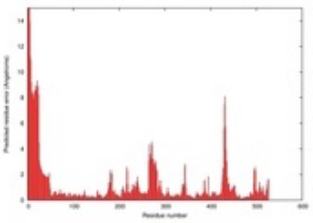
## H. Molecular modelling input and output files

### I.1 LmCYP51B homology modelling

I.1.1 Amino acid CYP51B sequences used for Modeller input.

```
>5EAD:A|PDBID|CHAIN|SEQUENCE
MSATKSIVGEALEYVNIGLSHFLALPLAQRISLIIIIPFIYNIVWQLLYSLRKDRPPLVFYWIPWVGSAVVYGMKPYEFF
EECQKKYGDIFSFVLLGRVMTVYLGPKGHEFVFNAKLADVSAEAAYAHLTTPVFGKGVIYDCPNSRLMEQKKFVKGALTK
EAFKSYVPLIAEEVYKYFRDSKNFRLNERTTGTIDVMVTQPEMTIFTASRSLLGKEMRAKLDTDFAYLYSDLDKGFTPIN
FVFPNLPLEHYRKRDHAQKAISGTYMSLIKERRKNNDIQDRDLIDSLMKNSTYKDGVKMTDQEIANLLIGVLMGGQHTSA
ATSAWILLHLAERPDVQQELYEEQMRVLDGGKKELTYDLLQEMPLLNQTIKETLRMHHPLHSLFRKVMKDMHVPNTSYVI
PAGYHVLVSPGYTHLRDEYFPNAHQFNIHRWNNDSASSYSVGEEVDYGFGAISKGVSSPYLPFGGGRHRCIGEHFAYCQL
GVLMSIFIRTLKWHYPEGKTVPPPDFTSMVTLPTGPAKIIWEKRNPEQKIGGR

>3LD6:A|PDBID|CHAIN|SEQUENCE
MAKKTLPAGVKSPPYIFSPIPFLGHAIAFGKSPIEFLENAYEKYGPVFSFTMVGKTFTYLLGSDAAALLFNSKNEDLNAE
DVYSRLTTPVFGKGVAYDVPNPVFLEQKKMLKSGLNIAHFKQHVSIIEKETKEYFESWGESGEKNVFEALSELIILTASH
CLHGKEIRSQLNEKVAQLYADLDGGFSHAAWLLPGWLPLPSFRRRDRAHREIKDIFYKAIQKRRQSQEKIDDILQTLLDA
TYKDGRPLTDDEVAGMLIGLLLAGQHTSSTTSAWMGFFLARDKTLQKKCYLEQKTVCGENLPPLTYDQLKDLNLLDRCIK
ETLRLRPPIMIMMRMARTPQTVAGYTIPPGHQVCVSPTVNQRLKDSWVERLDFNPDRYLQDNPASGEKFAYVPFGAGRHR
CIGENFAYVQIKTIWSTMLRLYEFDLIDGYFPTVNYTTMIHTPENPVIRYKRRSTHHHHHH

>4UYM:A|PDBID|CHAIN|SEQUENCE
KTPPVVFHWFPFIGSTISYGIDPYKFFFDCRAKYGDIFTFILLGKKTTVYLGTKGNDFILNGKLRDVCAEEVYSPLTTPV
FGRHVVYDCPNAKLMEQKKFVKYGLTSDALRSYVPLITDEVESFVKNSPAFQGHKGVFDVCKTIAEITIYTASRSLQGKE
VRSKFDSTFAELYHNLDMGFAPINFMLPWAPLPHNRKRDAAQRKLTETYMEIIKARRQAGSKKDSEDMVWNLMSCVYKNG
TPVPDEEIAHMMIALLMAGQHSSSSTASWIVLRLATRPDIMEELYQEQIRVLGSDLPPLTYDNLQKLDLHAKVIKETLRL
HAPIHSIIRAVKNPMAVDGTSYVIPTSHNVLSSPGVTARSEEHFPNPLEWNPHRWDENIAASAEDDEKVDYGYGLVSKGT
NSPYLPFGAGRHRCIGEQFAYLQLGTITAVLVRLFRFRNLPGVDGIPDTDYSSLFSKPLGRSFVEFEKRH

>LM_CYP51
MAVLATVAGPLGDFTAKSSNFVLAITGFASFILVAVVLNVLKQLLFKKANEPPVVFHWLPIIGSTVTYGMDPYAFFFRNQ
KKYGNVFTFILLGRKMTVCLDTTGNNFILNGKIKDVNAEEIYSPLTTPVFGKDVVYDCPNSKLMEQKKFVKFGLTQEALR
SYVVIITQETEDFLKRHKAFKGQKGTFDVTKVMAELTIYTASRSLQGEEIRRSFDSKFAELYHDLDMGFSPVNFMLSWAP
LPHNRARDHARETMIELYSNIVRKRRAGVAKDSHDMIWHLMDCKYKDGTPVPEHEIAGIMIALLMAGQHSSSSTIAWILL
RLAQNPHIIEELFEEQKTVLGADLPALTYEDLQKLPLHAQVVKETLRIHAPIHSIMRRVKQPLVVDGTNFVVPTSHTLMS
SPGFSAQLDSHFANPSHWDPHRWDAGNSQYDEEADEKDDEKIDYGWGVVSKGTNSPYLPFGAGRHRCIGEQFAYLQLQTI
LVAFVREFKLRNVGGSRDIIGTDYTSLFSRPLAPGIIEWERREKVE
```

I.1.2 ModFOLD results for LmCYP51B models

| Graphical ModFOLD4 results for LmCYP51 I Help | | | | |
|---|---|---|---|---|
| Model name | Confidence and P-value | Global model quality score | Residue error plot (click image for large version) | 3D view of residue error (click image for large version) |
| lm_cyp51_model_final_LM_CYP51model5.pdb | CERT: 8.24E-5 | 0.8947 |  |  |
| lm_cyp51_model_final_LM_CYP51model1.pdb | CERT: 8.295E-5 | 0.8941 |  |  |
| lm_cyp51_model_final_LM_CYP51model3.pdb | CERT: 8.41E-5 | 0.8928 |  |  |
| lm_cyp51_model_final_LM_CYP51model4.pdb | CERT: 8.475E-5 | 0.8920 |  |  |
| lm_cyp51_model_final_LM_CYP51model2.pdb | CERT: 8.504E-5 | 0.8917 |  |  |

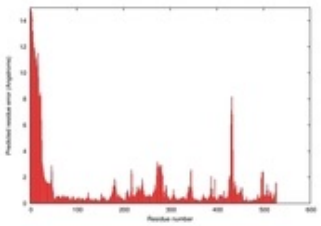
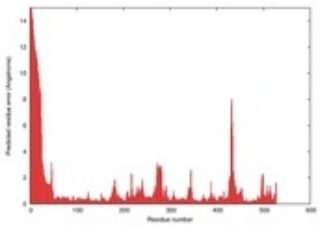
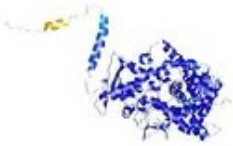
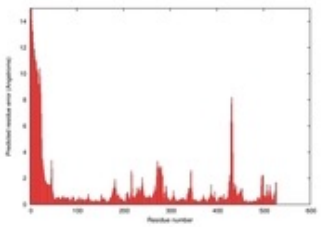
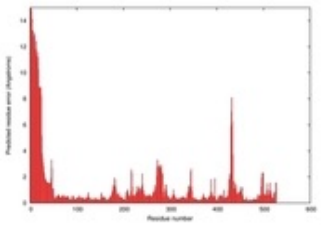
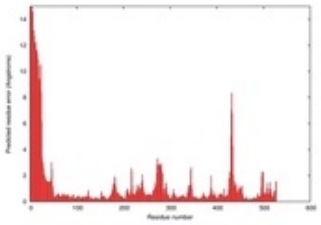
## **I.2** LbCYP51B **homology modelling**

I.1.2 Amino acid CYP51B sequences used for Modeller input.

```
>5EAD:A|PDBID|CHAIN|SEQUENCE
MSATKSIVGEALEYVNIGLSHFLALPLAQRISLIIIIPFIYNIVWQLLYSLRKDRPPLVFYWIPWVGSAVVYGMKPYEFF
EECQKKYGDIFSFVLLGRVMTVYLGPKGHEFVFNAKLADVSAEAAYAHLTTPVFGKGVIYDCPNSRLMEQKKFVKGALTK
EAFKSYVPLIAEEVYKYFRDSKNFRLNERTTGTIDVMVTQPEMTIFTASRSLLGKEMRAKLDTDFAYLYSDLDKGFTPIN
FVFPNLPLEHYRKRDHAQKAISGTYMSLIKERRKNNDIQDRDLIDSLMKNSTYKDGVKMTDQEIANLLIGVLMGGQHTSA
ATSAWILLHLAERPDVQQELYEEQMRVLDGGKKELTYDLLQEMPLLNQTIKETLRMHHPLHSLFRKVMKDMHVPNTSYVI
PAGYHVLVSPGYTHLRDEYFPNAHQFNIHRWNNDSASSYSVGEEVDYGFGAISKGVSSPYLPFGGGRHRCIGEHFAYCQL
GVLMSIFIRTLKWHYPEGKTVPPPDFTSMVTLPTGPAKIIWEKRNPEQKIGGR

>3LD6:A|PDBID|CHAIN|SEQUENCE
MAKKTLPAGVKSPPYIFSPIPFLGHAIAFGKSPIEFLENAYEKYGPVFSFTMVGKTFTYLLGSDAAALLFNSKNEDLNAE
DVYSRLTTPVFGKGVAYDVPNPVFLEQKKMLKSGLNIAHFKQHVSIIEKETKEYFESWGESGEKNVFEALSELIILTASH
CLHGKEIRSQLNEKVAQLYADLDGGFSHAAWLLPGWLPLPSFRRRDRAHREIKDIFYKAIQKRRQSQEKIDDILQTLLDA
TYKDGRPLTDDEVAGMLIGLLLAGQHTSSTTSAWMGFFLARDKTLQKKCYLEQKTVCGENLPPLTYDQLKDLNLLDRCIK
ETLRLRPPIMIMMRMARTPQTVAGYTIPPGHQVCVSPTVNQRLKDSWVERLDFNPDRYLQDNPASGEKFAYVPFGAGRHR
CIGENFAYVQIKTIWSTMLRLYEFDLIDGYFPTVNYTTMIHTPENPVIRYKRRSTHHHHHH

>4UYM:A|PDBID|CHAIN|SEQUENCE
KTPPVVFHWFPFIGSTISYGIDPYKFFFDCRAKYGDIFTFILLGKKTTVYLGTKGNDFILNGKLRDVCAEEVYSPLTTPV
FGRHVVYDCPNAKLMEQKKFVKYGLTSDALRSYVPLITDEVESFVKNSPAFQGHKGVFDVCKTIAEITIYTASRSLQGKE
VRSKFDSTFAELYHNLDMGFAPINFMLPWAPLPHNRKRDAAQRKLTETYMEIIKARRQAGSKKDSEDMVWNLMSCVYKNG
TPVPDEEIAHMMIALLMAGQHSSSSTASWIVLRLATRPDIMEELYQEQIRVLGSDLPPLTYDNLQKLDLHAKVIKETLRL
HAPIHSIIRAVKNPMAVDGTSYVIPTSHNVLSSPGVTARSEEHFPNPLEWNPHRWDENIAASAEDDEKVDYGYGLVSKGT
NSPYLPFGAGRHRCIGEQFAYLQLGTITAVLVRLFRFRNLPGVDGIPDTDYSSLFSKPLGRSFVEFEKRH

>LB_CYP51
MGVLATIAGPLGDFTAKSSYPVLAVTGFASFILIAVVLNVLKQLLLKKPNEPPVVFHWLPIIGSTVTYGMDPYAFFFRNHKKYGN
VFTFILLGRKMTVCLDTTGNNFILNGKIKDVNAEEIYSPLTTPVFGKDVVYDCPNSKLMEQKKFVKFGLTQEALRSYVVIITQET
EDFLKRHKSFKGQKGTFDVTKDMAELTIYTASRSLQGEEIRRSFDSKFAELYHDLDMGFSPVNFMLSWAPLPHNRARDHARETMI
ELYSNIVRKRRAGAAKKDSHDMIWHLMDCKYKDGTPVPEHEIAGIMIALLMAGQHSSSSTIAWILLRLAQNPHIVEELLEEQKNI
LGADLPALRYEDLQRLPLHSQVVKETLRIHAPIHSIMRKVKQPLVVDGTNFVVPTSHTLMSSPGFSAQLDSHFVNPSHWDPHRWD
AGNSQYDEEADEKDDEKIDYGWGVVSKGTNSPYLPFGAGRHRCIGEQFAYLQLQTILVAFVREFKLRNVGGSKDIIGTDYTSLFS
RPLAPGVVEWERREKVE
```

I.1.2 ModFOLD results for LbCYP51B models

**Graphical ModFOLD4 results for LbCYP51 I Help**

| Model name | Confidence and P-value | Global model quality score | Residue error plot (click image for large version) | 3D view of residue error (click image for large version) |
|---|---|---|---|---|
| lb_cyp51_model_4.pdb | CERT: 7.926E-5 | 0.8985 |  |  |
| lb_cyp51_model_2.pdb | CERT: 7.969E-5 | 0.8979 |  |  |
| lb_cyp51_model_1.pdb | CERT: 8.01E-5 | 0.8974 |  |  |
| lb_cyp51_model_5.pdb | CERT: 8.047E-5 | 0.8970 |  |  |
| lb_cyp51_model_3.pdb | CERT: 8.063E-5 | 0.8968 |  |  |

### I.3. Quantitative assessment of protein structure similarity (TM-Score)

```
 ***************************************************************************
 *                                 TM-SCORE                                *
 * A scoring function to assess the similarity of protein structures       *
 * Based on statistics:                                                    *
 *       0.0 < TM-score < 0.17, random structural similarity               *
 *       0.5 < TM-score < 1.00, in about the same fold                     *
 * Reference: Yang Zhang and Jeffrey Skolnick, Proteins 2004 57: 702-710   *
 * For comments, please email to: zhng@umich.edu                           *
 ***************************************************************************

Structure1: A277946     Length=  526
Structure2: B277946     Length=  527 (by which all scores are normalized)
Number of residues in common=  526
RMSD of  the common residues=    2.763

TM-score    = 0.9031  (d0= 8.12)
MaxSub-score= 0.5497  (d0= 3.50)
GDT-TS-score= 0.7239 %(d<1)=0.4554 %(d<2)=0.4725 %(d<4)=0.9696 %(d<8)=0.9981
GDT-HA-score= 0.5849 %(d<0.5)=0.4421 %(d<1)=0.4554 %(d<2)=0.4725 %(d<4)=0.9696

 -------- rotation matrix to rotate Chain-1 to Chain-2 ------
 i          t(i)         u(i,1)         u(i,2)         u(i,3)
 1   -202.3369953195  -0.1546339252   0.4265244058   0.8911595146
 2   -168.6119212248   0.9493684317  -0.1855293793   0.2535319117
 3    427.3635149659   0.2734738196   0.8852433455  -0.3762396700
```

## Publications

1. Research paper – European Journal of Plant Pathology – Effects of a penthiopyrad and picoxystrobin fungicide mixture on phoma stem canker (*Leptosphaeria* spp.) on UK winter oilseed rape
2. Research paper – Under review – Azole sensitivity in Leptosphaeria pathogens of oilseed rape: the role of lanosterol 14α-demethylase
3. Abstract – 14th International Rapeseed Congress 2015 – The effect of fungicides on *Leptosphaeria biglobosa* and *L. maculans*, phoma stem canker severity and oilseed rape yield
4. Abstract – 11th Conference of the European Foundation for Plant Pathology 2014 – Effects of different fungicides on development of phoma stem canker and oilseed rape yield
5. Abstract – BSPP 2014 Presidential Meeting – Effects of different fungicides on severity of phoma stem canker
6. Poster – Brassica 2016 – Azole sensitivity in *Leptosphaeria* species: The role of lanosterol 14α-demethylase
7. Poster – ADAS open day 2016 – Effects of a penthiopyrad and picoxystrobin fungicide mixture on phoma stem canker (*Leptosphaeria* spp.) on UK winter oilseed rape
8. Poster ADAS open day 2014 – Investigating the potential threat of *L. biglobosa* to UK oilseed rape yield.
9. Poster – University of Hertfordshire Life and Medical Science Conference 2014 – Effects of different fungicides on phoma stem canker pathogens and oilseed rape yield

Eur J Plant Pathol
DOI 10.1007/s10658-016-0916-8

# Effects of a penthiopyrad and picoxystrobin fungicide mixture on phoma stem canker (*Leptosphaeria* spp.) on UK winter oilseed rape

**Thomas R. Sewell · Steven Moloney · Mike Ashworth · Faye Ritchie · Alla Mashanova · Yong Ju Huang · Henrik U. Stotz · Bruce D. L. Fitt**

Accepted: 22 March 2016


**Abstract** In the UK, fungicides are often used to control phoma stem canker on winter oilseed rape. Field trials were established near Boxworth, Cambridgeshire for four cropping seasons (2011/2012, 2012/2013, 2013/2014 and 2014/15) to test the efficacy of a new fungicide mixture Refinzar® (penthiopyrad + picoxystrobin) by comparison to an existing fungicide Proline 275® (prothioconazole) against phoma stem canker (*Leptosphaeria* spp.) and the effect on winter oilseed rape (cv. Catana) yield. In each season, weather data were collected from a weather station at Boxworth and the release of ascospores was monitored using a nearby Burkard spore sampler. The patterns of ascospore release differed between seasons and related to weather conditions. Fungicides penthiopyrad + picoxystrobin and prothioconazole were applied in October/November when 10 % of plants had phoma leaf spotting (T1, early), 4/8 weeks after T1 (T2, late) or at both T1 and T2 (combined). When phoma leaf spot symptoms were assessed in autumn/winter, penthiopyrad + picoxystrobin and prothioconazole both decreased numbers of phoma leaf spots caused by *L. maculans*; there were few leaf spots caused by *L. biglobosa*. Penthiopyrad + picoxystrobin and prothioconazole both reduced phoma stem canker severity before harvest compared to the untreated control but did not increase yield in these seasons when epidemics were not severe. In 2013/2014, the presence of *L. maculans* and *L. biglobosa* in upper stem lesions or stem base cankers was determined by species-specific PCR. The proportions of stems with *L. maculans* DNA were much greater than those with *L. biglobosa* DNA for both upper stem lesions and basal stem cankers. These results suggest that both penthiopyrad + picoxystrobin and prothioconazole can decrease phoma stem canker severity on winter oilseed rape in severe disease seasons.

**Keywords** Phoma stem canker · Winter oilseed rape · Fungicides · DMI · QoI · SDHI

T. R. Sewell (✉) · S. Moloney · A. Mashanova · Y. J. Huang · H. U. Stotz · B. D. L. Fitt
Centre for Agriculture, Food and Environmental Management, University of Hertfordshire, College Lane, Hatfield, Hertfordshire AL10 9AB, UK
e-mail: t.sewell@herts.ac.uk

M. Ashworth
DuPont (UK) Ltd, Kings Court, London Road, Stevenage, Hertfordshire SG1 2NG, UK

F. Ritchie
ADAS UK Ltd, Battlegate Road, Boxworth, Cambridge CB23 4NN, UK

## Introduction

Phoma stem canker is a disease of oilseed rape, which is caused by closely related fungal species *Leptosphaeria maculans* and *L. biglobosa* (Fitt et al. 2006a; Stonard et al. 2010). Both pathogens follow a monocyclic disease cycle in the UK with phoma leaf spotting symptoms in autumn/winter and stem base canker in spring/summer. Severe cankers inhibit the flow of water and nutrients to the seed, and thus decrease seed yield and

Published online: 05 April 2016

quality. Oilseed rape is the third most valuable arable crop grown in the UK and has a total annual value of > £600 M and an average on-farm yield of 3.5–4.0 t/ha (AHDB Cereals and Oilseeds 2015). Globally, phoma stem canker has been calculated to annually cause approximately £700 M worth of losses, making it a significant threat to worldwide oilseed rape production and food security (Fitt et al. 2006b).

Generally, *L. maculans* forms damaging stem base cankers and *L. biglobosa* forms less damaging upper stem lesions on UK winter oilseed rape (West et al. 2002a; Fitt et al. 2006a; Huang et al. 2011). This difference is considered a result of differences in timing of ascospore release, with *L. maculans* spores released in early/mid-autumn and *L. biglobosa* spores released in early/mid-winter (Fitt et al. 2006b). More recently, however, *L. biglobosa* has been shown to cause severe upper stem lesions and lodging on crops in some growing seasons (Huang et al. 2014a). If this occurs regularly, *L. biglobosa* could become a more important threat to winter oilseed rape yield.

Together with conventional plant breeding strategies that adopt effective resistance (Delourme et al. 2006; Huang et al. 2014b), fungicides are commonly used in the UK to control phoma stem canker on winter oilseed rape. In 2014, 98.1 % of the total area of oilseed rape (674,580 ha) received fungicide treatment for control of diseases including phoma stem canker because growers generally expect such treatments to give a yield response (Garthwaite et al. 2012). UK winter oilseed rape experiments have often shown a yield response from fungicide application against phoma stem canker, although an increase in yield was registered only when canker severity in unsprayed plots was ≥ 3 on a 0–5 disease severity scale (West et al. 2002b). Typically, azole fungicides have been applied because of their effective action against *L. maculans* as well as their relatively low cost compared to alternatives. Examples include flusilazole, prothioconazole and tebuconazole (Eckert et al. 2010; Huang et al. 2011). Other fungicides are available to growers; these include quinone outside inhibitor (QoI) fungicides and succinate dehydrogenase inhibitor (SDHI) fungicides, both of which disrupt energy production in the fungal cell (Avenot and Michailides 2010; Bartlett et al. 2002); however, their efficacy against phoma stem canker has not been evaluated.

Legislation from the European Union has forced the withdrawal of some fungicides used to control fungal pathogens in arable crops (Marx-Stoelting et al. 2014). An example is the withdrawal of flusilazole, a chemical widely used for phoma stem canker control in the UK until 2014. Despite concluding that flusilazole fulfils safety requirements set by Member States, the European Commission, on review, withdrew usage of flusilazole across the entire European Union (European Commission 2007). Withdrawal of flusilazole reduced options available to growers for control of phoma stem canker, along with other crop diseases. It is thus imperative to obtain a complete understanding of the effects that novel fungicide mixtures have on phoma stem canker in winter oilseed rape crops.

This paper describes work investigating the efficacy of a new fungicide mixture Refinzar® (a.i. penthiopyrad plus picoxystrobin, an SDHI plus QoI, respectively) to reduce phoma leaf spotting, decrease phoma stem canker severity and improve oilseed rape yield.

## Materials and methods

### Weather conditions at the field site

Weather data for the 2011/12, 2012/13, 2013/14 and 2014/15 winter oilseed rape growing seasons were collected at Boxworth, Cambridgeshire, UK (52.259814, -0.025437); near the winter oilseed rape field experiments and the Burkard spore sampler in the 2014/15 cropping season, and approximately 15 km from the site of the Burkard spore sampler in 2011/12, 2012/13 and 2013/14. Temperature and rainfall data were collected daily using an automated weather station (Campbell Scientific, UK).

### Numbers of ascospores in the air

The numbers of *Leptosphaeria* ascospores in the air were estimated using a 7-day volumetric spore sampler (Burkard Manufacturing Co. Ltd, UK). For the 2011/12, 2012/13 and 2013/14 cropping seasons, the spore sampler was located at Whittlesford, Cambridgeshire, UK (52.109299, 0.156023). For the 2014/15 cropping season, the spore sampler was located at Boxworth, Cambridgeshire (52.270127, -0.027112). The spore sampler accommodated a rotating drum (2 mm per hour) that held a strip of Melinex tape. The tape was lined with a thin layer of petroleum jelly and hexane paste mixture (10 g petroleum jelly, 20 ml hexane). After 7 days of sampling, the rotating drum was removed and the Melinex tape was divided into seven 24-h segments.

Each segment was then cut horizontally, with one half stored at −20 °C for molecular analysis and one-half mounted for microscopy to count spore numbers. The slide-mounted tape was stained with trypan blue solution (0.4 % *w/v* in water, Sigma-Aldrich, UK) so that the ascospores were visible under a light microscope (100x total magnification). Counting was done in three longitudinal traverses across the slide and the number of ascospores recorded for each traverse. The concentration of ascospores in the air was calculated according to equation described by Lacey and West (2006).

## Winter oilseed rape field experiments

Field experiments were established near Boxworth, Cambridgeshire, UK for the 2011/12, 2012/13, 2013/14 and 2014/15 cropping seasons. The winter oilseed rape cultivar Catana (Dekalb, UK) was used because of its susceptibility to *L. maculans* (resistance rating of 4 in the UK North region on a 1–9 scale; where 9 is very resistant) but good resistance against *Pyrenopeziza brassicae* the cause of light leaf spot (AHDB Cereals and Oilseeds 2015).

In each growing season, seeds of cv. Catana were sown in mid/late August at a seed rate of 5 kg/ha and a drilling depth of 1 cm. To test the efficacy of a new fungicide mixture (penthiopyrad + picoxystrobin), by comparison to existing fungicides (flusilazole or prothioconazole), for control of phoma stem canker (*Leptosphaeria* spp.) and effect on winter oilseed rape yield, experiments were arranged in a randomised block design with three replicates. Each plot received one of 14 treatments (four different fungicides applied under three different timing regimes (T1, T2 or T1 and T2 combined), one untreated throughout the cropping season, one treated with a spring spray only, T3), thus totalling 42 plots (Table 1). The fungicide Refinzar® (DuPont UK Ltd; a.i. penthiopyrad 160 g/l plus picoxystrobin 80 g/l) was used in all four cropping seasons. The product has been marketed as a potential alternative to the azole fungicides that are used widely in the UK on winter oilseed rape. Sanction® (DuPont UK

**Table 1** Treatment list giving fungicides and spray timings used in field experiments at Boxworth, Cambridge over four winter oilseed rape (cv. Catana) cropping seasons[+]

| Spray timing | T1 (10% leaf spotting) | | T2 (T1 + 4 or 8 weeks) | |
|---|---|---|---|---|
| Treatment number | Chemical | Rate g a.i/ha | Chemical | Rate g a.i/ha |
| 1 | Untreated | – | Untreated | – |
| 2[*] | Untreated | – | Untreated | – |
| 3[^] | Flusilazole or Prothioconazole | 200 or 176 | Untreated | – |
| 4 | Penthiopyrad | 160 | Untreated | – |
| 5 | Picoxystrobin | 80 | Untreated | – |
| 6 | Penthiopyrad + Picoxystrobin | 160 + 80 | Untreated | – |
| 7[^] | Untreated | – | Flusilazole or Prothioconazole | 200 or 176 |
| 8 | Untreated | – | Penthiopyrad | 160 |
| 9 | Untreated | – | Picoxystrobin | 80 |
| 10 | Untreated | – | Penthiopyrad + Picoxystrobin | 160 + 80 |
| 11[^] | Flusilazole or Prothioconazole | 200 or 176 | Flusilazole or Prothioconazole | 200 or 176 |
| 12 | Penthiopyrad | 160 | Penthiopyrad[^] | 160 |
| 13 | Picoxystrobin | 80 | Picoxystrobin | 80 |
| 14 | Penthiopyrad + Picoxystrobin | 160 + 80 | Penthiopyrad + Picoxystrobin | 160 + 80 |

[+] Experiments were arranged in a randomised block design with three replicates. T1 spray was applied in the autumn when 10 % of the plants had phoma leaf spotting. T2 spray was applied in the autumn/winter 4 or 8 weeks after T1. A third fungicide spray (T3) targeting sclerotinia stem rot was applied to all treatments except treatment 1, which remained untreated throughout the cropping season. In 2011/12 and 2012/13 cropping seasons, prothioconazole was used as the flowering spray (T3) and in 2013/14 and 2014/15 picoxystrobin was used

[*] Received T3 flowering spray and therefore differs from treatment 1 which was untreated throughout cropping season

[^] Flusilazole was applied in 2011/12 and 2012/13 until its withdrawal and was replaced by prothioconazole in 2013/14 and 2014/15

Ltd; a.i. flusilazole 250 g/l) was used for the first two cropping seasons before its active ingredient flusilazole was withdrawn. It was replaced by another azole fungicide, Proline 275® (Bayer Crop Science UK Ltd; a.i. prothioconazole 275 g/l), for the 2013/14 and 2014/15 cropping seasons. To represent the components of Refinzar®, Galileo® (DuPont UK Ltd; a.i. picoxystrobin 250 g/l) and LEM17® (DuPont UK Ltd; a.i. penthiopyrad 200 g/l) were also applied but data are not presented. The fungicide spray timings differed from season to season, with the first application (T1) taking place in autumn when 10 % of plants were affected with phoma leaf spots. The second application (T2) was made 8 weeks after T1 in 2011/2012 season and 4 weeks after T1 in 2012/13, 2013/14 and 2014/15 seasons. All plots except the untreated control received a spring-flowering spray (T3) against the pathogen *Sclerotinia sclerotiorum*, the causal agent of sclerotinia stem rot.

### Phoma leaf spotting, stem canker and yield assessment

Phoma leaf spotting was assessed by randomly sampling ten plants per plot in the 2011/12 and 2012/13 cropping seasons and 15 plants per plot in the 2013/14 and 2014/15 cropping seasons; as described in Steed et al. (2007). The sampling was done regularly between November and February each cropping season. The total numbers of *L. maculans* (large grey lesions with pycnidia) and *L. biglobosa* (small dark lesions with few or no pycnidia) leaf spots on each leaf were recorded, together with growth stage of the plant.

Phoma stem canker severity assessment was done once in the 2011/12 and 2012/13 cropping seasons (25 July 2012 and 9 July 2013), twice in the 2013/14 cropping season (27 May and 1 July 2014) and twice in the 2014/15 cropping season (1 June and 29 June 2015). A random sample of either 10 (2011/12 and 2012/13), 25 (2013/14) or 15 (2014/15) plants was collected from each of the 42 plots using the method described in Steed et al. (2007). The severity of basal cankers was assessed by cutting the stem at the base of each sampled plant and scoring the cross-sectional area of necrotic tissue according to a 0–6 scale (Huang et al. 2011), modified from Lô-Pelzer et al. (2009). Upper stem lesions were cut at the centre point of the lesions and assessed on the same scale. Desiccated plots were harvested using a small plot harvester and yield (t/ha) recorded. Presence of light leaf spot on stems was also noted.

### Stem canker subsampling, DNA extraction and species-specific PCR

To investigate whether the phoma stem cankers were caused by *L. maculans* and/or *L. biglobosa*, stems with basal stem canker or upper stem lesion symptoms were subsampled for DNA extraction and *Leptosphaeria* species-specific PCR. Approximately three stems per plot were selected from basal stem canker and upper stem lesion samples from all 42 plots of the 2013/14 field experiment. Using a scalpel, thin shavings of the basal canker or upper stem lesion tissue were cut away from each stem and placed in 2-ml Eppendorf tubes (Sigma-Aldrich Co LLC, UK). The subsamples were stored at −20 °C after freeze-drying for 24 h. The subsamples were then ground into a powder using a mortar and pestle. Subsamples of the powdered stem material were transferred into 2-ml Eppendorf tubes and DNA was extracted using a DNA extraction kit (DNAMITE Plant kit; Microzone Ltd, UK) and quantified using a Nanodrop ND-1000 spectrophotometer (Labtech International, UK). Identification of species was done using end-point PCR with species-specific PCR primers LmacF/LmacR for *L. maculans* and LbigF/LmacR for *L. biglobosa* (Liu et al. 2006). Gel electrophoresis was done to identify the presence of *L. maculans* and/or *L. biglobosa* DNA.

### Statistical analysis

The R software was used for statistical analyses of data (Development Core Team and R 2011). Linear mixed effects models were done on phoma leaf spotting, canker severity and yield data. Two-way mixed effect ANOVA was done on spray timing and fungicide treatment data. One-way mixed effect ANOVA was done independently on spray timing and then fungicide treatment data. Residuals were tested for normality using the Shapiro-Wilk test of normality.

## Results

### Rainfall

Rainfall patterns differed between the four seasons during autumn/winter (phoma leaf spot development stage) and summer (phoma stem canker development stage). In the 2011/12 cropping season, the autumn and winter months were dry compared with the 2013/14 cropping

season. In August and September, 73 mm of rainfall was recorded. Periods of prolonged rainfall did not commence until December 2011 and there were never periods of heavy rainfall. In the summer, it was predominantly wet, with heavy rainfall in April (101 mm), June (103 mm) and July (115 mm) 2012 (Fig. 1b). In the 2012/13 cropping season, prolonged rainfall occurred much earlier, with periods of substantial rainfall commencing in mid-September and continuing to mid-February with the occasional short dry period. In August and September, 70 mm of rainfall was recorded. The spring and summer were dry with occasional periods of short-term rainfall (Fig. 1d). In the 2013/14 cropping season, rainfall pattern was similar to that of the 2012/13 growing season in the autumn/winter. Rainfall started in early autumn, with increases in August and September over a few days and then continued for a period between October and mid-November. In August and September, 91 mm of rainfall was recorded. A period of prolonged rainfall occurred between December and February (202 mm over 88 days) (Fig. 1f). In the 2014/15 cropping season, high rainfall commenced early (8 August) with a period of very heavy rainfall (112.6 mm) causing flash floods in the region. In August and September, 192 mm of rainfall was recorded although 58 % of this was on 8 August. Rainfall in the winter months was more sporadic than in the previous seasons, with no periods of particularly prolonged rainfall between December and February (Fig. 1h).

### Average temperature

Across the four seasons, average temperature followed a typical pattern, with temperature decreasing to ≤ 0 °C in December, January and February. Periods of particularly low temperatures differed among seasons. In the 2011/12 cropping season, a low temperature (−7.1 °C) occurred on 10 and 11 of February. Average temperature between 1 October and 31 May was 7.8 °C (Fig. 1b). In 2012/13, a similar pattern was observed, but low temperature (−4.4 °C) occurred a month earlier on 14 January. One notable difference in this cropping season was an uncharacteristic period of cold weather in mid-late March. Snowfall and temperatures < 0 °C were recorded during this period. Average temperature between 1 October and 31 May was 5.7 °C (Fig. 1d). In 2013/14, there was no period of particularly cold weather, with average daily temperature never < 0 °C. Average temperature between 1 October and 31 May was 8.3 °C (Fig. 1f). The 2014/2015 cropping season was similar to the previous season in that there was no period of particularly cold weather, with average daily temperature only < 0 °C on two occasions (−0.7 and −0.4 °C on 19 January and 22 January, respectively). Average temperature between 1 October and 31 May was 7.2 °C (Fig. 1h).

### Ascospore numbers

The numbers of ascospores in the air and the period in which most ascospores were released differed among growing seasons. In 2011/12 and 2012/13, there was a major discharge of spores in November and a large discharge of spores in January; the discharge in November was longer in 2012/13 (Fig. 1a, c). In 2013/14, the spore release pattern was similar to 2012/13 but differed in timing; ascospore dispersal occurred over a longer period in the autumn, with a large release in the winter of both seasons; however, in 2013/14, the autumn release of spores was a month before the equivalent release in 2012/13 (November in 2012/13 and October in 2013/14). Similarly, a large release of spores in the winter occurred a month earlier in 2013/14 than 2012/13 (January in 2012/13 and December in 2013/14) (Fig. 1c, e). Due to accessibility issues in the 2014/2015 cropping season, spore release data commenced at the start of November. Nonetheless, two large releases were recorded, at the end of November and mid/late January (Fig. 1g). A common pattern among all four seasons was the relationship between rainfall and spore release. In most seasons, spore release commenced in large numbers after a period of prolonged or heavy rainfall. For example, heavy rainfall at the start of November 2011 was associated with ascospore release later that month. However, some spores were also released after periods of light rainfall, such as in December 2013.

### Field experiments

In all four cropping seasons, the spring flowering spray had no effect on leaf spotting, canker severity or yield when compared to the control; therefore, the untreated control data presented are a mean of untreated plots and spring spray only (T3) plots. Penthiopyrad alone produced similar results to penthiopyrad + picoxystrobin and therefore has been excluded from the analysis. Picoxystrobin alone produced similar results to the untreated control and therefore has been excluded from the analysis and data are not presented.

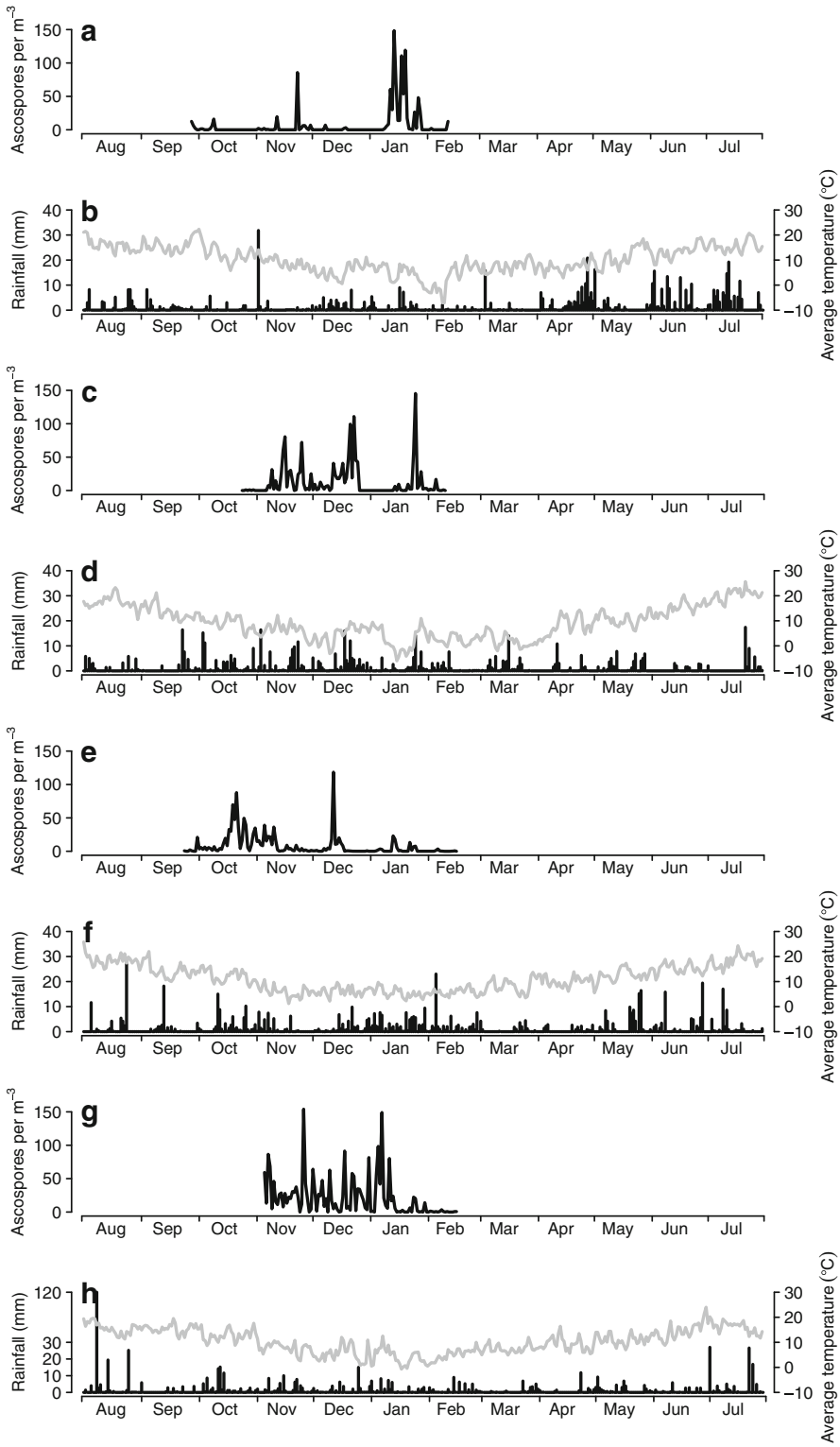



**Fig. 1** Numbers of ascospores of *Leptosphaeria* spp. (**a**, **c**, **e**, **g**), average temperature and daily rainfall (**b**, **d**, **f**, **h**) monitored over four cropping seasons. **a**–**b**) 2011/12 cropping season; **c**–**d**) 2012/13; **e**–**f**) 2013/14; **g**–**h**) 2014/15. Weather data were collected at Boxworth, Cambridgeshire, using a day interval automated weather station. The grey line represents average temperature (°C) and black bars represent total daily rainfall (mm). Airborne ascospores (number m$^{-3}$) were collected using a Burkard spore sampler that was situated at Whittlesford, Cambridgeshire (15 km from site of the field experiment) in 2011/12, 2012/13 and 2013/14 and Boxworth, Cambridgeshire in 2014/15

## Phoma leaf spotting

In the 2011/12, 2012/13 and 2014/15 cropping seasons, phoma leaf spotting in unsprayed plots did not increase in severity on winter oilseed rape leaves until March and phoma leaf spotting was never severe during the autumn/winter; therefore, data are not shown. In 2013/14, the phoma leaf spotting started earlier and incidence (% plants affected) was much greater in unsprayed plots in the autumn/winter months compared to the previous two winter oilseed rape cropping seasons (Fig. 2). Experimental plots treated with penthiopyrad + picoxystrobin or prothioconazole had significantly less *L. maculans* type leaf lesions per plant when compared with the untreated control, except when fungicides had only just been applied (T2 only plots at December 2013 assessment) or when their activity had decreased over time (T1 application at February 2014 assessment). The penthiopyrad alone treatment was statistically similar to the picoxystrobin + penthiopyrad treatment.

The two fungicides significantly decreased number of *L. biglobosa* type lesions, compared with the untreated control, in December 2013 on T1 only and on T1 plus T2 plots, and in February 2014 on T2 only treated plots. When comparing the efficacy of the two fungicides, there was no significant difference in the numbers of *L. maculans* type lesions present between penthiopyrad + picoxystrobin and prothioconazole treated plots (Fig. 2a, c, e). Furthermore, there was no significant difference in the numbers of *L. biglobosa* type leaf lesions present between penthiopyrad + picoxystrobin and prothioconazole treated plots (Fig. 2b, d, f).

## Stem canker severity

In the 2011/12, 2012/13 and 2014/15 cropping seasons, stem canker was not severe (Fig. 3). Severity was never more than 1.5 on a 0–6 scale for either upper stem lesions or basal stem cankers in these three cropping seasons. Fungicide application did not significantly decrease stem canker severity in 2011/12 and only prothioconazole at the combined T1/T2 application timing significantly reduced severity compared to the control in 2012/13. In the 2013/14 cropping season (Fig. 3c), canker was more severe than in other seasons. There were significant differences in the severity of basal stem cankers between fungicide treatments and between timings ($P<0.05$, 12 df); however, there was no significant difference in upper stem lesion

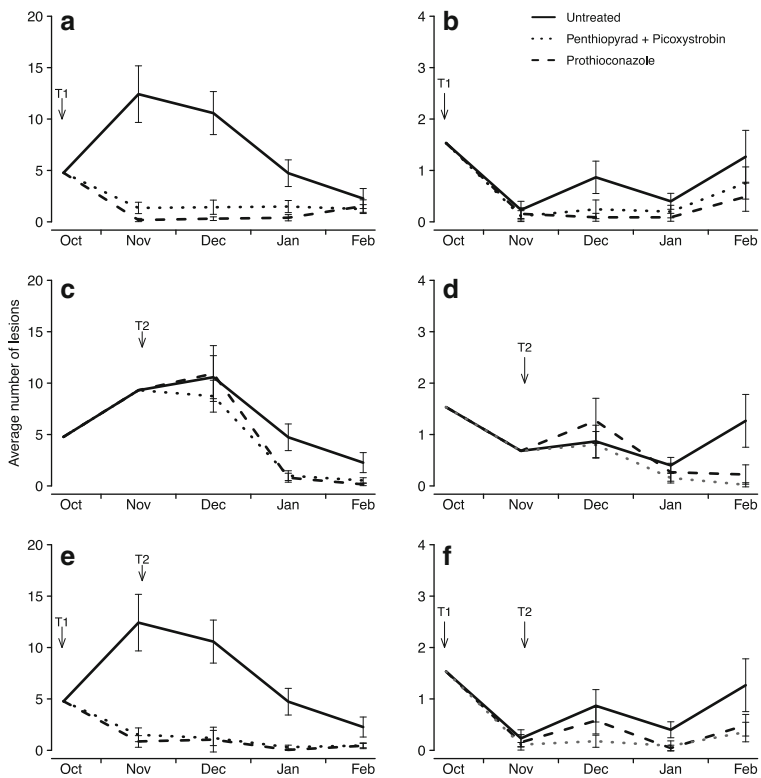



**Fig. 2** Incidence of phoma leaf spotting associated with *Leptosphaeria maculans* (**a**, **c**, **e**) or *L. biglobosa* (**b**, **d**, **f**) type leaf lesions on winter oilseed rape (cv. Catana) plots sprayed with fungicide at T1 (early) (**a**, **b**), T2 (late) (**c**, **d**) or T1 & T2 (combined) (**e**, **f**) in the 2013/14 cropping season near Boxworth, Cambridgeshire. Fifteen winter oilseed rape plants were collected from each plot and assessed for numbers of *L. maculans* and *L. biglobosa* type leaf lesions. Plots were treated with penthiopyrad + picoxystrobin (dotted line), prothioconazole (dashed line) or untreated (solid line). Average number of leaf lesions per leaf was calculated. Standard errors of the means are represented as error bars. Details of spray timings are given in Table 1

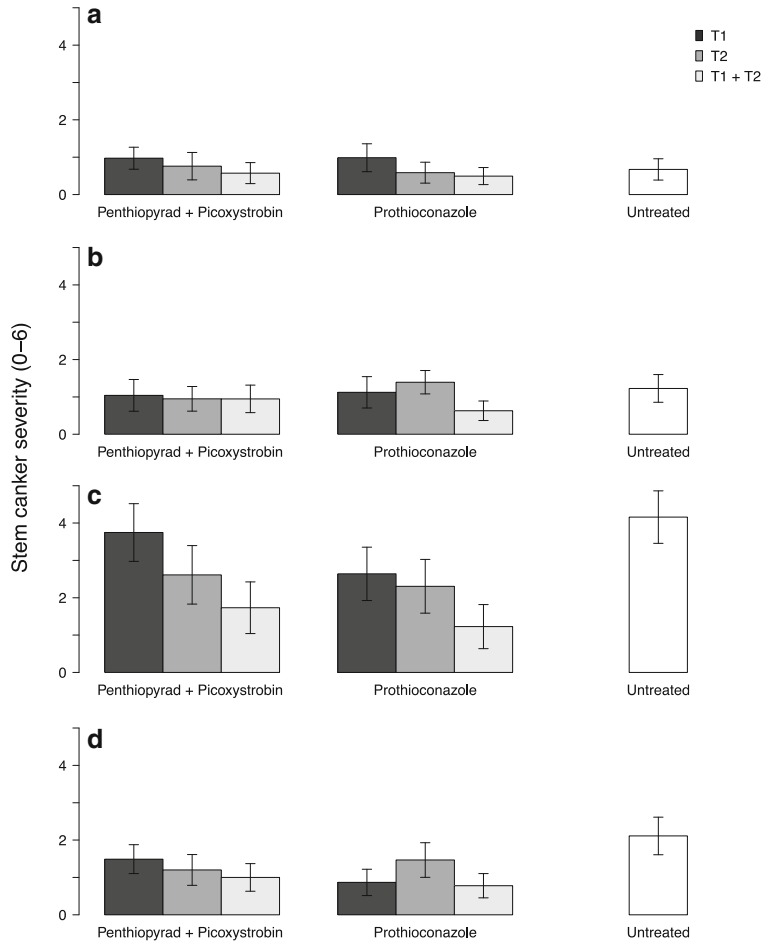



**Fig. 3** Basal stem canker severity on experimental winter oilseed rape (cv. Catana) plots in **a**) 2011/12, **b**) 2012/13, **c**) 2013/14 and **d**) 2014/15 cropping seasons near Boxworth, Cambridgeshire. Plots received sprays of penthiopyrad + picoxystrobin or prothioconazole at T1 (early), T2 (late) or T1 & T2 (combined). Basal stem canker severity (scale 0–6; Lô-Pelzer et al. 2009) was scored on 25 plant stems sampled from each plot. Standard errors of the means are represented as error bars (6 df). Details of spray timings are given in Table 1

severity between fungicide treatments and timings (data not shown). Unlike prothioconazole, penthiopyrad + picoxystrobin did not decrease the severity of basal stem cankers when applied at the T1 spray timing only when compared to untreated ($P < 0.05$, 4 df). Nonetheless, at T2 and T1/T2 timings, both penthiopyrad + picoxystrobin and prothioconazole reduced severity equally. Penthiopyrad + picoxystrobin at T1/T2 and prothioconazole at T1/T2 performed similarly, reducing basal stem canker severity more than if they were applied at T1 only or T2 only. Although there were significant differences between fungicide treatments and between timings, the interactions were not significant and were removed from the final model.

No other diseases were severe in the field experiments across all four growing seasons; in 2014/15, cabbage stem flea beetle affected winter oilseed rape establishment in the Cambridgeshire region and may have had an affect on the field experiments. Light leaf spot was present but not severe.

## Yield

Improvement in yield of fungicide-treated plots was sometimes positive and sometimes negative when compared with the control over the four cropping seasons (Fig. 4). Despite effects of treatment on stem canker severity across all cropping seasons, there was no significant effect of fungicide treatment on yield in any season.

## Proportion of stems with *L. maculans* or *L. biglobosa*

A total of 133 basal stem canker samples and 74 upper stem lesion samples was analysed by PCR. The proportions of upper stem lesions and basal stem cankers with *L. maculans* DNA detected in the sample were much greater than those with *L. biglobosa* DNA detected (Table 2). Out of 74 samples of upper stem lesions, 45 had only *L. maculans* DNA detected,

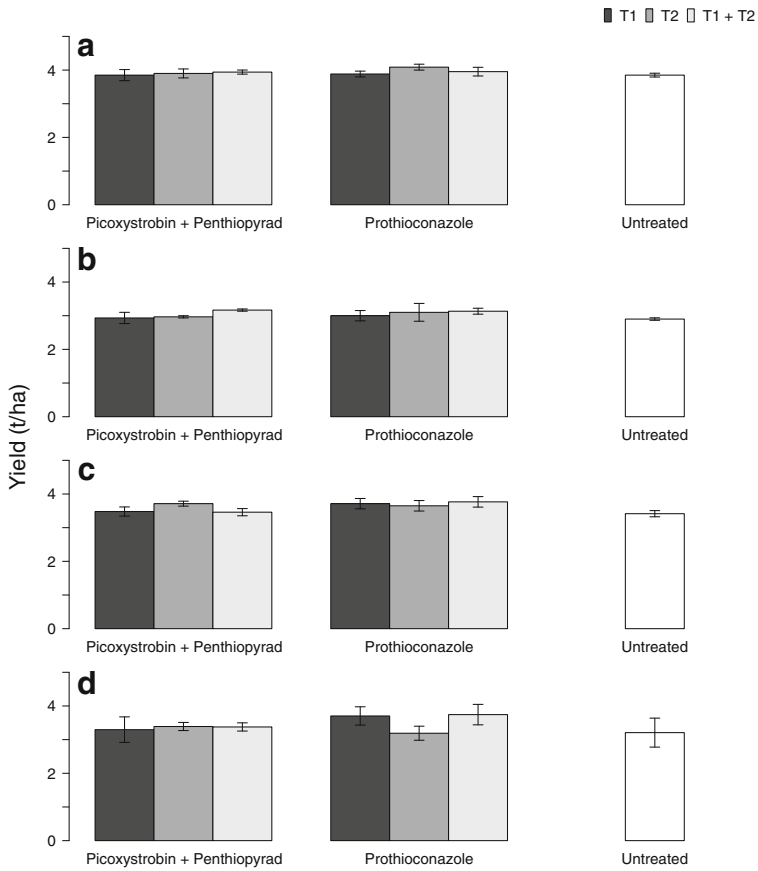



**Fig. 4** Average yield (t/ha) from experimental winter oilseed rape (cv. Catana) plots in **a**) 2011/12, **b**) 2012/13, **c**) 2013/14 or **d**) 2014/15 cropping seasons near Boxworth, Cambridgeshire. Plots received sprays of penthiopyrad + picoxystrobin or prothioconazole at early (T1), late (T2) or combined (T1 & T2) timings. Desiccated plots were harvested using a small plot harvester and yield was calculated. Standard deviations are represented as error bars (6 df). Details of spray timings are given in Table 1

two samples had only *L. biglobosa* DNA detected and 11 samples had DNA of both species detected. No *L. maculans* or *L. biglobosa* DNA was detected in 16 upper stem samples. Of 133 basal stem canker samples, 102 had only *L. maculans* DNA detected and four samples had both species detected. No samples had only *L. biglobosa* DNA recorded. No *L. maculans* or *L. biglobosa* DNA was detected in 27 basal stem canker samples.

## Discussion

These results suggest that in cropping seasons when there are moderately severe phoma stem canker epidemics, penthiopyrad + picoxystrobin and prothioconazole are both effective at reducing phoma stem canker severity. Severe canker results in yield loss because transport of water and nutrients up the stem is decreased by girdling, thus resulting in premature ripening and shrivelled seed

**Table 2** Numbers (percentage) of winter oilseed rape (cv. Catana) phoma stem canker subsamples with *L. maculans* or *L. biglobosa* DNA present determined by species-specific PCR for *L. maculans* and *L. biglobosa* (subsamples collected from stem base cankers or upper stem lesions sampled from all plots on 1 July 2014 were ground into a powder before DNA was extracted)

| | Number (%) of stem canker subsamples with | | | |
|---|---|---|---|---|
| | *L. maculans* only | *L. biglobosa* only | Both | Neither |
| Upper stem lesion ($n = 74$) | 45 (60.8 %) | 2 (2.7 %) | 11 (14.9 %) | 16 (21.6 %) |
| Basal stem canker ($n = 133$) | 102 (77 %) | 0 | 4 (2.7 %) | 27 (20.3 %) |

Three stem base cankers or upper stem lesions per plot

pods (West et al. 2002a). These results show that penthiopyrad + picoxystrobin or prothioconazole both prevent the formation of severe cankers, potentially allowing good pod development.

Furthermore, they show that foliar application of penthiopyrad + picoxystrobin or prothioconazole in the autumn reduced the number of *L. maculans* type leaf lesions that formed on leaves. Application of either fungicide when incidence of *L. maculans* leaf spotting reached 10 % of plants affected (T1) significantly reduced the number of lesions; a further application 1 or 2 months later (T2) appears to have had a smaller but still significant effect on the number of lesions. Work with GFP-labelled *L. maculans* has shown that if the phoma leaf spot stage is prevented, the pathogen does not grow along the leaf petiole to form stem cankers (Huang et al. 2006). Thus, this early stage inhibition stops the later development of cankers; exemplified here by the T1 and T2 application of either penthiopyrad + picoxystrobin or prothioconazole, which significantly reduced the number of lesions on leaves in November and December and significantly reduced stem canker severity in the following July.

By contrast, in seasons when there is little early phoma leaf spotting (e.g., 2011/12 and 2012/13), the data suggest that fewer fungicide sprays are needed since canker severity was very low and it did not affect yield. The timing and severity of basal stem cankers and upper stem lesions has previously been reported to affect the potential yield of winter oilseed rape crops (Zhou et al. 1999). Early, severe basal cankers or upper stem lesions are more likely to cause yield loss than later/slight basal stem cankers or upper stem lesions. The development of later, less severe stem cankers can be associated with a later release of ascospores, as shown by the 2011/12 and 2012/13 cropping seasons, when a large release of ascospores occurred later in the season compared to 2013/14. When there was less rainfall in August and September, the release of ascospores was delayed, resulting in a later onset of phoma leaf spotting. Disease severity has previously been linked to yield loss in winter oilseed rape; only when disease severity is high (≥3 on a 0–5 severity scale) does a yield response occur in fungicide treated plots (West et al. 2002b).

The results for timing of ascospore release and leaf spotting suggest that the optimum fungicide application regime differs between seasons. In 2013/14, ascospore release was earlier, due to greater rainfall in August/September, than in the previous two seasons, thus resulting in a more severe canker prior to harvest. These observations are in general agreement with the UK phoma stem canker disease model published by Evans et al. (2008), based on many seasons of data, since the model predicts an earlier date for 10 % phoma leaf spotting when rainfall and/or temperature are high during summer. Furthermore, the model predicts the date of onset and severity of canker using thermal time, with greater thermal time between 10 % phoma leaf spotting and harvest resulting in more severe cankers. This explains why canker severity was less in 2011/2012 and 2012/13, when winter temperatures were less than in 2013/14.

The low incidence of *L. biglobosa* leaf spots, and small amount of *L. biglobosa* DNA in stem canker samples suggests that the disease was caused predominantly by *L. maculans* in these experiments. It has been suggested that *L. maculans* and *L. biglobosa* have a north–south divide (Stonard et al. 2010), so a smaller amount of *L. biglobosa* in these southern sites was not unexpected. A multiple site study over several years is required to establish more information on the threat that *L. biglobosa* poses to UK oilseed rape production.

**Acknowledgments** This work was financially supported by the DuPont UK Ltd, Perry Foundation, Felix Thornley Cobbold Agricultural Trust, AHDB Cereals and Oilseeds and the Biotechnology and Biological Sciences Research Council (BBSRC). This research was also supported by ADAS UK Ltd and University of Hertfordshire. The authors thank Aiming Qi for statistical advice, Avice Hall for supervision, Fay Newbury for advice on modelling and Coretta Klöppel and Georgia Mitrousia for assisting with data collection.

## References

AHDB Cereals & Oilseeds. (2015). *Oilseed rape guide* (p. 31). Warwickshire: Agriculture and Horticulture Development Board.

Avenot, H. F., & Michailides, T. J. (2010). Progress in understanding molecular mechanisms and evolution of resistance to succinate dehydrogenase inhibiting (SDHI) fungicides in phytopathogenic fungi. *Crop Protection, 29*, 643–651.

Bartlett, D. W., Clough, J. M., Godwin, J. R., Hall, A. A., Hamer, M., & Parr-Dobrzanski, B. (2002). The strobilurin fungicides. *Pest Management Science, 58*, 649–662.

Delourme, R., Chevre, A. M., Brun, H., Rouxel, T., Balesdent, M. H., Dias, J. S., et al. (2006). Major gene and polygenic resistance to *Leptosphaeria maculans* in oilseed rape (*Brassica napus*). *European Journal of Plant Pathology, 114*, 41–52.

Eckert, M. R., Rossall, S., Selley, A., & Fitt, B. D. L. (2010). Effects of fungicides on *in vitro* spore germination and mycelial growth of the phytopathogens *Leptosphaeria maculans* and *L. biglobosa* (phoma stem canker of oilseed rape). *Pest Management Science, 66*, 396–405.

European Commission. (2007). *Review report for the active substance flusilazole* (p. 24). Brussels: EC Review Reports 6850/VI/97 final.

Evans, N., Baierl, A., Semenov, M. A., Gladders, P., & Fitt, B. D. L. (2008). Range and severity of a plant disease increased by global warming. *Journal of the Royal Society Interface, 5*, 525–531.

Fitt, B. D. L., Huang, Y.-J., van den Bosch, F., & West, J. S. (2006a). Coexistence of related pathogen species on arable crops in space and time. *Annual Review of Phytopathology, 44*, 163–182.

Fitt, B. D. L., Brun, H., Barbetti, M. J., & Rimmer, S. R. (2006b). World-wide importance of phoma stem canker (*Leptosphaeria maculans* and *L. biglobosa*) on oilseed rape (*Brassica napus*). *European Journal of Plant Pathology, 114*, 3–15.

Garthwaite, D. G., Hudson, S., Barker, I., Parrish, G., Smith, L., & Pietravalle, S. (2012). *Pesticide Usage Survery Report* (p. 87). York: Arable Crops in the United Kingdom 2012.

Huang, Y.-J., Evans, N., Li, Z., Eckert, M., Chèvre, A., Renard, M., & Fitt, B. D. L. (2006). Temperature and leaf wetness duration affect phenotypic expression of *Rlm6*-mediated resistance to *Leptosphaeria maculans* in *Brassica napus*. *New Phytologist, 170*, 129–141.

Huang, Y.-J., Hood, J. R., Eckert, M. R., Stonard, J. F., Cools, H. J., King, G. J., et al. (2011). Effects of fungicide on growth of *Leptosphaeria maculans* and *L. biglobosa* in relation to development of phoma stem canker on oilseed rape (*Brassica napus*). *Plant Pathology, 60*, 607–620.

Huang, Y.-J., Karandeni-Dewage, C. S., & Fitt, B. D. L. (2014a). Importance of *Leptosphaeria biglobosa* as a cause of phoma stem canker on winter oilseed rape in the UK. *Aspects of Applied Biology, 127*, 117–122.

Huang, Y.-J., Qi, A., King, G. J., & Fitt, B. D. L. (2014b). Assessing quantitative resistance against *Leptosphaeria maculans* (phoma stem canker) in *Brassica napus* (oilseed rape) in young plants. *PloS One, 9*, e84924.

Lacey, M. E., & West, J. S. (2006). *The air spora: a manual for catching and identifying airborne biological particles* (p. 156). United States of America: Springer US.

Liu, S. Y., Liu, Z., Fitt, B. D. L., Evans, N., Foster, S. J., Huang, Y.-J., et al. (2006). Resistance to *Leptosphaeria maculans* (phoma stem canker) in *Brassica napus* (oilseed rape) induced by *L. biglobosa* and chemical defence activators in field and controlled environments. *Plant Pathology, 55*, 401–412.

Lô-Pelzer, E., Aubertot, J. N., Bousset, L., Pinochet, X., & Jeuffroy, M. H. (2009). Phoma stem canker (*Leptosphaeria maculans*/*L. biglobosa*) of oilseed rape (*Brassica napus*): is the G2 Disease Index a good indicator of the distribution of observed canker severities? *European Journal of Plant Pathology, 125*, 515–522.

Marx-Stoelting, P., Niemann, L., Ritz, V., Ulbrich, B., Gall, A., Hirsch-Ernst, K. I., et al. (2014). Assessment of three approaches for regulatory decision making on pesticides with endocrine disrupting properties. *Regulatory Toxicology and Pharmacology, 70*, 590–604.

R Development Core Team, R. (2011). R: A Language and Environment for Statistical Computing. (R. D. C. Team, Ed.) *R Foundation for Statistical Computing*. doi:10.1007/978-3-540-74686-7.

Steed, J. M., Baierl, A., & Fitt, B. D. L. (2007). Relating plant and pathogen development to optimise fungicide control of phoma stem canker (*Leptosphaeria maculans*) on winter oilseed rape (*Brassica napus*). *European Journal of Plant Pathology, 118*, 359–373.

Stonard, J. F., Latunde-Dada, A. O., Huang, Y.-J., West, J. S., Evans, N., & Fitt, B. D. L. (2010). Geographic variation in severity of phoma stem canker and *Leptosphaeria maculans*/*L. biglobosa* populations on UK winter oilseed rape (*Brassica napus*). *European Journal of Plant Pathology, 126*, 97–109.

West, J. S., Balesdent, M., Rouxel, T., Narcy, J. P., Huang, Y.-J., Roux, J., et al. (2002a). Colonization of winter oilseed rape tissues by A/Tox$^{+}$ and B/Tox$^{0}$ *Leptosphaeria maculans* (phoma stem canker) in France and England. *Plant Pathology, 51*, 311–321.

West, J. S., Fitt, B. D. L., Leech, P. K., Biddulph, J. E., Huang, Y., & Balesdent, M. (2002b). Effects of timing of *Leptosphaeria maculans* ascospore release and fungicide regime on phoma leaf spot and phoma stem canker development on winter oilseed rape (*Brassica napus*) in southern England. *Plant Pathology, 51*, 454–463.

Zhou, Y., Fitt, B. D. L., Welham, S. J., Gladders, P., Sansford, C. E., & West, J. S. (1999). Effects of severity and timing of stem canker (*Leptosphaeria maculans*) symptoms on yield of winter oilseed rape (*Brassica napus*) in the UK. *European Journal of Plant Pathology, 105*, 715–728.

# Azole sensitivity in *Leptosphaeria* pathogens of oilseed rape: the role of lanosterol 14α-demethylase.

[a]Thomas R Sewell, [b]Nichola J Hawkins, [a]YongJu Huang, [a]Henrik U Stotz, [c]Steven L Kelly, [c]Diane E Kelly, [b]Bart Fraaije, [a]Bruce DL Fitt

[a] Crop Protection Group, Centre for Agriculture, Food and Environmental Management, University of Hertfordshire, Hatfield, UK

[b] Biological Chemistry and Crop Protection, Rothamsted Research, Harpenden, Hertfordshire, United Kingdom.

[c] Centre for Cytochrome P450 Biodiversity, Institute of Life Science, College of Medicine, Swansea University, Swansea, UK.

## Abstract

Lanosterol 14-α demethylase (*erg11*, CYP51) is a key enzyme intermediating the biosynthesis of ergosterol in fungi, and the target of azole fungicides. Previously, it had been suggested that *Leptosphaeria maculans* and *L. biglobosa,* the causal agents of phoma stem canker on oilseed rape (*Brassica napus*), differ in their sensitivity to some azoles used to control the disease. We sought to determine the role CYP51 has in the fungicide-sensitivity differences previously described between the two *Leptosphaeria* species. Heterologous yeast expression of LmCYP51B and LbCYP51B with fungicide sensitivity testing of the yeast transformants suggests that LmCYP51B and LbCYP51B are similarly sensitive to azole fungicides flusilazole, prothioconazole-desthio and tebuconazole. These

findings are supported by homology protein modelling, which predicts that LmCYP51B and LbCYP51B are structurally very similar, specifically at the azole-binding site. Supplementary analysis into the differential response of the two species to azole fungicides shows that they sometimes have a very minor difference in sensitivity *in vitro* but not *in planta* and at present both species remain very sensitive to azole fungicides.

## Importance

Globally, phoma stem canker is a damaging disease of oilseed rape, causing an estimated £700 million worth of damage each cropping season. Fungicides are a key component of integrated control strategies used to reduce the severity of the disease epidemics and increase the potential yield of the crop. A more detailed understanding of the efficacy of azole fungicides on the two *Leptosphaeria* species that cause phoma stem canker will improve crop protection, increase the lifespan of an important class of fungicides and advance global food security.

## Introduction

Antifungal demethylation inhibitors (DMIs), e.g. triazoles and imidazoles (azoles), target lanosterol 14α-demethylase (CYP51), a member of the cytochrome P450 superfamily and key regulatory enzyme in the ergosterol biosynthetic pathway (1, 2). Ergosterol is an essential structural component of the fungal plasma membrane and is required to maintain membrane integrity, fluidity and

permeability. Inhibition of CYP51 blocks the synthesis of ergosterol, which subsequently increases cytotoxic sterol precursors. The lack of membrane-bound ergosterol and the accumulation of toxic molecules in the cytosol have a combined fungistatic effect on the cell, decreasing membrane integrity and permeability (3).

The azoles are used to control fungal pathogens in both clinical and agricultural situations. In the clinical setting, voriconazole, itraconazole and posaconazole are commonly used to treat the opportunistic human pathogen *Aspergillus fumigatus,* which can cause invasive pulmonary infections amongst immunocompromised patients (4), along with other invasive pathogens, e.g. *Candida* spp (5). In agriculture, a larger variety of azole fungicides have been used to control numerous fungal plant pathogens since their inception in the 1970s (6). Since 2005, azole fungicides have maintained approximately 20% share of the global fungicide market, which in total (including seed treatments) was worth $13.3 billion in 2011 (6–8).

Agricultural azole fungicides are often used as a component of fungicide programmes (often in mixtures) to control phoma stem canker, a damaging disease of winter oilseed rape (*Brassica napus*), which is caused by coexisting fungal plant pathogens *Leptosphaeria maculans* and *L. biglobosa* (9). Globally, phoma stem canker causes approximately £700 million worth of yield losses,

making it a considerable threat to sustainable oilseed rape production and food security (10).

Recent studies have suggested that the two *Leptosphaeria* spp. pathogens differ in their sensitivity to azole fungicides, both *in vitro* (11) and *in planta* (12), with *L. biglobosa* exhibiting a less sensitive phenotype in the presence of flusilazole and tebuconazole. Differences in sensitivity between coexisting plant pathogens have previously been shown to affect population structure (13) and may explain the recent increase in *L. biglobosa* incidence in some UK locations (14).

Increased exposure to azole fungicides has led to the evolution of insensitivity in some plant pathogen populations. Currently, three mechanisms of insensitivity to DMIs have been identified in agricultural crop pathogens; these are 1) target site polymorphisms 2) CYP51 overexpression or presence of CYP51 paralogs (CYP51A, CYP51B, CYP51C) and 3) increased expression of efflux pump genes (6, 15, 16). Missense mutations in the coding sequence of *erg11*, the gene that encodes CYP51, and subsequent alterations in the secondary structure of CYP51, correlate with an insensitivity to DMI fungicides and are currently the most common mechanism of azole-insensitivity (6, 15, 17–19). Using predictive homology modelling, polymorphisms in the CYP51 coding sequence have been shown to increase the size of the azole binding pocket, thus decreasing the affinity of the bound fungicide and reducing its effectiveness as an anti-fungal (20).

Using heterologous expression in a *Saccharomyces cerevisiae* mutant, together with homology protein modelling, we have investigated the role lanosterol 14α-demethylase plays in the phenotypic fungicide-sensitivity differences previously described between *Leptosphaeria maculans* and *L. biglobosa* populations. Moreover, we further investigate fungicide sensitivity of *L. maculans and L. biglobosa* populations both *in vitro* and *in planta.*

## Materials and methods

### Characterisation of *erg11* and CYP51B in *L. biglobosa*

*L. biglobosa* CYP51B (LbCYP51B) characterisation began with a BLASTp search of GenBank using the published *L. maculans* CYP51B (LmCYP51B) protein sequence (AAN28927.1). A Clustal Omega multiple sequence alignment was generated using six protein sequences with similar percentage identity: *Pyrenophora tritici-repentis* (XP_001939023.1), *Alternaria alternata* (OAG21175.1)*, Stagonosporopsis caricae* (AMM76225.1)*, Botrytis cinerea* (CCD54835.1)*, Sclerotinia sclerotiorum* (XP_001594997.1) and *Aspergillus nidulans* (XP_681552.1) (21, 22). Using the alignment, a 13 amino acid region (DVVYDCPNSKLME) conserved across five of the six aligned species was selected for a custom BLASTp search using Geneious R9.1.4 (Biomatters Limited, New Zealand), which was executed against a database of translated

115 open reading frames generated from published *L. biglobosa* genome data
116 provided by INRA-Bioger, France (23). The corresponding nucleotide sequence
117 was manually identified using an annotations text search; BLASTn search was
118 performed to corroborate the putative LbCYP51 nucleotide sequence.
119
120 LmCYP51B and LbCYP51B sequencing
121
122 Total DNA from 12 *L. maculans* isolates and eight *L. biglobosa* isolates was
123 extracted using DNAMITE Plant DNA extraction kit (Microzone) according to
124 manufacturer's specifications. PCR amplification was completed using
125 sequencing primers for both species (ST 1) and REDTaq DNA polymerase
126 (Sigma). Amplification conditions for both species were 1 cycle at 95 °C for 2
127 min; 35 cycles at 95 °C for 40 sec, 53.5 °C for 30 sec and 72 °C for 1 min;
128 followed by a final extension at 72 °C for 5 min. PCR products were purified
129 using QIAquick PCR Purification kit (Qiagen, USA) according to the
130 manufacturer's guidelines. For sequencing, samples were prepared to a final
131 concentration of 20 - 30 ng $\mu l^{-1}$ and sent to GATC Biotech for Sanger
132 sequencing; sequence data were analysed using Geneious R9.1.4 and contigs
133 assembled using Cap3 (24), with low quality ends trimmed manually.
134 Constructed contigs were aligned alongside the published LmCYP51B gene
135 sequence (AY142146) as the reference sequence. Sequences were manually
136 trimmed to the start (ATG) and stop codon (TAG). The recognised location of a
137 single intron in LmCYP51B was removed manually. The predicted location of a

single intron in LbCYP51B, inferred from the alignment with *L. maculans* reference sequence, was also removed. Finally, nucleotide sequences were checked using ORF Finder (NCBI 2016) and translated into amino acid sequence using Geneious R9.14.

Phylogenetic analysis was carried out using Molecular Evolutionary Genetics Analysis (MEGA7) software (25). Firstly, several notable CYP51B orthologs were obtained using a BLASTn search, which was executed on the published LmCYP51B nucleotide coding sequence. Sequences were translated and a Clustal Omega alignment performed on the translated CYP51B protein sequences from *L. maculans*, *L. biglobosa*, *P. tritici-repentis*, *A. alternata, Phaeosphaeria nodorum* (XM_001938988.1)*, Stagonosporopsis cucurbitacearum* (ANP43771.1), *B. cinerea*, *S. sclerotiorum*, *A. fumigatus* CYP51A (JX283445.1) and *A. fumigatus* CYP51B (XM_744041.1), *A. flavus* (KOC13803.1)*, A. niger* (GAQ46773.1) and *Saccharomyces cerevisiae* (NP_011817.1), with *Homo sapiens* (NP_000777.1) as an outgroup. The corresponding codon alignment was generated and phylogenetic relations were inferred by maximum-likelihood with 1000 bootstrap replications.

Heterologous expression of LmCYP51 and LbCYP5 in *Saccharomyces cerevisiae*

Total RNA was extracted from one *L. maculans* (Hrox 12-2-1) and one *L. biglobosa* ($F_2$ dm 11-5) isolate using the RNeasy Plant Mini Kit (Qiagen). Mycelium of each species was grown in Sabouraud dextrose broth for 10 days, harvested through centrifugation, flash-frozen in liquid nitrogen, freeze-dried for 24 h and then ground into a fine powder. The remaining RNA extraction procedure was done according to manufacturer's instructions. Complementary DNA (cDNA) was synthesised from 1 µg of total RNA using the SuperScript® IV First-Strand Synthesis System (SSIV) (Invitrogen) according to manufacturer's guidelines. The complete LmCYP51B and LbCYP51B coding sequences were amplified using primer pairs LmCYP51res (forward/reverse) and LbCYP51res (forward/reverse) (ST 1). Restriction primers introduced a *HindIII* restriction site to the 5" end and a *NotI* restriction site to the 3" of the amplified CYP51B sequences.

A touchdown-PCR (26) was completed using EasyA High Fidelity PCR Cloning Enzyme (Agilent, USA). Amplification conditions for LmCYP51 were 1 cycle at 94°C for 2 min; 7 cycles at 94 °C for 30 sec, 59 to 53°C each for 1 min (1 °C decrease per cycle) and 72 °C for 2 min; 28 cycles at 94 °C for 30 sec, 52°C for 1 min and 72°C for 1 min; followed by a final extension at 72 °C for 7 min. Amplification conditions for LbCYP51 were 1 cycle at 94 °C for 2 min; 7 cycles at 94 °C for 30 sec, 58 to 52 °C each for 1 min (1 °C decrease per cycle) and 72 °C for 2 min; 28 cycles at 94 °C for 30 sec, 56 °C for 1 min and 72 °C for 1 min; followed by a final extension at 72 °C for 7 min. PCR products were purified,

cloned into the pGEM-T easy vector (Promega, USA) and transformed into high efficiency JM109 competent cells (Promega) using ampicillin selection and blue-white screening, all according to the manufacturer's specification. Colony PCR used primers LmCYP51res (forward/reverse) and LbCYP51res (forward/reverse) to determine successful ligation of LmCYP51B or LbCYP51B. PCR was carried out using REDTaq DNA polymerase (Sigma) with a 52 $^{o}$C or 51$^{o}$C annealing temperature, respectively, and 40 seconds extension time. Plasmids were extracted using the QIAprep Spin Miniprep Kit (Qiagen).

Restriction digestions of pGEM-T-LmCYP51 and pGEM-T-LbCYP51 constructs were prepared using *HindIII* and *NotI* high fidelity enzymes (New England Biolabs) according to manufacturer's double-digestion specification. The yeast vector, pYES2/CT (Thermo Fisher, USA) was digested under the same conditions. Digested pYES2/CT, LmCYP51 and LbCYP51 were purified by gel electrophoresis (0.7 % agarose) using QIAquick gel extraction kit according to manufacturer's specification. Purified samples were then ligated and transformed into high efficiency JM109 competent *E. coli* cells. Colony PCR was used to check which *E. coli* colonies had the correct insertion. Positive transformants underwent plasmid extraction and the purified pDNA was used for transformation of *S. cerevisiae* strain YUG37: *erg11* (MATa ura3-52 trp1-63 LEU2::tTA tetO-CYC1::erg11) (27) using the S.c EasyComp transformation kit (Invitrogen, USA). YUG37:LmCYP51B and YUG37:LbCYP51B transformants were plated out onto synthetic dropout minimal medium (SD) containing 4 g $l^{-1}$ yeast nitrogen base

206 without amino acids (Sigma), 3.92 g $l^{-1}$ dropout medium supplement without
207 uracil (Sigma), 2 % galactose (GAL) and 2 % raffinose (RAF).
208
209 YUG37::LmCYP51B, YUG37::LbCYP51B and YUG37::pYES2/CT (negative
210 control) transformants were grown in 10 ml liquid SD medium for 24 h at 30 $^{O}$C
211 with shaking (250 RPM). Cell suspensions were diluted to a concentration of $10^6$
212 cells $ml^{-1}$ and inoculated onto SD GAL+RAF agar plates with or without 3 µg $ml^{-1}$
213 doxycycline (5 µl of five 5-fold dilutions). Plates were incubated at 30 $^{o}$C and
214 photographed after 5 days.
215
216 Fungicide sensitivity of YUG37::LmCYP51B and YUG37::LbCYP51B
217 transformants was tested using flusilazole, tebuconazole and prothioconazole-
218 desthio fungicides. A single transformant colony was grown in 10 ml liquid SD
219 GAL+RAF for 24 h at 30 $^{o}$C with shaking. The resultant cell suspensions were
220 diluted to a concentration of $10^6$ cells $ml^{-1}$ and 100 µl aliquots were added to a
221 flat-bottomed 96-well microtitre plate. The microtitre plate was subsequently
222 populated with 100 µl of SD GAL+ RAF media with doxycycline (6 µg $ml^{-1}$), which
223 had been amended with decreasing concentrations of fungicide (5, 1.7, 0.56,
224 0.19, 0.062, 0.021, 0.0069, 0.0023, 0.00076, 0.00025, 8.47 x $10^{-5}$, 2.82 x $10^{-5}$, 0
225 µg $ml^{-1}$). Plates were incubated at 30 $^{O}$C for 6 days. Fungal growth was
226 measured by absorbance using a FLUOstar OPTIMA microplate reader (BMG
227 Labtech, Offenburg, Germany) set to 630 nm in endpoint mode. Fungicide
228 sensitivities for each isolate were calculated as 50% effective concentrations

229 ($EC_{50}$) using a dose-response relationship curve generated by the FLUOstar
230 OPTIMA microplate software. Distribution of data was determined using a
231 Shapiro-Wilk normality test in R (28). Significant differences in the distribution of
232 $EC_{50}$ values between species were determined using a One-Way ANOVA in R.
233
234 Comparative homology modelling
235
236 Predictive homology protein modelling was processed in Chimera (29) using
237 Modeller v9.14 (30). Firstly, to identify similar CYP51B sequences that have been
238 structurally solved, a BLASTp search of the protein data bank (PDB) database
239 was done individually on LmCYP51B and LbCYP51B amino acid sequences.
240
241 A Clustal Omega multiple sequence alignment was performed on the three most
242 homologous CYP51B protein sequences, together with either LmCYP51B or
243 LbCYP51B. For both *Leptosphaeria* spp., the closest solved CYP51B structures
244 were from *A. fumigatus, S. cerevisiae* and *H. sapiens* (SF 1a-b). The three
245 homologous CYP51B structures used for modelling were automatically loaded
246 into the software using annotations attached to the multiple sequence alignment
247 files. Bound ligands within these structures were manually removed and the
248 heme cofactor, which is a feature of CYP51B, was manually removed from the *S.*
249 *cerevisiae* and *H. sapiens* structures but conserved from *A. fumigatus*.
250

The Advanced options in Modeller v9.14 were used to build the model with hydrogens and the number of output models was set to five. Selection of the most reliable model was done using ModFOLD4 (31). Prothioconazole-desthio, in the PDB: 5EAD binding confirmation was superimposed into the predicted structures using Matchmaker in Chimera with parameters set to default. Hydrogen bonds were predicted using H-bond finder in Chimera with parameters set to default. A structural alignment (TM-align) using a residue-to-residue comparison was generated with a 0 to 1 (32).

Fungicide sensitivity *in vitro*

Sensitivity assays, modified from those of Pijls et al. (1994)(33), consisted of 2x sabouraud dextrose media amended with decreasing concentrations of technical grade flusilazole, tebuconazole (20, 10, 5, 2.5, 1.25, 0.63, 0.31, 0.16, 0.078, 0.039, 0.020, 0 μg $ml^{-1}$ final concentration) or prothioconazole-desthio (20, 6.67, 2.22, 0.74, 0.25, 0.082, 0.027, 0.009, 0.003, 0.001, 0.0003, 0 μg $ml^{-1}$ final concentration). Aliquots (100 μl) of the fungicide-amended media were added to the wells of flat-bottomed 96-well microtitre plates (NUNC™, Thermo Fisher, USA). Aliqouts (100 μl) of conidial suspensions ($10^6$ conidia $ml^{-1}$) of 23 *L. maculans* isolates and 22 *L. biglobosa* isolates were added to each well of a single row (in duplicate). Plates were incubated at 20 °C for 4 days and fungal growth measured by absorbance using a FLUOstar OPTIMA microplate reader (BMG Labtech, Offenburg, Germany) set to wavelength 630 nm in endpoint

mode. Fungicide sensitivities for each isolates were calculated as $EC_{50}$ using a dose-response relationship curve generated by the FLUOstar OPTIMA microplate software. Distribution of data was determined using a Shapiro-Wilk normality test in R. Significant differences in the distribution of $EC_{50}$ values between species were determined using a Mann-Whitney *U*-test in R.

Prothioconazole efficacy *in planta*

Seeds of *B. napus*, cv. Catana (Dekalb, Monsanto, UK), were pre-germinated in a shallow rectangular germination tray (50 x 30 x 8 cm) containing a mixture of 50% enriched general-purpose compost (Miracle-Gro, UK) and 50% John Innes No 3 compost (JA Bower, UK). After 10 days growth, plants were arranged in an alternate block design (two replicate blocks each containing six treatments with eight plants per treatment) located in a plant growth chamber (Conviron Europe, UK). The chamber was set to a 12-hour light/12-hour dark cycle with a light intensity of 210 μE $m^2$ $s^{-1}$. Under regular growing conditions, relative humidity in the chamber was set at 70% and temperature was set at 20 $^oC$ when light and 18 $^oC$ when dark.

After 12 days growth, the cotyledons were inoculated with the conidial suspension of one *L. maculans* ($H_{ROX}$ 12-2-1) or one *L. biglobosa* ($F_2$ Exc dm 11-5) isolate by wounding. Conidial suspension (10 μl), at a concentration of $10^6$ conidia $ml^{-1}$, was applied directly to each wound site. After inoculation, the plants

were left for 24 hours in darkness and 100% humidity, before re-establishing normal growing conditions. At 6 DPI, commercial grade prothioconazole was applied at a concentration of 2 µg $ml^{-1}$ or 20 µg $ml^{-1}$ using a water atomiser. Untreated control (0 µg $ml^{-1}$ prothioconazole) cotyledons were sprayed with water.

Disease assessments were completed by measuring lesion diameter (mm) at 14 DPI. The cotyledons were subsequently removed, instantly frozen in liquid nitrogen and stored at -80 $^{O}$C for qPCR analysis. Frozen leaves were ground into a fine powder and DNA was extracted and diluted to a concentration of 25 ng $µl^{-1}$. Amplification of *L. maculans* or *L. biglobosa* DNA was done using LmacF/LmacR or LbigF/LmacR primers, respectively. The PCR reactions used Brilliant III Ultra-fast SYBR Green qPCR master mix with low Rox at a final volume of 20 µl and a primer concentration of 0.3 µM. PCR parameters were as follows: initial denaturation at 95 °C for 2 min, followed by 40 cycles of 95 °C for 15 sec, 60 °C (*L. maculans*) or 55 °C (*L. biglobosa*) for 30 sec and 72 °C for 36 sec.

Distribution of data was determined using a Shapiro-Wilk normality test in R. Significant differences in lesion sizes between species and between fungicide concentrations were determined independently using a Kruskal-Wallis rank sum test in R. Significant differences in DNA quantity between species were determined using One-Way ANOVA and *post hoc* Tukey honest significant test in R.

## Results

### Characterisation and sequencing of Lm*erg11*/CYP51B and Lb*erg11*/CYP51B

All 12 *L. maculans* isolate sequence assemblies (GenBank accession numbers KY500978 – KY500989) encompassed Lm*erg11*, which was 1635 bp in length and consisted of two exons and one 54 bp intron; nucleotide sequence similarity between all isolates was 100%. LmCYP51B was identical in all 12 isolates but differed by 1 amino acid (K337N) when compared with the published LmCYP51B sequence (AAN28927.1) from Australia. Lb*erg11* was encompassed within all *L. biglobosa* sequence assemblies (GenBank accession numbers KY500970 – KY500977), was 1642 bp in length and consisted of two exons and one 58 bp intron. Of the eight isolates sequenced, six had an identical *erg11* gene, while two isolates (F2 dm 11-5 and H Dr 12 12) contained the same single nucleotide polymorphism (SNP), namely C1467T. Nonetheless, the SNP proved synonymous and all LbCYP51B sequences were 100 % similar. Neither *Leptospaheria* spp. contained amino acid alterations previously recognised as conferring reduced azole sensitivity in other plant pathogenic fungi (6).

A pair-wise alignment between LmCYP51B and LbCYP51B identified 24 amino acid alterations, one amino acid deletion and a pair-wise percentage identity of 97.5 % (Score matrix = BLOSUM80, threshold = 1) (SF 2). Of the 24 alterations,

12 were considered a change to amino acids with similar physical-chemical properties and 12 considered a change to amino acids with dissimilar physical-chemical properties (Score matrix = BLOSUM80, threshold = 1).

Phylogenetic analysis by maximum likelihood clearly shows that CYP51B from *L. maculans* and *L. biglobosa* share a similar evolutionary trajectory but are distinct from one another. Moreover, they share a clade with other asomycete plant pathogen fungi, namely *P. tritici-repentis*, *A alternate, P.nodorum* and *S. cucurbitacearum* (Fig 1).

(Fig 1 here)

Heterologous expression of LmCYP51B and LbCYP51B in *Sacharomyces cerevisiae*

The vector-only transformed strain YUG37::pYES2/CT, which lacked a complementary CYP51B protein, was unable to grow on the doxycycline amended SD GAL+RAF medium due to the repression of native CYP51B expression in *S. cerevisiae*. Complementation of native CYP51B was achieved in YUG37::LmCYP51B and YUG37::LbCYP51B *S. cerevisiae* transformants, which both showed robust growth across five 5-fold dilutions on SD GAL+RAF medium amended with doxycycline; demonstrating that expression of the predicted wild-

type CYP51B protein from *L. maculans* or *L. biglobosa* can support ergosterol synthesis in *S. cerevisiae* (Fig 2)

(Fig 2 here)

All YUG37 transformants expressing LmCYP51B or LbCYP51B were very sensitive to tebuconazole, flusilazole and prothioconazole-desthio (Table 1). There was a marginal, 1.35-fold (1 df, $P$ <0.05) difference between the $EC_{50}$ values of YUG37::LmCYP51B and YUG37::LbCYP51B transformants for tebuconazole, and no significant difference in sensitivity to flusilazole or prothioconazole-desthio.

(Table 1 here)

Comparative homology modelling

For both species, all five CYP51B models that were predicted by Modeller had a very high structural fold confidence (P <0.001) and a global model quality score of 0.89 (0 to 1 scale). The best models for each species, Lm_cyp51_model_final_5.pdb and Lb_cyp51_model_final_4.pdb, were selected for further analysis.

The chosen predicted structures of LmCYP51B and LbCYP51B were very similar (TM-score = 0.90). The root-mean-square deviation (RMSD) of the common residues was 2.76 Å on a 526 amino acid alignment length (Fig 3). The globular active sites of the proteins appear to have the highest structural conservation. Noteworthy differences in the predicted structures were distinguishable only in the trans-membrane tail, although some amino acid substitutions did slightly alter the predicted fold of the globular structure. The heme cofactor and subsequently bound prothioconazole-desthio ligand were shown to be located inside the binding pocket of the proteins. The surrounding amino acids, which form this pocket, were all highly conserved (Fig 4). Moreover, important residues involved in the development of a water-mediated hydrogen bond network that facilitates CYP51B/azole interaction were structurally conserved; specifically Tyr122 and Tyr136 in both *L. maculans* and *L. biglobosa* (Fig 4).

(Figure 3 here)

(Figure 4 here)

Detailed two-dimensional schematics of protein-ligand interactions identified further similarities between the predicted structures of LmCYP51B (SF 3a) and LbCYP51B (SF 3b). A water-mediated hydrogen bond network was maintained in both structures, specifically facilitated by the presence of Tyr136, a water molecule and the relative location of the heme cofactor. Furthermore, five other residues, Tyr122, Ala306 (Ala307 in LbCYP51B), Ile372 (Ile373 in LbCYP51B),

410 Ser374 (Ser375 in LbCYP51B) and Phe508 (Phe509 in LbCYP51B), which were
411 interacting with the bound ligand (prothioconazole-desthio), were correspondingly
412 located in the binding pocket. The predicted structure of LbCYP51B had two
413 extra residues, Ser311 and Leu508, both located sufficiently close to interact with
414 the ligand.
415
416 Fungicide sensitivity *in vitro*
417
418 Both species were very sensitive to prothioconazole-desthio, flusilazole and
419 tebuconazole (Fig 5). Comparison between *L. maculans* (n = 23) and *L.*
420 *biglobosa* (n = 21) isolates identified a significant difference in sensitivity to
421 flusilazole (Figure 5a) ($W = 373$, $P < 0.01$) and prothioconazole-desthio (Figure
422 5b) ($W = 39$, $P < 0.001$). *L. maculans* isolates were 2.07 times less sensitive than
423 *L. biglobosa* isolates to prothioconazole desthio, whereas *L. biglobosa* was 1.31
424 times less sensitive to flusilazole than *L. maculans*. There was no significant
425 difference in sensitivity to tebuconazole (Figure 5c).
426
427 (Figure 5 here)
428
429 Prothioconazole efficacy *in planta*
430
431 At 14 DPI, treatment with prothioconazole showed a significant effect on *L.*
432 *maculans* or *L. biglobosa* lesion diameter (Kruskal-Wallis chi-squared = 124.4, 2

433 df, $P < 0.05$) (Fig 6), with lesion diameter decreasing as concentration increased.
434 A concentration of 20 µg $ml^{-1}$ prothioconazole reduced both *L. maculans* and *L.*
435 *biglobosa* lesion size by 3 fold compared to the untreated control. Moreover, *L.*
436 *biglobosa* and *L. maculans* lesions were not statistically different in their size on
437 prothioconazole-treated leaves.
438
439 (Figure 6 here)
440
441 DNA quantity for each treatment was determined using qPCR to quantify the
442 effect of fungicide treatment on pathogen growth (Fig 7). Increased concentration
443 of fungicide decreased the amount of *L. maculans* DNA (2 df, $P <0.05$) but did
444 not significantly decrease amount of *L. biglobosa* DNA, which remained low. A
445 concentration of 20µg $ml^{-1}$ prothioconazole reduced the amount of *L. maculans*
446 DNA by 23 times and the amount of *L. biglobosa* DNA by 6 times compared to
447 the untreated controls.
448
449 (Figure 7)
450
451 **Discussion**
452 Heterologous yeast expression of lanosterol 14α-demethylase (CYP51B) from
453 *Leptosphaeria maculans* (LmCYP51B) and *L. biglobosa* (LbCYP51B), together
454 with subsequent fungicide sensitivity assays on the yeast transformants, suggest
455 that LmCYP51B and LbCYP51B interact similarly with azole fungicides

456 flusilazole, prothioconazole-desthio and tebuconazole, which exhibited a minor
457 difference in $EC_{50}$ between strains expressing the two different proteins.
458 Homology modelling supports these findings, which predicts that LmCYP51B and
459 LbCYP51B, a key enzyme in the synthesis of ergosterol in fungi, share a high
460 level of structural similarities, specifically at the azole binding site.
461
462 Sequence analysis confirmed that both LmCYP51B and LbCYP51B contained no
463 previously identified CYP51B mutations that confer azole resistance or azole
464 insensitivity in other plant and human pathogenic fungi (8, 34–36). Therefore,
465 evolution of resistance to azole fungicides, which has been strongly linked to the
466 gradual development of missense mutations in the CYP51B amino acid
467 sequence (17, 18, 20, 37, 38), has not yet occurred in either the *L. maculans* or
468 the *L. biglobosa* populations sampled. Nonetheless, there were multiple amino
469 acid substitutions between LmCYP51B and LbCYP51B that could potentially
470 affect the tertiary structure of the proteins and thus the azole binding potential.
471
472 Changes to the structure of CYP51B have previously been proposed to alter the
473 affinity of azoles, such as tebuconazole or triadimenol; this was determined
474 through predictive homology modelling of wild type (MgCYP51B) and mutant
475 CYP51B proteins found in insensitive *Mycosphaerella graminicola* populations
476 (20). More recently, analysis of a high resolution *S. cerevisiae* CYP51B structure
477 (PDB:4WMZ), co-crystallised with medical azole fluconazole, identified two water
478 molecules within the active site of the proteins that form a network of hydrogen

bonds adhering the azole to the protein (39). The presence of Y136F/H substitution (ScCYP51B) breaks one part of this water-mediated hydrogen bond network due to the loss of the hydroxyl group incurred by the tyrosine to phenylalanine or histidine substitution. This reduces the binding capacity of the proteins, acknowledged through spectral characterisation of fluconazole binding with the wildtype ScCYP51B protein or Y140F/H mutants (40).

Here the predicted structures of LmCYP51B and LbCYP51B have been compared through the structural alignment of homology models. Prothioconazole-desthio, the predicted *in planta* metabolite of agricultural azole prothioconazole (41), was superimposed using its confirmation in co-crystallised ScCYP51B structure PDB:5EAD. Analysis of the alignment (Fig 3) noticeably illustrates that structural conservation is maintained throughout the globular pocket. Only in the transmembrane tail is there any noticeable distance between the aligned residues.

Locality of residues: Y136, K147 and *G461/G462 (*LmCYP51B/LbCYP51B, respectively), which correspond to substitutions Y132F, K143R and G464S in resistant *Candida albicans* isolates (Y140F, K151R and G464S in ScCYP51B) (36), are all conserved in *L. maculans* and *L. biglobosa*. In addition to this, Y122 (Y126 in ScCYP51B) and *S374/S375 (S383 in ScCYP51B), which form a water-mediated hydrogen bond network with another water molecule when fluconazole is bound, are also structurally conserved. In fact, according to the multiple

sequence alignment with other plant and human pathogenic fungi, many residues interacting with the azole, heme cofactor and/or the water-meditated hydrogen bond network are uniformly conserved, along with that of the *S. cerevisiae* and the *H. sapiens* orthologs.

Consequentially, it can be postulated that the wild type LmCYP51B and LbCYP51B proteins would interact with azole fungicides similarly due to the likeness of key structural locations and the preservation of a water-mediated hydrogen bond network in the example shown. Testing of this hypothesis was done using heterologous yeast expression and subsequent fungicide sensitivity assays, to investigate the efficacy of various azole fungicides on LmCYP51B and LbCYP51B within an isogenic background; previous studies have used this method to investigate the effects of CYP51B mutations on fungicide efficacy, showing a causal link between key mutations and resistant phenotypes (16, 17, 38). Both LmCYP51B and LbCYP51B yeast transformants responded similarly to azole treatment, suggesting that the affinity of various azole fungicides to both LmCYP51B and LbCYP51B is similar, with the only significant difference between the two transformants a 1.35-fold change in tebuconazole $EC_{50}$. A minor difference when compared to MgCYP51B harbouring mutations L50S and Y461H, which conferred a resistance factor (fold-change against wild-type) of 63.2 to tebuconazole and 192 to triadimenol, when expressed in yeast (38).

Despite a difference in tebuconazole sensitivity at the protein level, the same difference was not observed between organisms; both *L. maculans* and *L. biglobosa* isolates had a similar response to tebuconazole *in vitro.* Interestingly, the two species did differ in their sensitivity to flusilazole and prothioconazole-desthio, both of which did not exhibit a differing effect on LmCYP51B and LbCYP51B when heterologously expressed in yeast. It is possible that these minor differences in sensitivity could be due to natural variation in the uptake, efflux or metabolism of fungicide compounds, however to determine this would require further investigation.

Ultimately, all *L. maculans* and *L. biglobosa* isolates sampled were sensitive to tebuconazole, flusilazole and prothioconazole-desthio. Moreover, the distribution of $EC_{50}$ values for each fungicide were constrained, suggesting that no isolate had developed increased sensitivity to fungicide treatment prominently distinctive from the wild-type population. This evidence supports previous historical findings that proposed there to be no evidence of azole-insensitivity among populations of either *L. maculans* or *L. biglobosa* (11, 12), together with implications determined from CYP51B sequence analysis and corroboration with current literature. Similarities in sensitivity are also observed *in planta*, with prothioconazole treatment having a similar affect on the lesion size of *L. maculans* or *L. biglobosa* when colonising oilseed rape cotyledons. Despite this similarity, however, DNA quantities varied greatly between species and only *L. maculans* was significantly affected by increasing prothioconazole concentrations. Difference in DNA

amounts could be an artefact of the different colonisation strategies adopted by the two *Leptosphaeria* spp. For example, *L. biglobosa* lesions may appear large on cotyledons, not because of pathogen colonisation but due to the necrotrophic characteristics of the species, namely expression of cell wall degrading enzymes during infection (42).

Fungicides are an essential component of integrated disease management, and improved understanding of their efficacy on crop diseases is imperative. Knowledge that phoma stem canker pathogens *L. maculans* and *L. biglobosa* have yet to evolve insensitivity to some azole fungicides reinforces confidence in the durability of these chemicals for the management of phoma stem canker and their suitability as mixing partners for higher risk fungicide groups. This coupled with evidence suggesting that there is no great difference in sensitivity to azoles between the two species, suggests that currently neither pathogen will be actively selected through the prolonged usage of these fungicides. Further investigation on the potential evolution of these pathogens, through the generation of lab-based mutants, will help enhance the longevity of fungicides for the control of these pathogens.

**Acknowledgements**

This work was part of a PhD studentship financially supported by the Biotechnology and Biological Sciences Research Council (BBSRC), DuPont UK Ltd, Perry Foundation, Felix Thornley Cobbold Agricultural Trust and the

University of Hertfordshire. The authors thank Gala Ramón Zamorano (University of Herfordshire) and Pryank Patel (University of Hertfordshire) for advice on experimental procedure and Alla Mashanova (University of Hertfordshire) for statistical advice. They also thank the laboratory technicians at the University of Hertfordshire and Rothamsted Research.

## Bibliography

1. **Kelly SL**, **Lamb DC**, **Corran AJ**, **Baldwin BC**, **Kelly DE**. 1995. Mode of action and resistance to azole antifungals associated with the formation of 14α-methylergosta-8, 24 (28)-dien-3β, 6α-diol. Biochem Biophys Res Commun **207**:910–915.
2. **Lepesheva GI**, **Waterman MR**. 2007. Sterol 14α-demethylase cytochrome P450 (CYP51), a P450 in all biological kingdoms. Biochim Biophys Acta (BBA)-General Subj **1770**:467–477.
3. **Marichal P**, **Gorrens J**, **Laurijssens L**, **Vermuyten K**, **Van Hove C**, **Le Jeune L**, **Verhasselt P**, **Sanglard D**, **Borgers M**, **Ramaekers FCS**. 1999. Accumulation of 3-ketosteroids induced by itraconazole in azole-resistant clinical *Candida albicans* isolates. Antimicrob Agents Chemother **43**:2663–2670.
4. **Walsh TJ**, **Anaissie EJ**, **Denning DW**, **Herbrecht R**, **Kontoyiannis DP**, **Marr KA**, **Morrison VA**, **Segal BH**, **Steinbach WJ**, **Stevens DA**. 2008. Treatment of aspergillosis: clinical practice guidelines of the Infectious Diseases Society of America. Clin Infect Dis **46**:327–360.

593 5. **Pappas PG**, **Rex JH**, **Sobel JD**, **Filler SG**, **Dismukes WE**, **Walsh TJ**,
594 **Edwards JE**. 2004. Guidelines for treatment of candidiasis. Clin Infect Dis
595 **38**:161–189.
596 6. **Price CL**, **Parker JE**, **Warrilow AGS**, **Kelly DE**, **Kelly SL**. 2015. Azole
597 fungicides–understanding resistance mechanisms in agricultural fungal
598 pathogens. Pest Manag Sci **71**:1054–1058.
599 7. **Morton V**, **Staub T**. 2008. A short history of fungicides. Online, APSnet
600 Featur. doi:10.1094/APSnetFeature-2008-0308.
601 8. **Oliver RP**, **Hewitt HG**. 2014. Fungicides in crop protection. 2nd ed. CABI,
602 Oxon, UK.
603 9. **Sewell TR**, **Moloney S**, **Ashworth M**, **Ritchie F**, **Mashanova A**, **Huang**
604 **YJ**, **Stotz HU**, **Fitt BDL**. 2016. Effects of a penthiopyrad and picoxystrobin
605 fungicide mixture on phoma stem canker (*Leptosphaeria* spp.) on UK
606 winter oilseed rape. Eur J Plant Pathol **145**:675–685.
607 10. **Fitt BDL**, **Brun H**, **Barbetti MJ**, **Rimmer SR**. 2006. World-wide importance
608 of phoma stem canker (*Leptosphaeria maculans* and *L. biglobosa*) on
609 oilseed rape (*Brassica napus*). Eur J Plant Pathol **114**:3–15.
610 11. **Eckert MR**, **Rossall S**, **Selley A**, **Fitt BDL**. 2010. Effects of fungicides on
611 in vitro spore germination and mycelial growth of the phytopathogens
612 *Leptosphaeria maculans* and *L. biglobosa* (Phoma stem canker of oilseed
613 rape). Pest Manag Sci **66**:396–405.
614 12. **Huang YJ**, **Hood JR**, **Eckert MR**, **Stonard JF**, **Cools HJ**, **King GJ**,
615 **Rossall S**, **Ashworth M**, **Fitt BDL**. 2011. Effects of fungicide on growth of

*Leptosphaeria maculans* and *L. biglobosa* in relation to development of phoma stem canker on oilseed rape (*Brassica napus*). Plant Pathol **60**:607–620.

13. **Bierman SM**, **Fitt BDL**, **Van Den Bosch F**, **Bateman GL**, **Jenkyn JF**, **Welham SJ**. 2002. Changes in populations of the eyespot fungi *Tapesia yallundae* and *T. acuformis* under different fungicide regimes in successive crops of winter wheat, 1984–2000. Plant Pathol **51**:191–201.
14. **Huang Y-J**, **Karandeni-Dewage CS**, **Fitt BDL**. 2014. Importance of *Leptosphaeria biglobosa* as a cause of phoma stem canker on winter oilseed rape in the UK. Asp Appl Biol **127**:117–122.
15. **Parker JE**, **Warrilow AGS**, **Price CL**, **Mullins JGL**, **Kelly DE**, **Kelly SL**. 2014. Resistance to antifungals that target CYP51. J Chem Biol **7**:143–161.
16. **Hawkins NJ**, **Cools HJ**, **Sierotzki H**, **Shaw MW**, **Knogge W**, **Kelly SL**, **Kelly DE**, **Fraaije BA**. 2014. Paralog re-emergence: a novel, historically contingent mechanism in the evolution of antimicrobial resistance. Mol Biol Evol **31**:1793–1802.
17. **Carter HE**, **Fraaije BA**, **West JS**, **Kelly SL**, **Mehl A**, **Shaw MW**, **Cools HJ**. 2014. Alterations in the predicted regulatory and coding regions of the sterol 14α-demethylase gene (CYP51) confer decreased azole sensitivity in the oilseed rape pathogen *Pyrenopeziza brassicae*. Mol Plant Pathol **15**:513–522.
18. **Cools HJ**, **Fraaije BA**. 2008. Are azole fungicides losing ground against

639 Septoria wheat disease? Resistance mechanisms in *Mycosphaerella*
640 *graminicola*. Pest Manag Sci **64**:681–684.
641 19. **Cools HJ**, **Fraaije BA**. 2013. Update on mechanisms of azole resistance
642 in *Mycosphaerella graminicola* and implications for future control. Pest
643 Manag Sci **69**:150–155.
644 20. **Mullins JGL**, **Parker JE**, **Cools HJ**, **Togawa RC**, **Lucas JA**, **Fraaije BA**,
645 **Kelly DE**, **Kelly SL**. 2011. Molecular modelling of the emergence of azole
646 resistance in *Mycosphaerella graminicola*. PLoS One **6**:e20973
647 21. **Sievers F**, **Wilm A**, **Dineen D**, **Gibson TJ**, **Karplus K**, **Li W**, **Lopez R**,
648 **McWilliam H**, **Remmert M**, **Söding J**. 2011. Fast, scalable generation of
649 high-quality protein multiple sequence alignments using Clustal Omega.
650 Mol Syst Biol **7**:539.
651 22. **NCBI RC**. 2016. Database resources of the National Center for
652 Biotechnology Information. Nucleic Acids Res **44**:D7–D19.
653 23. **Grandaubert J**, **Lowe RG**, **Soyer JL**, **Schoch CL**, **Van de Wouw AP**,
654 **Fudal I**, **Robbertse B**, **Lapalu N**, **Links MG**, **Ollivier B**. 2014.
655 Transposable element-assisted evolution and adaptation to host plant
656 within the *Leptosphaeria maculans-Leptosphaeria biglobosa* species
657 complex of fungal pathogens. BMC Genomics **15**:891.
658 24. **Huang X**, **Madan A**. 1999. CAP3: A DNA sequence assembly program.
659 Genome Res **9**:868–877.
660 25. **Kumar S**, **Stecher G**, **Tamura K**. 2016. MEGA7: Molecular Evolutionary
661 Genetics Analysis version 7.0 for bigger datasets. Mol Biol Evol

662 **22**:msw054.
663 26. **Akagi A**, **Dandekar AM**, **Stotz HU**. 2011. Resistance of *Malus domestica*
664 fruit to *Botrytis cinerea* depends on endogenous ethylene biosynthesis.
665 Phytopathology **101**:1311–1321.
666 27. **Revankar SG**, **Fu J**, **Rinaldi MG**, **Kelly SL**, **Kelly DE**, **Lamb DC**, **Keller**
667 **SM**, **Wickes BL**. 2004. Cloning and characterization of the lanosterol 14α-
668 demethylase (ERG11) gene in *Cryptococcus neoformans*. Biochem
669 Biophys Res Commun **324**:719–728.
670 28. **R Development Core Team R**. 2011. R: A Language and Environment for
671 Statistical Computing. R Found Stat Comput. R Foundation for Statistical
672 Computing, Vienna, Austria.
673 29. **Yang Z**, **Lasker K**, **Schneidman-Duhovny D**, **Webb B**, **Huang CC**,
674 **Pettersen EF**, **Goddard TD**, **Meng EC**, **Sali A**, **Ferrin TE**. 2012. UCSF
675 Chimera, MODELLER, and IMP: an integrated modeling system. J Struct
676 Biol **179**:269–278.
677 30. **Šali A**, **Blundell TL**. 1993. Comparative protein modelling by satisfaction
678 of spatial restraints. J Mol Biol **234**:779–815.
679 31. **McGuffin LJ**, **Buenavista MT**, **Roche DB**. 2013. The ModFOLD4 server
680 for the quality assessment of 3D protein models. Nucleic Acids Res
681 **25**:gkt294.
682 32. **Zhang Y**, **Skolnick J**. 2005. TM-align: a protein structure alignment
683 algorithm based on the TM-score. Nucleic Acids Res **33**:2302–2309.
684 33. **Pijls CFN**, **Shaw MW**, **Parker A**. 1994. A rapid test to evaluate in vitro

sensitivity of *Septoria tritici* to flutriafol, using a microtitre plate reader. Plant Pathol **43**:726–732.

34. **Howard SJ**, **Cerar D**, **Anderson MJ**, **Albarrag A**, **Fisher MC**, **Pasqualotto AC**, **Laverdiere M**, **Arendrup MC**, **Perlin DS**, **Denning DW**. 2009. Frequency and evolution of azole resistance in *Aspergillus fumigatus* associated with treatment failure. Emerg Infect Dis **15**:1068–1076.

35. **Lucas JA**, **Hawkins NJ**, **Fraaije BA**. 2015. The Evolution of Fungicide Resistance. Adv Appl Microbiol **90**:29–92.

36. **Flowers SA**, **Colón B**, **Whaley SG**, **Schuler MA**, **Rogers PD**. 2015. Contribution of clinically derived mutations in ERG11 to azole resistance in *Candida albicans*. Antimicrob Agents Chemother **59**:450–460.

37. **Fraaije BA**, **Cools HJ**, **KIM S**, **Motteram J**, **Clark WS**, **Lucas JA**. 2007. A novel substitution I381V in the sterol 14α-demethylase (CYP51) of *Mycosphaerella graminicola* is differentially selected by azole fungicides. Mol Plant Pathol **8**:245–254.

38. **Cools HJ**, **Parker JE**, **Kelly DE**, **Lucas JA**, **Fraaije BA**, **Kelly SL**. 2010. Heterologous expression of mutated eburicol 14α-demethylase (CYP51) proteins of *Mycosphaerella graminicola* to assess effects on azole fungicide sensitivity and intrinsic protein function. Appl Environ Microbiol **76**:2866–2872.

39. **Sagatova AA**, **Keniya M V**, **Wilson RK**, **Monk BC**, **Tyndall JDA**. 2015. Structural insights into binding of the antifungal drug fluconazole to *Saccharomyces cerevisiae* lanosterol 14α-demethylase. Antimicrob Agents

Chemother **59**:4982–4989.

40. **Sagatova AA**, **Keniya M V**, **Wilson RK**, **Sabherwal M**, **Tyndall JDA**, **Monk BC**. 2016. Triazole resistance mediated by mutations of a conserved active site tyrosine in fungal lanosterol 14α-demethylase. Sci Rep **6**.

41. **Parker JE**, **Warrilow AGS**, **Cools HJ**, **Fraaije BA**, **Lucas JA**, **Rigdova K**, **Griffiths WJ**, **Kelly DE**, **Kelly SL**. 2013. Prothioconazole and prothioconazole-desthio activities against *Candida albicans* sterol 14-α-demethylase. Appl Environ Microbiol **79**:1639–1645.

42. **Lowe RGT**, **Cassin A**, **Grandaubert J**, **Clark BL**, **Van de Wouw AP**, **Rouxel T**, **Howlett BJ**. 2014. Genomes and transcriptomes of partners in plant-fungal interactions between Canola (*Brassica napus*) and two *Leptosphaeria* species. PLoS One **9**:e103098.

43. **Tamura K**, **Nei M**. 1993. Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. Mol Biol Evol **10**:512–526.

Table 1. Fungicide sensitivity (EC50 µg $ml^{-1}$) of YUG37::LmCYP51B and YUG37::LbCYP51B *Sacharomyces cerevisiae* transformants. A 96-well plate was populated with decreasing concentrations (5, 1.7, 0.56, 0.19, 0.062, 0.021, 0.0069, 0.0023, 0.00076, 0.00025, 8.47 x $10^{-5}$, 2.82 x $10^{-5}$, 0 µg $ml^{-1}$) of fungicide-amended SD + GAL + RAF media with doxycycline and cell suspension ($10^6$ cells $ml^{-1}$). Established assays were incubated at 28 $^{O}C$ for 6 days and absorbance was recorded by well-scanning (12 locations per well; 630 nm). $EC_{50}$ values were calculated automatically. Standard deviations are given in brackets and were calculated from two biological replicates. Asterisks denote significance when compared with the control (* $P < 0.05$, ** $P < 0.01$ *** $P < 0.001$).

| Transformant | Fungicide sensitivity ($EC_{50}$ µg $ml^{-1}$) | | | | | |
|---|---|---|---|---|---|---|
| | Tebuconazole | Fold difference | Flusilazole | Fold difference | Prothioconazole-desthio | Fold difference |
| YUG37::LmCYP51B | 0.0084 (0.0017) | | 0.036 (0.014) | | 0.0007 (0.0006) | |
| YUG37::LbCYP51B | 0.0062 (0.0005) | **1.35 *** | 0.032 (0.0056) | **1.13** | 0.0004 (0.0002) | **1.75** |

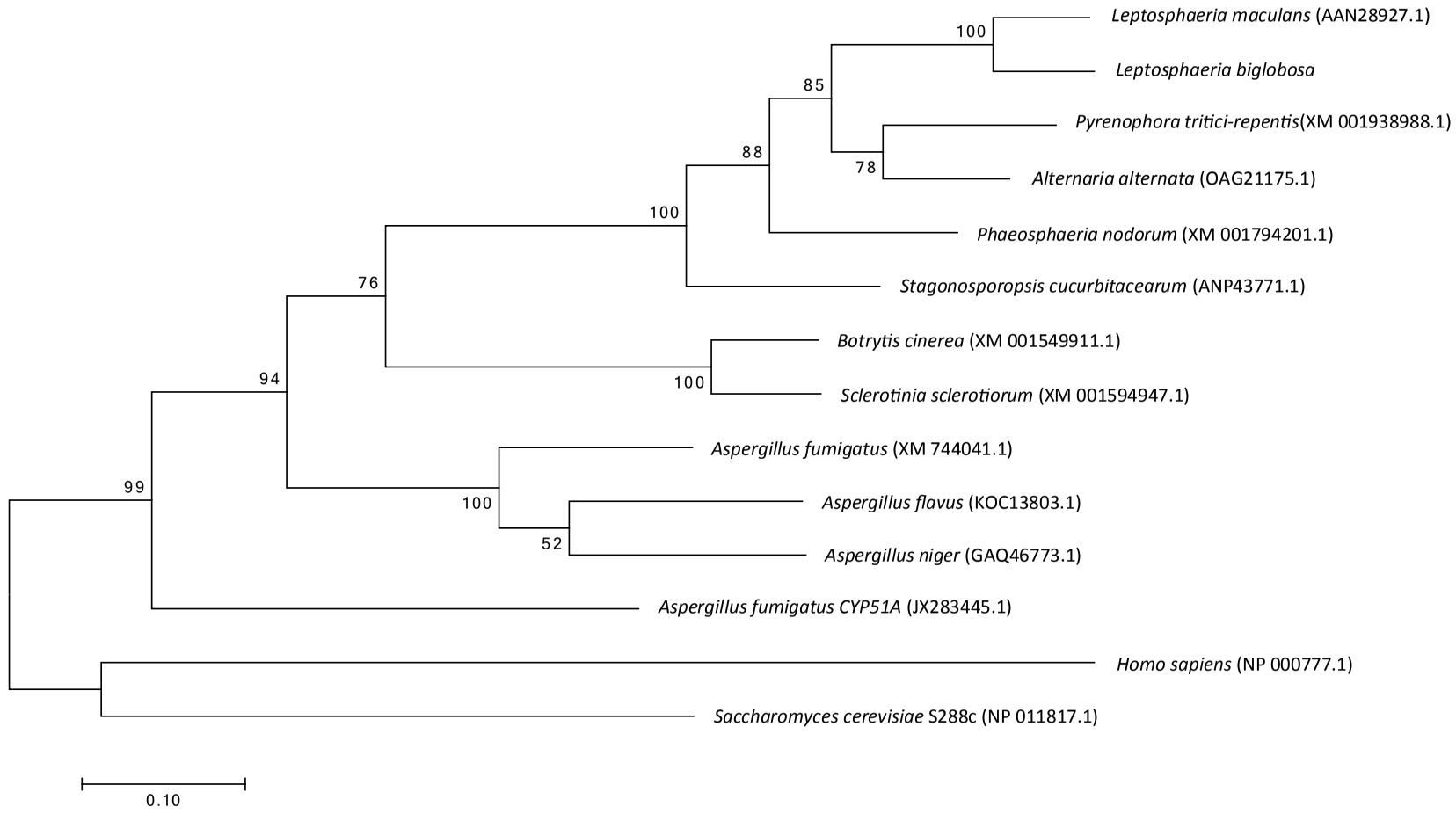

Leptosphaeria maculans (AAN28927.1)
Leptosphaeria biglobosa
Pyrenophora tritici-repentis(XM 001938988.1)
Alternaria alternata (OAG21175.1)
Phaeosphaeria nodorum (XM 001794201.1)
Stagonosporopsis cucurbitacearum (ANP43771.1)
Botrytis cinerea (XM 001549911.1)
Sclerotinia sclerotiorum (XM 001594947.1)
Aspergillus fumigatus (XM 744041.1)
Aspergillus flavus (KOC13803.1)
Aspergillus niger (GAQ46773.1)
Aspergillus fumigatus CYP51A (JX283445.1)
Homo sapiens (NP 000777.1)
Saccharomyces cerevisiae S288c (NP 011817.1)
100
85
88
78
100
76
100
94
99
100
52
0.10

Fig 1.

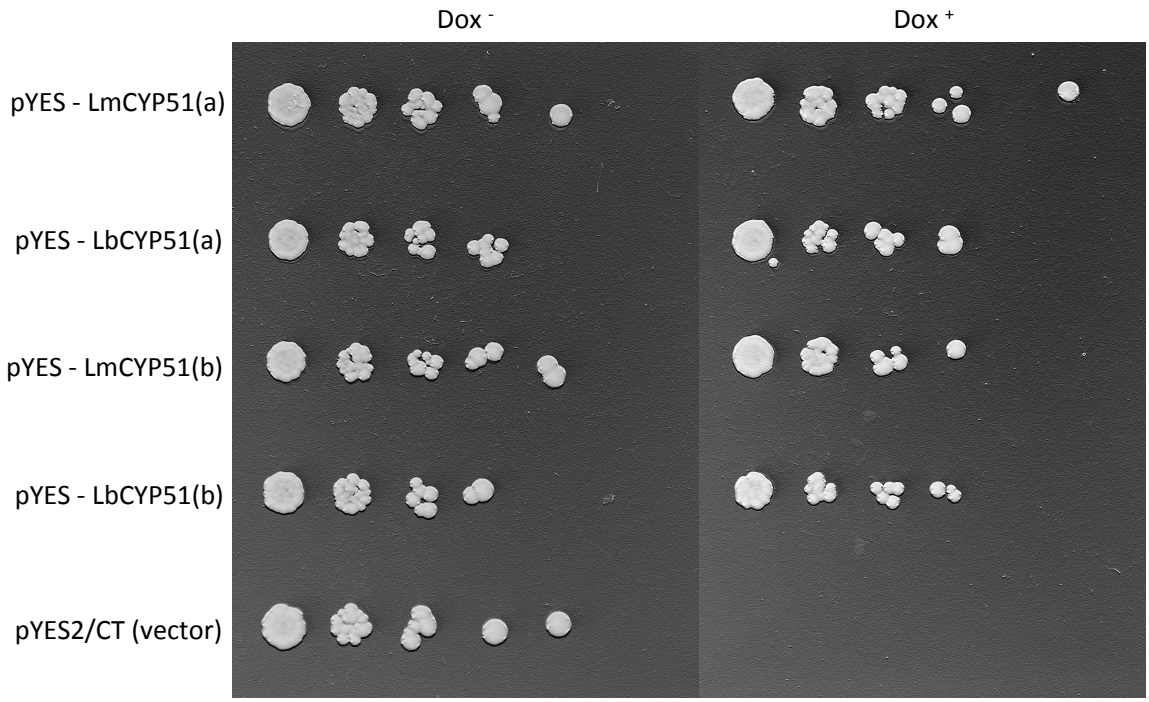

Dox -
Dox +
pYES - LmCYP51(a)
pYES - LbCYP51(a)
pYES - LmCYP51(b)
pYES - LbCYP51(b)
pYES2/CT (vector)

Fig 2

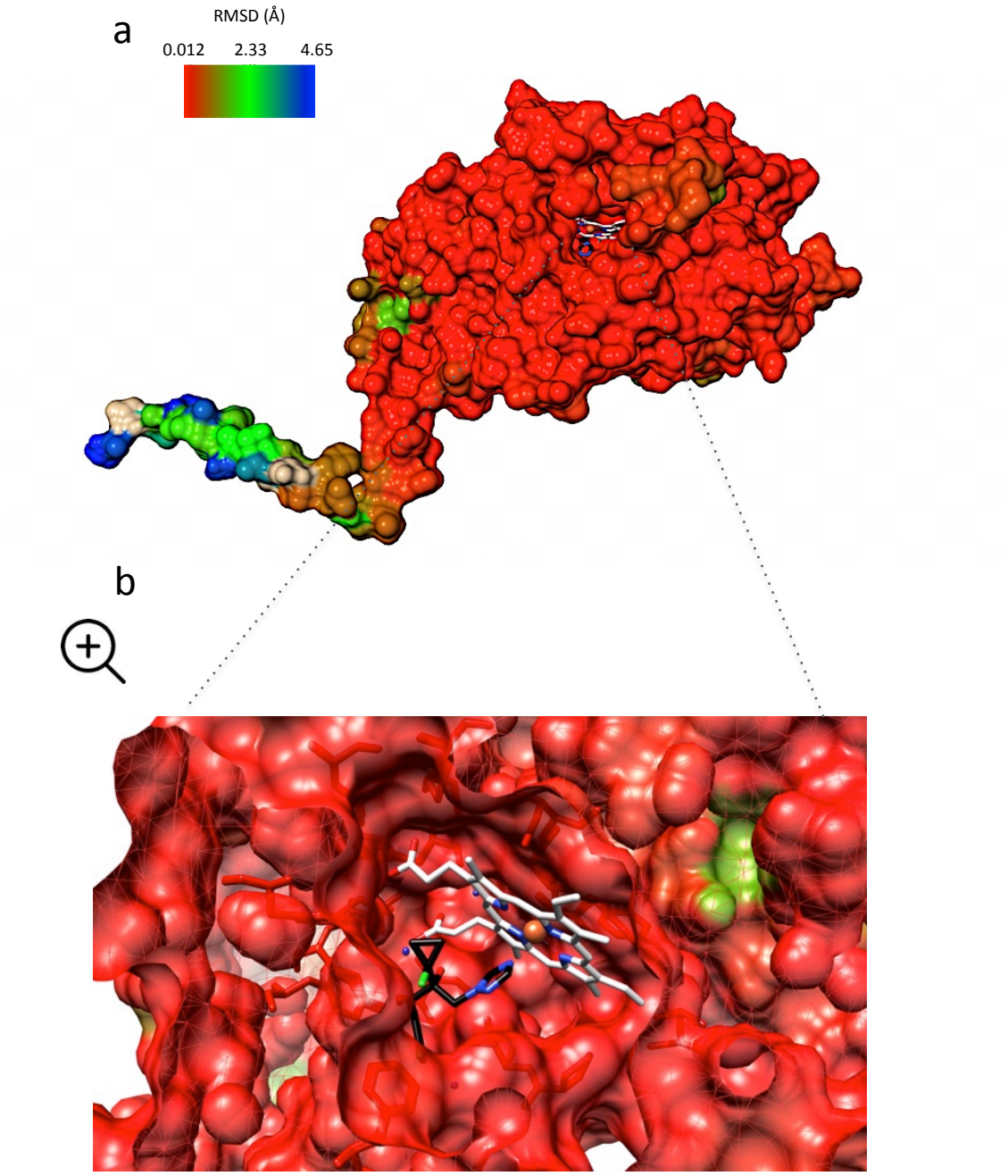



Fig. 3

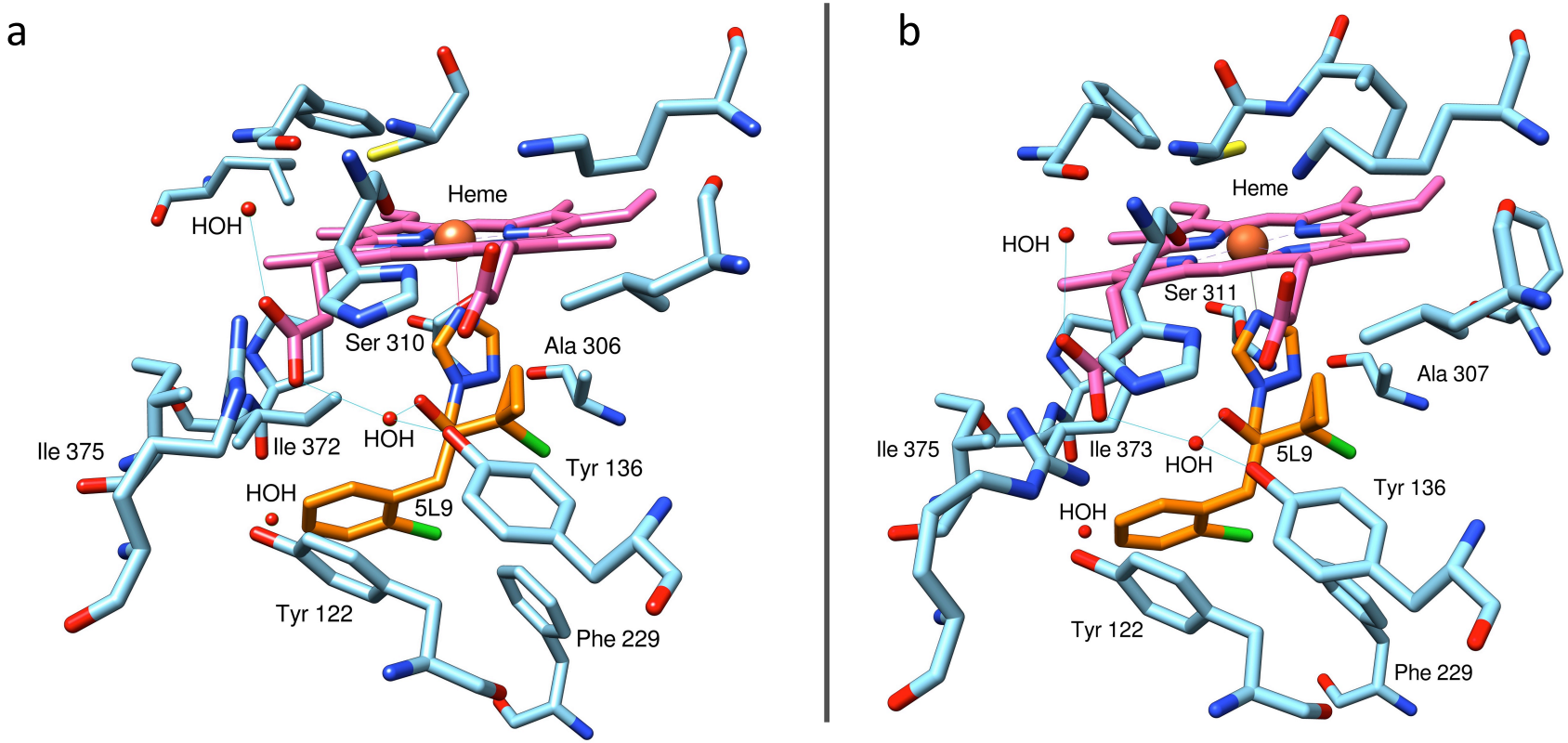
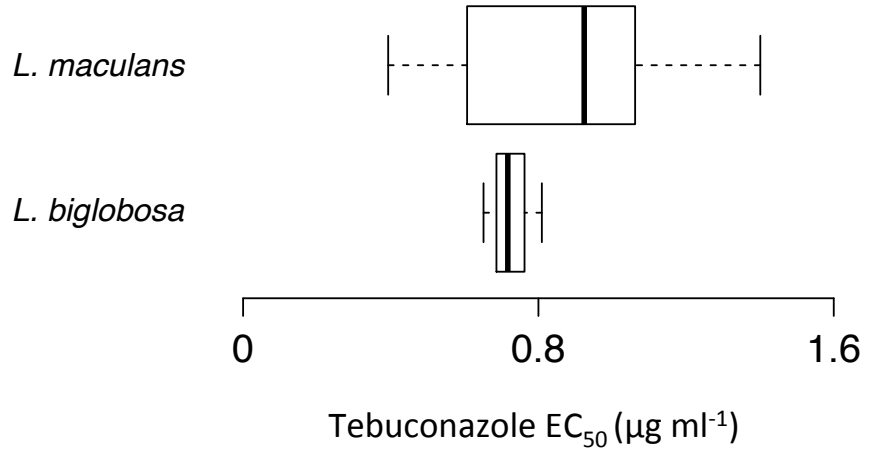
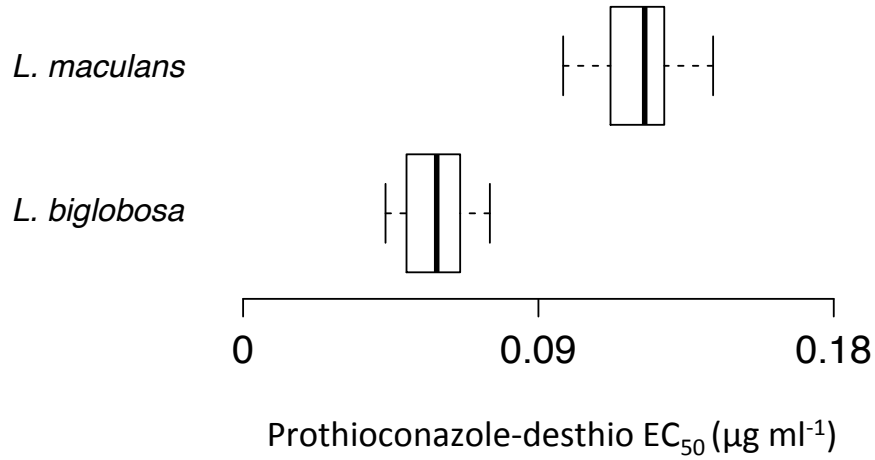

a
Heme
HOH
Ser 310
Ala 306
Ile 375
Ile 372
HOH
Tyr 136
HOH
5L9
Tyr 122
Phe 229
b
Heme
HOH
Ser 311
Ala 307
Ile 375
Ile 373
HOH
5L9
Tyr 136
HOH
Tyr 122
Phe 229

Fig 4.


L. maculans
L. biglobosa
0
0.8
1.6
Tebuconazole EC50 (µg ml-1)


L. maculans
L. biglobosa
0
0.09
0.18
Prothioconazole-desthio EC50 (µg ml-1)

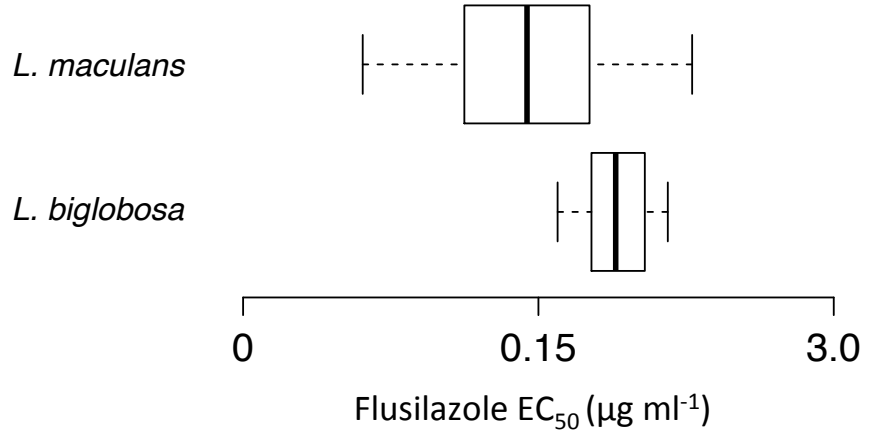
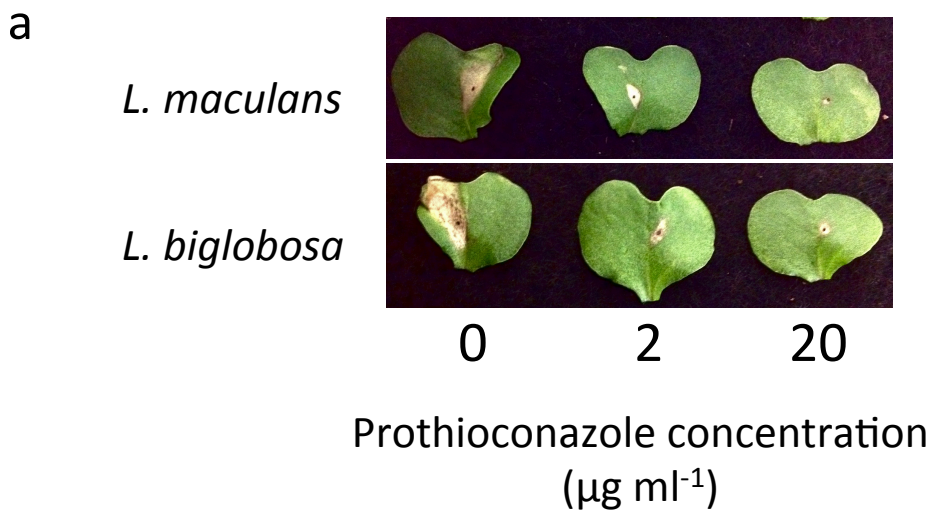

L. maculans
L. biglobosa
0
0.15
3.0
Flusilazole EC50 (µg ml-1)

Fig 5.


a
L. maculans
L. biglobosa
0
2
20
Prothioconazole concentration
(µg ml-1)

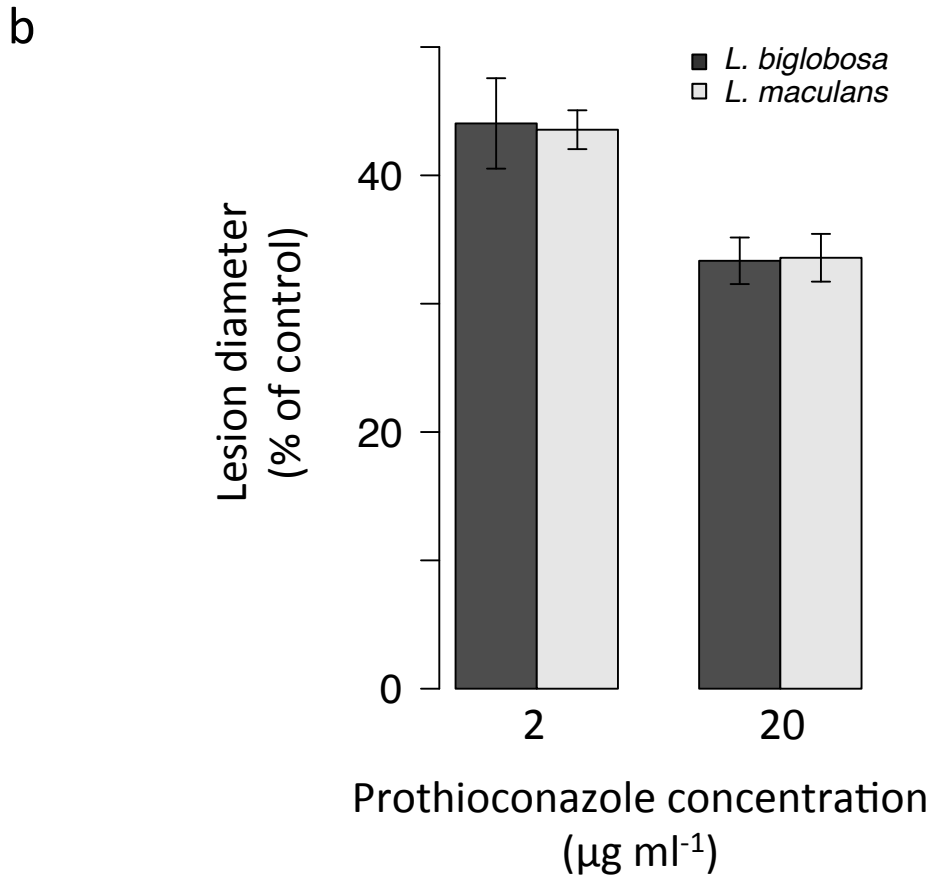

b
L. biglobosa
L. maculans
Lesion diameter
(% of control)
40
20
0
2
20
Prothioconazole concentration
(µg ml-1)

Figure 6.

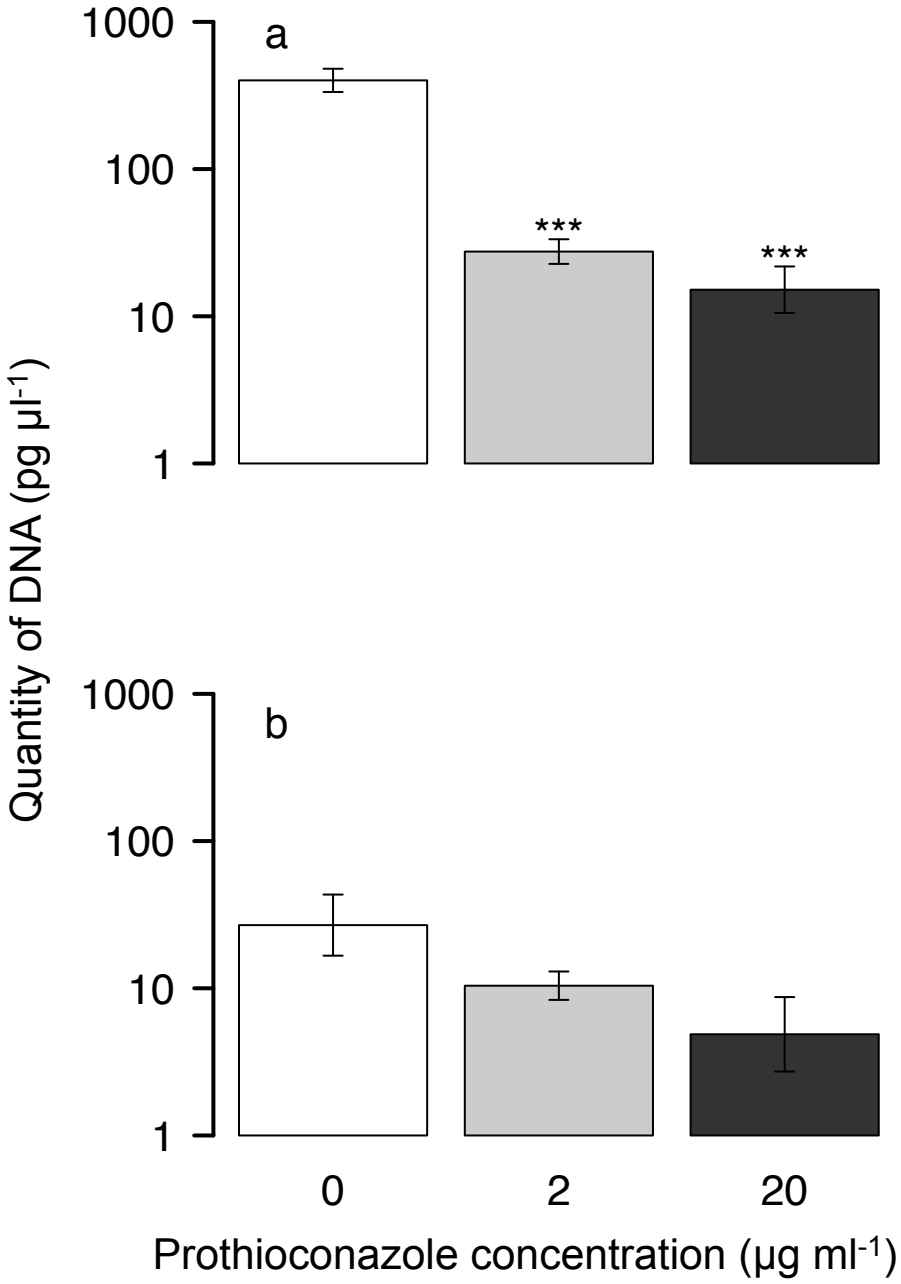

1000
100
10
1
a
***
***
b
Quantity of DNA (pg µl-1)
0
2
20
Prothioconazole concentration (µg ml-1)

Fig 7.

Fig 1. Maximum Likelihood phylogenetic tree of fungal *CYP51* sequences, with *H. sapiens CYP51* outgroup The evolutionary history was inferred by using the maximum likelihood method based on the Tamura-Nei model (43). Branch lengths scaled by number of substitutions per site. Node labels indicate percentage bootstrap support (1000 replicates).

Fig 2. Complementation of *Saccharomyces cerevisiae* strain *YUG37::erg11* with *Leptosphaeria maculans* CYP51B and *Leptosphaeria biglobosa* CYP51B. Yeast transformants grown for 16 hours in SD + GAL + RAF media were inoculated onto SD + GAL + RAF agar without ($DOX^-$) or with ($DOX^+$) doxycycline, which represses the native *S. cerevisiae* CYP51B. The YUG37::pYES2/CT vector-only strain was used as a control. Lettering (a,b) refers to biological replicates.

Fig 3. Structural pair-wise alignment (Match > Align; Chimera) on the predicted homology models of LmCYP51B and LbCYP51B (a). Difference in colour represents distance (Å) between aligned residues (RMSD), with red representing residues close in structural locality and blue representing residues that are further apart. Zoomed image (b) is a cross-section of the binding pocket of aligned proteins. Images were generated using Chimera.

Fig 4. Azole binding sites in the *Leptosphaeria maculans CYP51B* (a) and *L. biglobosa CYP51B* (b) proteins inferred by homology. Superimposed

prothioconazole-desthio (5L9) is highlighted in orange with residues < 5 Å from ligand (5L9) labelled. The heme cofactor is highlighted in pink and the metal-ligand bond in purple. Hydrogen bonds are highlighted in blue and water molecules (HOH) in red. Images were generated in Chimera.

Fig 5. Box plot depicting sensitivities of *Leptosphaeria maculans* isolates (n = 23) and *L. biglobosa* isolates (n = 21) to flusilazole (a), prothioconazole-desthio (b) and tebuconazole (c) . Thick line denotes median value, box edges signify lower and upper quartiles and error bars represent minimum and maximum values.

Fig 6. Efficacy of prothioconazole on the leaf lesions of *Leptosphaeria maculans* and *L. biglobosa.* Plants were arranged in an alternate block design consisting of two replicate blocks each containing six treatments (eight plants per treatment). Oilseed rape cotyledons (cv. Catana) were inoculated with either *L. maculans* or *L. biglobosa* conidial suspension ($x10^6$). At 6 days post inoculation (DPI), cotyledons were sprayed with prothioconazole at 0 μg $ml^{-1}$, 2 μg $ml^{-1}$ or 20 μg $ml^{-1}$ concentration. At 14 DPI, lesion sizes were measured (a). Lesion diameter (mm) was recorded and average lesion diameter calculated as percentage compared to control (b). Error bars represent standard errors of the mean.

Fig 7. Quantification of DNA from prothioconazole-treated oilseed rape cotyledons inoculated with either *L. maculans* (a) or *L. biglobosa* (b). Plants were arranged in an alternate block design consisting of two replicate blocks each

containing six treatments (eight plants per treatment). Oilseed rape cotyledons (cv. Catana) were inoculated with either *L. maculans* or *L. biglobosa* conidial suspension (x$10^6$ conidia $ml^{-1}$). At 6 days post inoculation (DPI), cotyledons were sprayed with prothioconazole at either 0 µg $ml^{-1}$, 2 µg $ml^{-1}$ or 20 µg $ml^{-1}$ concentration.

Cotyledons were sampled at 14 days post incoulation (DPI) and DNA was extracted and then diluted to a concentration of 25 ng $µl^{-1}$. Quantitative PCR was done on duplicate samples and run alongside a standard curve, which was used for automated quantification of DNA (pg $ul^{-1}$). Data were $log_{10}$-transformed. Asterisks denote significance when compared with the control (* $P < 0.05$, ** $P < 0.01$ *** $P < 0.001$).

**110**
**ORAL PRESENTATION THEME B**

**T.R. Sewell**[1]
**Y.J. Huang**[1]
**H. Stotz**[1]
**F. Ritchie**[2]
**M. Ashworth**[3]
**B.D.L. Fitt**[1]

1. School of Life and Medical Sciences, University of Hertfordshire, Hatfield, Herts, AL10 9AB UK,

2. ADAS, Battlegate Road, Boxworth, Cambridge, CB23 4NN UK;
3Dupont (UK) Ltd, Wedgewood Way, Stevenage, Herts, SG14QN UK

t.sewell@herts.ac.uk

# The effect of fungicides on *Leptosphaeria biglobosa* and *L. maculans*, phoma stem canker severity and oilseed rape yield

**Background:** Phoma stem canker, a disease of oilseed rape *(Brassica napus)* caused by closely related pathogens *Leptosphaeria biglobosa* and *L. maculans*, is an economically important disease causing annual yield losses of approximately £1000M worldwide (Fitt et al., 2008). Both pathogens follow a monocylic disease cycle causing leaf spotting in autumn/winter and stem cankers in spring/summer. Severe cankers decrease transportation of water and nutrients to the developing seeds, resulting in reduced yield (Eckert et al., 2009). When colonising oilseed rape, *L. biglobosa* and *L. maculans* exist in close proximity on the leaf - competing for resources as they move down the main leaf vein and into the plant stem (Fitt et al., 2006). Fungicides are commonly used to decrease severity of phoma stem canker on oilseed rape. However, the efficacy and longevity of active chemicals is under threat from continuously evolving pathogen types (Carter et al., 2014).

**Objective:** To identify what effect commercially applied fungicides have on *L. biglobosa* and *L. maculans* interactions, phoma stem canker severity and oilseed rape yield

**Methods:** Winter oilseed rape field trials were done for three cropping season and phoma leaf spotting and phoma stem canker severity were assessed. Species composition in the stems of both upper stem lesions and basal stem cankers in the 2013/2014 cropping season was assessed using QPCR. The airspora for ispecies was monitored using a Burkard spore sampler, and species-specific DNA was quantified using QPCR. Fungicide sensitivity tests in vitro were done using fungicide amended agar plates at differing concentrations.

**Results:** The two pathogens differed greatly in their growth rates in vitro, with *L. biglobosa* growing much faster than *L. maculans*. The EC50 values show that *L. biglobosa* is significantly more tolerant of azole-amended media than *L. maculans*. Refinzar, containing active ingridents (a.i) penthiopyrad and picoxystorbin, was as effective at controlling phoma leaf spotting and phoma stem canker in the field as Proline (a.i. prothioconazole).

**Conclusions:** Differing sensitivities to azole fungicides could be selecting *L. biglobosa* resulting in an epidemic where current fungicides do not fully control the disease. A combination of SDHI + QoI fungicides could be used to control epidemics caused by a *L. biglobosa*.

### References:

CARTER, H. E., FRAAIJE, B. A., WEST, J. S., KELLY, S. L., MEHL, A., SHAW, M. W. & COOLS, H. J. 2014. Alterations in the predicted regulatory and coding regions of the sterol 14α-demethylase gene (CYP51) confer decreased azole sensitivity in the oilseed rape pathogen *Pyrenopeziza brassicae*. Molecular Plant Pathology.

ECKERT, M. R., ROSSALL, S., SELLEY, A. & FITT, B. D. L. 2010. Effects of fungicides on in vitro spore germination and mycelial growth of the phytopathogens *Leptosphaeria maculans* and *L. biglobosa* (Phoma stem canker of oilseed rape). Pest Management Science, 66, 396-405.

FITT, B. D., HU, B., LI, Z., LIU, S., LANGE, R., KHARBANDA, P., BUTTERWORTH, M. & WHITE, R. 2008. Strategies to prevent spread of *Leptosphaeria maculans* (phoma stem canker) onto oilseed rape crops in China; costs and benefits. Plant pathology, 57, 652-664.

FITT, B. D., HUANG, Y.-J., VAN DEN BOSCH, F. & WEST, J. S. 2006. Coexistence of related pathogen species on arable crops in space and time. Phytopathology, 44.

t.sewell@herts.ac.uk

# Effects of different fungicides on development of phoma stem canker and oilseed rape yield

**Thomas Sewell**[1], Steven Moloney[1], Yongju Huang[1], Henrik U. Stotz[1], Mike Ashworth[2], Peter Walker[3], Faye Ritchie[3], Bruce D.L. Fitt[1]

[1] School of Life and Medical Sciences, University of Hertfordshire, Hatfield, United Kingdom
[2] Dupont (UK) LTD, Wedgewood Way, Stevenage, Hertfordshire, United Kingdom
[3] ADAS, Battle Gate Road, Boxworth, Cambridge, United Kingdom

Phoma stem canker, a disease of oilseed rape (*Brassica napus*) caused by pathogens *Leptosphaeria maculans* and *L. biglobosa*, is an economically important disease causing annual yield losses of approximately £1000M worldwide (Fitt et al., 2008). Both pathogens follow a monocylic disease cycle causing leaf spotting in autumn/winter and stem cankers in spring/summer. Most severe cankers decrease transportation of water and nutrients to the developing seeds, resulting in reduced yield (Eckert et al., 2009). The use of fungicides is an important tool for controlling phoma stem canker in the UK. The triazole fungicides currently dominate the market, although reduced sensitivity in some plant pathogen populations is an emergent concern (Carter et al., 2013). This current study aims to examine the efficacy of a novel fungicide when compared with flusilazole, a commonly used fungicide at the time of assessment. It also aims to investigate whether *L. biglobosa* is less sensitive to triazole fungicides in comparison to *L. maculans*. Field trials were established in Boxworth (Cambridgeshire) in the 2012/2013 and 2013/2014 cropping seasons. Four fungicides were applied: penthiopyrad, picoxystrobin, flusilazole and a novel fungicide. Spray timings were divided into three sprays T1, T2 and T3, with T1 being applied in autumn when phoma leaf spotting incidence was ≥ 10% per plant and T2 sprayed four weeks later. T3 was applied in spring against Sclerotinia sclerotiorum. Phoma leaf spotting incidence, stem canker severity and yield were recorded. *In vitro* sensitivity testing was done on one *L. maculans* and one *L. biglobosa* isolate. Flusilazole, at various concentrations, was applied to PDA medium, which was later inoculated with a mycelium plug. Flusilazole showed no noteworthy advantage over the novel fungicide in both the canker severity scoring or yield results. A significant difference in growth inhibition was observed between *L. biglobosa* and *L. maculans* isolates ($P \leq 0.05$). Field trials indicate that the novel fungicide is as equally effective as the more commonly used triazole fungicides and could replace flusilazole for commercial use. Fungicide sensitivity testing showed that *L. biglobosa* does have an increased level of resistance to triazole chemistry when compared with *L. maculans*. This interaction confirms previous studies that have also determined a reduced sensitivity to triazole fungicides in *L. biglobosa* populations (Eckert et al., 2009; Huang et al., 2011).

# 'Some Like It Hot'
# BSPP 2014 Presidential Meeting
**1st-2nd September 2014, St Andrew University, UK**

## Form for Abstracts

**Please use this form if you wish to make an oral presentation. The text (excluding title, authors, and affiliations) should not exceed 250 words. Please use Calibri, 12 pt font.**

**Title:** Effects of different fungicides on the severity of phoma stem canker

**Author(s) (please use following style)**
Thomas Sewell[1], Yongju Huang[1,] Henrik Stotz[1], Mike Ashworth[2], Philip Walker[3], Faye Ritchie[3] and Bruce D.L. Fitt[1]

**Affiliation(s) (please use following style)**
[1] School of Life and Medical Sciences, University of Hertfordshire, Hatfield, Herts, AL10 9AB, UK; [2] Dupont (UK) LTD, Wedgewood Way, Stevenage, Herts, SG14QN; [3] ADAS, Battle Gate Road, Boxworth, Cambridge, CB23 4NN.

**Presenting Author email**
t.sewell@herts.ac.uk

**Abstract Text (250 words maximum)**
Phoma stem canker, a disease of oilseed rape (*Brassica napus*) caused by sibling pathogens *Leptosphaeria maculans* and *L. biglobosa*. Both pathogens follow a monocylic disease cycle that causes leaf spotting in autumn/winter and stem cankers in spring/summer. Most severe cankers decrease transportation of water and nutrients. Fungicides are important for phoma stem canker control. Triazole fungicides currently dominate the market, although reduced sensitivity in some plant pathogen species is a concern. Moreover, *L. maculans* and *L. biglobosa* have shown differing level of sensitivity to triazole fungicides. Therefore, increased knowledge on controlling phoma stem canker using non-triazole based fungicides is essential. Field trials were established for 2013/2014 cropping season. Four fungicides were applied: penthiopyrad, picoxystrobin, prothioconazole and a novel fungicide. Spray timings were divided into three sprays T1 (phoma leaf spotting incidence ≥10%), T2 (3/4 weeks post T1) and T3 (Sclerotinia). Phoma leaf spotting incidence and stem canker severity were recorded. In vitro sensitivity testing was done on one *L. maculans* (ME24) and one *L. biglobosa* (68) isolate. Prothioconazole showed no noteworthy advantage over the novel fungicide in canker severity scoring. No significant difference in growth inhibition was observed between *L. biglobosa* and *L. maculans* ($P \leq 0.05$) when treated with novel fungicide. Canker severity indicates that the novel fungicide has a similar efficacy to triazole fungicides. Fungicide sensitivity testing shows that *L. biglobosa* does not have an increased sensitivity to non-triazole fungicides. This interaction suggests that the novel fungicide could be used to control both *L. maculans* and *L. biglobosa*.

When complete please submit via email to the Programme Secretary:
meetings@bspp.org.uk

# Azole sensitivity in *Leptosphaeria* species: The role of lanosterol 14-α demethylase

Thomas R. Sewell[1], Nichola Hawkins[2] Yongju Huang[1], Henrik U. Stotz[1], Bart Fraaije[2] and Bruce D.L. Fitt[1]

[1] School of Life and Medical Sciences, University of Hertfordshire, Hatfield, Herts, AL10 9AB, UK; [2] Rothamsted Research, Harpenden, Herts, AL5 2JQ, UK

## Introduction:

Antifungal demethylation inhibitors (DMIs), e.g. triazoles and imidazoles, target lanosterol 14α-demethylase (*ERG11, CYP51B*) in the ergosterol biosynthetic pathway (Kelly et al. 1995).

DMI fungicides disrupt membrane permeability through reduced ergosterol levels and the subsequent increase in toxic sterol precursors (Marichal et al. 1999).

Missense mutations in coding sequence of *ERG11* (gene), and subsequent alterations in secondary structure of *CYP51B* (protein), correlate with a desensitivity to DMI fungicides (Mullins et al. 2011).

Co-existing plant pathogens *Leptosphaeria maculans* and *L. biglobosa* differ in their sensitivity to some azoles used to control phoma stem canker (Eckert et al. 2010; Huang et al. 2011).

## Aim:

To clarify differential fungicide sensitivity through the molecular characterisation of *CYP51B* in coexisting phoma stem canker pathogens *L. maculans* (*LmCYP51B*) and *L. biglobosa* (*LbCYP51B*).

## Materials and methods:

Genome sequence data were used to identify *LmERG11* and *LbERG11* gene for cloning into *E. coli.*

*ERG11* was sequenced in 10 *L. maculans* isolates and 8 *L. biglobosa* isolates.

Predictive homology modelling of *LmCYP51B* and *LbCYP51B* was done using MODELLER v9.14 and subsequent structural alignments were used to determine structural differences.

Heterologous expression in *Saccharomyces cerevisiae* (with a doxycycline-repressible native *ERG11* gene) and subsequent fungicide-sensitivity assays were used to compare *LmCYP51B* and *LbCYP51B* interaction with tebuconazole, flusilazole, prothioconazole and prothioconazole-desthio (prothioconazole metabolite).

Liquid media with decreasing concentrations of tebuconazole, flusilazole and prothioconazole-desthio were used to determine fungicide sensitivities ($EC_{50}$) of 23 *L. maculans* and 22 *L. biglobosa isolates.*

Fungicide-sensitivity *in planta* was studied using *L. maculans*- or *L. biglobosa*-infected cotyledons treated with a prothioconazole based fungicide.

## *LmCYP51B* and *LbCYP51B* amino acid sequences are highly homologous, suggesting a similar secondary structure.

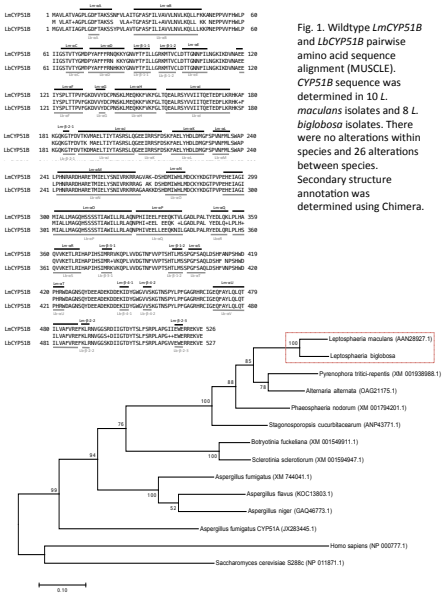


Fig. 1. Wildtype *LmCYP51B* and *LbCYP51B* pairwise amino acid sequence alignment (MUSCLE). *CYP51B* sequence was determined in 10 *L. maculans* isolates and 8 *L. biglobosa* isolates. There were no alterations within species and 26 alterations between species. Secondary structure annotation was determined using Chimera.




Fig. 2. Molecular phylogenetic analysis of *CYP51B* sequences using Maximum Likelihood with 1000 bootstrap. The evolutionary history was inferred using the Tamura-Nei model in MEGA7.

## Both *LmCYP51B* and *LbCYP51B* complement *S. cerevisiae* strain *YUG37::erg11* repressed with doxycycline

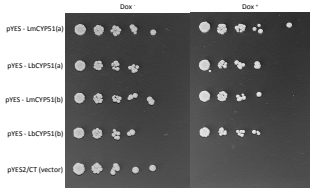



Fig. 5. Complementation of *S. cerevisiae* strain YUG37::erg11 with *LmCYP51B* and *LbCYP51B*. Galactose induced yeast transformants (pYES-LmCYP51Ba/b and pYES-LbCYP51Ba/b) were grown in the presence ($DOX^+$) or absence ($DOX^-$) of doxycycline, which represses the native *S. cerevisiae CYP51B*. The YUG37::pYES2/CT vector-only strain was used as a control.

## Subsequent fungicide sensitivity assays on *LmCYP51B* and *LbCYP51B* yeast transformants suggest minor difference in azole fungicide interaction (*P* = < 0.05)

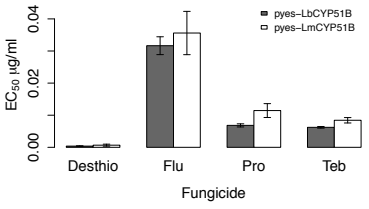



Fig. 6. Azole sensitivity ($EC_{50}$ µg/ml) of pYES-LmCYP51 and pYES-LbCYP51 *S. cerevisiae* (YUG37::erg11) transformants. $EC_{50}$ determined using decreasing concentrations of prothioconazole (Pro), prothioconazole-desthio (Desthio), flusilazole (Flu) or tebuconazole (Teb) amended media with galactose and doxycycline (native *CYP51B* repressor) and 1 x $10^6$ cell suspension.

## Predicted structures of *LmCYP51B* and *LbCYP51B* are highly similar with conservation around the haem cofactor and azole binding site

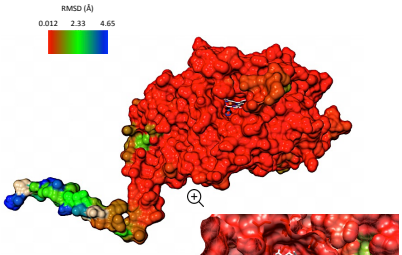



Fig. 3. Structural alignment of *LmCYP51B* and *LbCYP51B* predicted structures inferred through homology (MODELLER v9.14). Color describes structural similarity with red more similar and blue less similar.

## Water mediated hydrogen bond network is maintained in both predicted structures when superimposed with prothioconazole-desthio

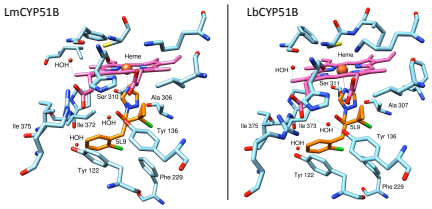



Fig. 4. Azole binding site in *LmCYP51B* and *LbCYP51B* proteins (light blue) inferred by homology. Superimposed prothioconazole-desthio (5L9) is highlighted in orange with residues < 5 Å from ligand (5L9) labelled. The haem cofactor is highlighted in pink and the metal-ligand bond in purple. Hydrogen bonds are highlighted in blue and water molecules (HOH) in red.

## *L. maculans* and *L. biglobosa* differ in their sensitivity to prothioconazole-desthio *in vitro*...

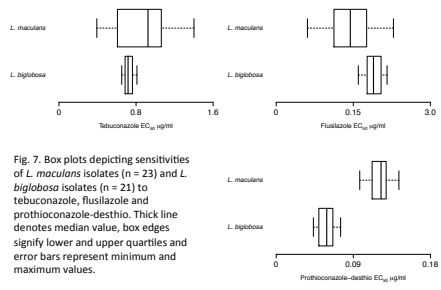



Fig. 7. Box plots depicting sensitivities of *L. maculans* isolates (n = 23) and *L. biglobosa* isolates (n = 21) to tebuconazole, flusilazole and prothioconazole-desthio. Thick line denotes median value, box edges signify lower and upper quartiles and error bars represent minimum and maximum values.

## ...but not *in planta*

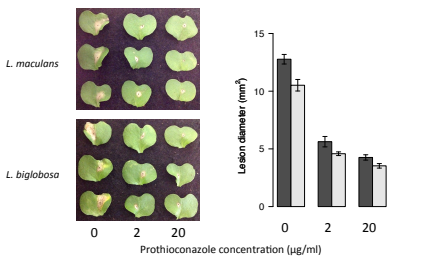



Fig. 8. Sensitivity of one *L. maculans* and one *L. biglobosa* isolate to prothioconazole 14 days after inoculation of oilseed rape cotyledons (cv. Catana). Cotyledons were inoculated with either *L. maculans* or *L. biglobosa* conidial suspension before being sprayed with fungicides at either 2 µg/ml or 20 µg/ml concentrations.

## Conclusion:

*CYP51B* structures conserved or similar and azole binding site and hydrogen bond network maintained in both species.

Minor differences in azole interactions between *LmCYP51B* and *LbCYP51B* yeast transformants exist.

Differential fungicide sensitivity is apparent *in vitro* but not *in planta.*

**References:**

Eckert, M. R., Rossall, S., Selley, A., & Fitt, B. D. L. (2010). Effects of fungicides on in vitro spore germination and mycelial growth of the phytopathogens *Leptosphaeria maculans* and *L. biglobosa* (Phoma stem canker of oilseed rape). *Pest Management Science*

Huang, Y.-J., Hood, J. R., Eckert, M. R., Stonard, J. F., Cools, H. J., King, G. J., et al. (2011). Effects of fungicide on growth of *Leptosphaeria maculans* and *L. biglobosa* in relation to development of phoma stem canker on oilseed rape (Brassica napus). *Plant pathology*

Kelly, S. L., Lamb, D. C., Corran, A. J., Baldwin, B. C., & Kelly, D. E. (1995). Mode of action and resistance to azole antifungals associated with the formation of 14α-methylergosta-8, 24 (28)-dien-3β, 6α-diol. *Biochemical and biophysical research communications*

Marichal, P., Gorrens, J., Laurijssens, L., Vermuyten, K., Van Hove, C., Le Jeune, L., et al. (1999). Accumulation of 3-ketosteroids induced by itraconazole in azole-resistant clinical *Candida albicans* isolates. *Antimicrobial agents and chemotherapy*

Mullins, J. G. L., Parker, J. E., Cools, H. J., Togawa, R. C., Lucas, J. A., Fraaije, B. A., et al. (2011). Molecular modeling of the emergence of azole resistance in *Mycosphaerella graminicola*.

# Effects of a penthiopyrad and picoxystrobin fungicide mixture on phoma stem canker (*Leptosphaeria spp.*) on UK winter oilseed rape

Thomas Sewell[1], Steven Moloney[1], Alla Mashanova[1], Yongju Huang[1], Henrik Stotz[1], Mike Ashworth[2], Faye Ritchie[3] and Bruce D.L. Fitt[1]

[1] School of Life and Medical Sciences, University of Hertfordshire, Hatfield, Herts, AL10 9AB, UK; [2] DuPont (UK) Ltd, Kings Court, London Road, Stevenage, Herts, SG1 2NG; [3] ADAS UK Ltd, Battle Gate Road, Boxworth, Cambridge, CB23 4NN.

## Introduction

- Phoma stem canker is a disease of oilseed rape, which is caused by closely related fungal species *Leptosphaeria maculans* and *L. biglobosa.*[1]
- Typically, *L. maculans* is considered the more damaging of the two pathogens due to the development of basal stem cankers on oilseed rape. Although, in recent years, *L. biglobosa* has increased in severity. [1,2]
- Fungicides are used in the UK to control phoma stem canker, with a typical application rate of two or three sprays per cropping season. [4]
- Legislation has forced the withdrawal of some fungicides used to control fungal pathogens in arable crops. [4]
- Novel fungicide mixtures could be used to control phoma stem canker on winter oilseed rape. [4]
- Penthiopyrad (SDHI) and picoxystrobin (QoI) target complex II and complex III on the electron transport chain, respectively; starving the fungal cell of ATP. [3]

**AIM: Evaluate the efficacy of penthiopyrad plus picoxystrobin on *Leptosphaeria* spp. and phoma stem canker severity**

## Methods

- Field trials were established at Boxworth (Cambridgeshire) in the 2011/2012, 2012/13, 2013/14 and 2014/15 cropping seasons. Fungicides applied were: penthiopyrad, picoxystrobin, prothioconazole and a penthiopyrad plus picoxystrobin mixture (in commerical forms).
- Phoma leaf spotting, upper stem lesions and basal stem canker severity was recorded.
- Fungicide efficacy was tested *in planta,* under controlled conditions, on winter oilseed rape cotyledons. Penthiopyrad plus picoxystrobin or prothioconazole were applied at a rate of 2 or 20 µg/ml.


Figure 1: Sampled leaves illustrating difference in phoma leaf spotting incidence due to fungicide application in the 2013/14 cropping season.

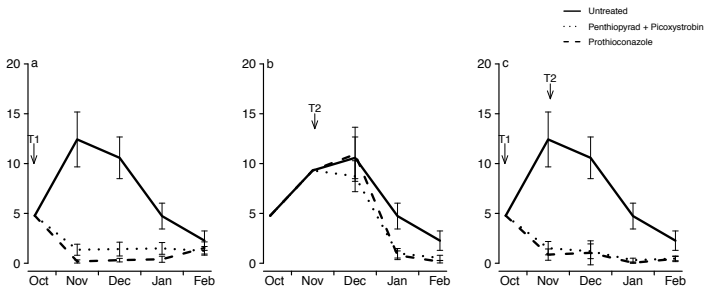



Figure 2: Incidence of phoma leaf spotting associated with *L. maculans* type leaf lesions on winter oilseed rape (cv. Catana) plots sprayed with fungicide at T1 (early) (a), T2 (late) (b) or T1 & T2 (combined) (c) in the 2013/14 cropping season.

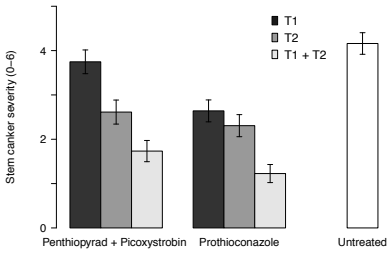



Figure 3: Basal stem canker severity on experimental winter oilseed rape (cv. Catana) plots in the 2013/14 cropping


Figure 4: Basal stem canker severity on treated and untreated oilseed rape stems in 2013/14

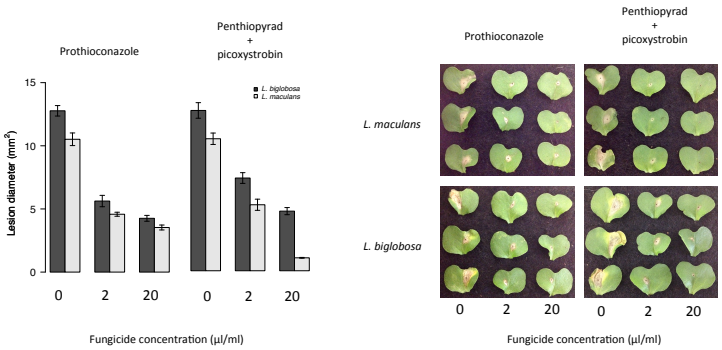



Figure 5: Efficacy of penthiopyrad plus picoxystrobin or prothioconazole on winter oilseed rape cotyledons infected with either *L. maculans* or *L. biglobosa* under controlled conditions.

## Results

- In a severe disease year (2013/14 only), plots treated with penthiopyrad plus picoxystrobin or prothioconazole had less *L. maculans* type leaf lesions when compared to the untreated control ($P$ = <0.05).
- The severity of basal stem cankers was reduced when treated with either penthiopyrad plus picoxystrobin or prothioconazole. The combined spray applied at T1 and T2 reduced severity the greatest ($P$ = <0.05).
- At 2 µg/ml and 20 µg/ml, both penthiopyrad plus picoxystrobin and prothioconazole reduced *L. maculans* and *L. biglobosa* lesion size.
- Penthiopyrad plus picoxystrobin had a differing effect on the lesion size of both species; *L. biglobosa* was less affected by the application.

## Discussion

- A fungicide mixture of penthiopyrad plus picoxystrobin is equally effective at reducing phoma stem leaf lesions and basal stem cankers in a severe disease year (2013/14), when compared with an azole based fungicide.
- Lesion sizes of both *L. maculans* and *L. biglobosa* appear to be equally affected by prothioconazole. However, penthiopyrad plus picoxystrobin appears to be less affective on *L. biglobosa* type lesions.
- Both penthiopyrad plus picoxystrobin and prothioconazole are effective fungicides that could be employed to control phoma stem canker caused by both *Leptosphaeria* spp.

## References

1. Fitt, B. D. L., Huang, Y.-J., van den Bosch, F., & West, J. S. (2006). Coexistence of related pathogen species on arable crops in space and time. *Phytopathology*, *44*, 163–82.
2. Huang, Y.-J., Karandeni-Dewage CS, & Fitt, B. D. L. (2014). Importance of *Leptosphaeria biglobosa* as a cause of phoma stem canker on winter oilseed rape in the UK. *Aspects of Applied Biology*, *127*, 117–122.
3. Oliver, R. P., & Hewitt, H. G. (2014). *Fungicides in crop protection.* CABI.
4. **Sewell, T. R., Moloney, S., Ashworth, M., Ritchie, F., Mashanova, A., Huang, Y. J., et al. (2016). Effects of a penthiopyrad and picoxystrobin fungicide mixture on phoma stem canker (*Leptosphaeria* spp.) on UK winter oilseed rape. *European Journal of Plant Pathology*, 1–11.**

University of Hertfordshire

Acknowledgements

DUPONT ADAS Perry Foundation FELIX COBBOLD TRUST

# Investigating the Potential Threat of *L. biglobosa* to UK Oilseed Rape Yield

Thomas Sewell[1], Chinthani Karandeni Dewage[1], Yongju Huang[1], Henrik Stotz[1], Mike Ashworth[2], Philip Walker[3], Faye Ritchie[3] and Bruce D.L. Fitt[1]

[1] School of Life and Medical Sciences, University of Hertfordshire, Hatfield, Herts, AL10 9AB, UK; [2] Dupont (UK) LTD, Wedgewood Way, Stevenage, Herts, SG14QN; [3] ADAS, Battle Gate Road, Boxworth, Cambridge, CB23 4NN.

## Introduction

- Phoma stem canker, a damaging disease of oilseed rape, is caused by the *Leptosphaeria* species-complex which currently consists of *Leptosphaeria maculans* and *L. biglobosa,* along with many sub-clades that appear to be either host or locality specific (Figure 2).[3,4]
- Typically, *L. maculans* is considered the more damaging of the two pathogens due to the development of basal stem cankers on oilseed rape. Although, in recent years *L. biglobosa* has also shown to cause basal stem cankers. [2,6]
- Fungicides are used in the UK to control phoma stem canker with a typical application rate of two or three sprays per cropping season. [2,6]
- Other economically important plant pathogens, such as *Pyrenopeziza brassicae*, the causal agent of light leaf spot, have shown a decrease sensitivity to fungicides.[1]
- Mutational changes in the gene coding for 14$^{\alpha}$-demethylase (CYP51), the target site for triazole fungicides, has been highlighted as the possible cause of reduced fungicide efficacy *in vitro.*[1]

**AIM: To identify which pathogen is causing phoma stem canker and what effect fungicides are having on the pathogen population structure**

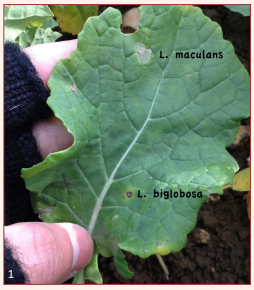



Figure 1: Pathogen lesions on oilseed rape differ: *L. maculans* develops a large grey lesion with visible pycnidia while *L. biglobosa* forms a more constrict dark lesion with very little visible pycnidia

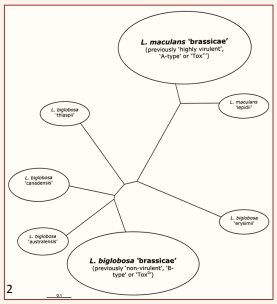



Figure 2: Unrooted dendrogram of the *Leptosphaeria* species-complex. Modified from: Eckert MR., 2005 *PhD Thesis*

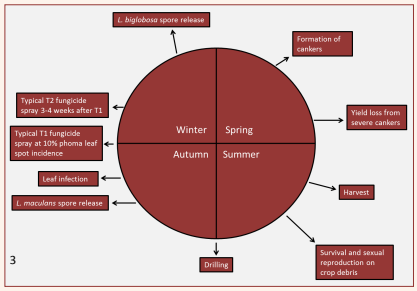



Figure 3: *L. maculans* and *L. biglobosa* have different spore-release timings. Current practice targets only *L. maculans* as the causal agent of Phoma stem canker


Figure 4: The two pathogens display differing phenotypes *in vitro.* Growth on V8 medium

## Methods

- *L. maculans* and *L. biglobosa* DNA Quantification using QPCR was carried out on stem canker samples taken from 9 cultivars during cropping season 2011/2012.
- *In vitro* sensitivity testing was done on one *L. maculans* and one *L. biglobosa* isolate. Flusilazole, at concentrations 0.001, 0.003, 0.01, 0.03, 0.1, 0.3, 1.0 µg/ml, was applied to PDA medium, which was later inoculated with a mycelium plug.

## Results

- A higher-level of *L. biglobosa,* compared to *L. maculans,* DNA was identified in basal stem cankers in various cultivars except Drakkar (Figure 5 b,d).
- A significant difference ($P \leq 0.05$) in growth inhibition was observed between *L. biglobosa* and *L. maculans* isolates when treated with flusilazole (Figure 6).

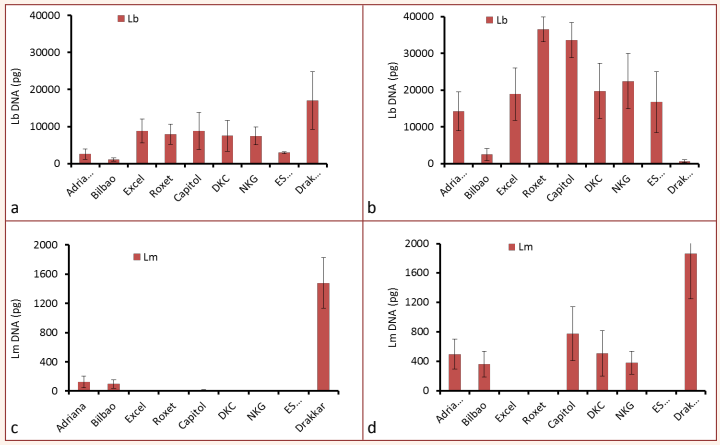



Figure 5: DNA quantification, using QPCR, of diseased upper stem (a, c) and stem base samples (b,d) from a selection of oilseed rape cultivars. Samples were taken during the 2011/2012 cropping season. Lm = *L. maculans*; Lb = *L. biglobosa*. Graph modified from HGCA project, data courtesy of Chinthani Karandeni Dewage.

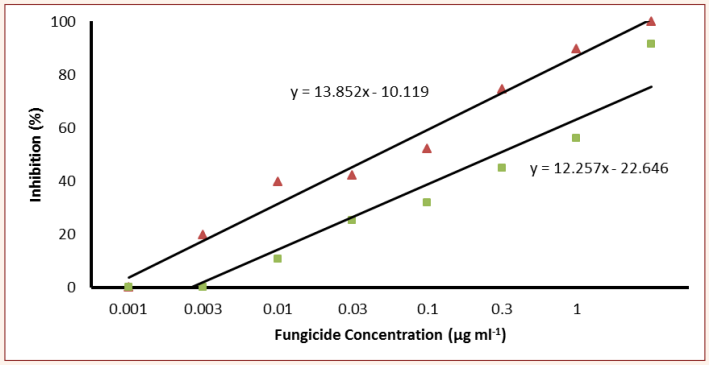



Figure 6: Percentage of inhibited growth of *L. maculans* and *L. biglobosa* isolates by Sanction (containing 250 µg ml-1 flusilazole). Both pathogens show a linear response to an increase in fungicide concentration, with *L. biglobosa* having a smaller response than *L. maculans*. Data courtesy of Stephen Moloney.

## Discussion

- Varietal control differs between pathogen species. Current cultivars target *L. maculans* growth allowing *L. biglobosa* to prosper. Countrywide planting of some cultivars may be selecting *L. biglobosa* populations (Figure 5).
- Typically, *L. maculans* is considered the more damaging of the two pathogens. However, this data shows that *L. biglobosa* can cause basal stem cankers (Figure 5b), which could have a negative effect on yield.
- A difference in sensitivity to current commercial fungicides, combined with the fact that control strategies target *L. maculans*, suggests that current spraying practice could be selecting *L. biglobosa* populations (Figure 6).
- This data highlights the potential threat of closely related 'sibling pathogens' to arable agriculture.

## References

1. CARTER, H. E., FRAAIJE, B. A., WEST, J. S., KELLY, S. L., MEHL, A., SHAW, M. W. & COOLS, H. J. 2014. Alterations in the predicted regulatory and coding regions of the sterol 14α-demethylase gene (CYP51) confer decreased azole sensitivity in the oilseed rape pathogen Pyrenopeziza brassicae. *Molecular Plant Pathology*.
2. ECKERT, M. R., ROSSALL, S., SELLEY, A. & FITT, B. D. L. 2010. Effects of fungicides on in vitro spore germination and mycelial growth of the phytopathogens *Leptosphaeria maculans* and *L. biglobosa* (Phoma stem canker of oilseed rape). *Pest Management Science*, 66, 396-405.
3. FITT, B. D., BRUN, H., BARBETTI, M. & RIMMER, S. 2006a. World-wide importance of phoma stem canker (Leptosphaeria maculans and L. biglobosa) on oilseed rape (Brassica napus). *Sustainable strategies for managing Brassica napus (oilseed rape) resistance to Leptosphaeria maculans (phoma stem canker)*. Springer.
4. FITT, B. D., HUANG, Y.-J., VAN DEN BOSCH, F. & WEST, J. S. 2006b. Coexistence of related pathogen species on arable crops in space and time. *Phytopathology*, 44.
5. HUANG, Y. J., HOOD, J. R., ECKERT, M. R., STONARD, J. F., COOLS, H. J., KING, G. J., ROSSALL, S., ASHWORTH, M. & FITT, B. D. L. 2011. Effects of fungicide on growth of Leptosphaeria maculans and L. biglobosa in relation to development of phoma stem canker on oilseed rape (Brassica napus). *Plant Pathology*, 60, 607-620.
6. STONARD, J. F., LATUNDE-DADA, A. O., HUANG, Y.-J., WEST, J. S., EVANS, N. & FITT, B. D. 2010. Geographic variation in severity of phoma stem canker and *Leptosphaeria maculans/L. biglobosa* populations on UK winter oilseed rape (Brassica napus). *European journal of plant pathology*, 126, 97

Acknowledgements

University of Hertfordshire

HGCA DUPONT ADAS Royal Agricultural University Felix Cobbold Trust

# Effects of Different Fungicides on Development of Phoma Stem Canker and Oilseed Rape Yield

Thomas Sewell[1], Steven Moloney[1], Yongju Huang[1], Henrik Stotz[1], Mike Ashworth[2], Philip Walker[3], Faye Ritchie[3] and Bruce D.L. Fitt[1]
[1] School of Life and Medical Sciences, University of Hertfordshire, Hatfield, Herts, AL10 9AB, UK; [2] Dupont (UK) LTD, Wedgewood Way, Stevenage, Herts, SG14QN; [3] ADAS, Battle Gate Road, Boxworth, Cambridge, CB23 4NN.

## Introduction

- Phoma stem canker, a disease of oilseed rape (*Brassica napus*) caused by sibling pathogens *Leptosphaeria maculans* and *L. biglobosa*, is an economically important disease causing annual yield losses of approximately £1000M worldwide (Fitt *et al*., 2008).
- Both pathogens follow a monocylic disease cycle that causes leaf spotting in autumn/winter and damaging stem cankers in spring/summer. Most severe cankers decrease transportation of water and nutrients to the developing seeds, resulting in reduced yield and financial loss to the grower (Eckert *et al*., 2010).
- The use of fungicides is an important tool for controlling phoma stem canker in the UK. Triazole fungicides currently dominate the market, although reduced sensitivity in some plant pathogen populations is an emergent concern (Carter *et al*., 2014).

Figure 1: Field trial at the ADAS facility in Boxworth, Cambridgeshire.

## Aims

- To examine the efficacy of a novel fungicide when compared with flusilazole, a commonly used triazole fungicide at the time of assessment.
- To investigate whether *L. biglobosa* is less sensitive to triazole fungicides than *L. maculans*.

## Methods

- Field trials were established at Boxworth (Cambridgeshire) in the 2012/2013 cropping season (Figure 1). Fungicides applied were: penthiopyrad, picoxystrobin, flusilazole and a novel fungicide.
- There were three spray timings at T1, T2 and T3, with T1 applied in autumn when phoma leaf spotting incidence was ≥10% plants affected and T2 sprayed four weeks later. T3 was applied in spring against *Sclerotinia sclerotiorum*. Phoma leaf spotting incidence, stem canker severity and yield were recorded.
- *In vitro* sensitivity testing was done on one *L. maculans* and one *L. biglobosa* isolate. Flusilazole, at concentrations 0.001, 0.003, 0.01, 0.03, 0.1, 0.3, 1.0 µg/ml, was applied to PDA medium, which was later inoculated with a mycelium plug.

## Results

- Yield (t/ha) results indicated no difference between flusilazole and the novel fungicide (Figure 2).
- A significant difference ($P \leq 0.05$) in growth inhibition was observed between *L. biglobosa* and *L. maculans* isolates when treated with flusilazole (Figure 3).

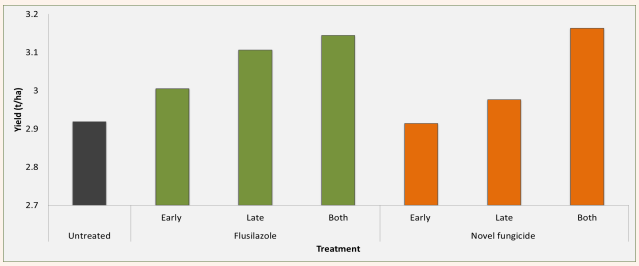



Figure 2: Yield (t/ha) results from field trials for plots treated with flusilazole or the novel fungicide; 2012/2013 cropping season.

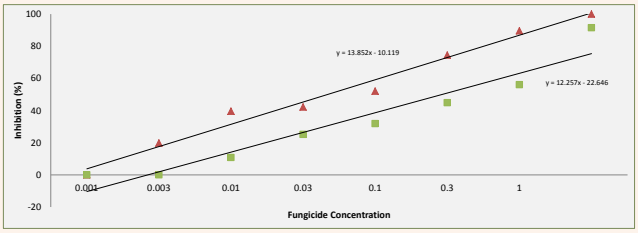



Figure 3: Percentage of inhibited growth of *L. maculans* and *L. biglobosa* isolates by Sanction (containing 250 µg ml-1 flusilazole). Both pathogens show a linear response to an increase in fungicide concentration, with *L. biglobosa* having a smaller response than *L. maculans*.

## Discussion

- Field trials indicate that the novel fungicide is equally as effective as the more commonly used triazole fungicides and could replace flusilazole for commercial use.
- Sensitivity testing showed that *L. biglobosa* does have an increased level of insensitivity to triazole fungicides than *L. maculans*.
- This interaction confirms previous evidence of a reduced sensitivity to triazole fungicides in *L. biglobosa* populations (Eckert et al., 2010; Huang *et al*., 2011).

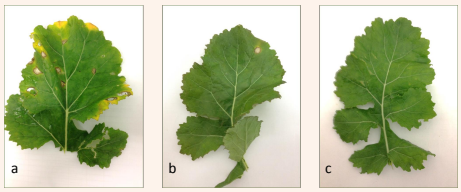



Figure 4: Sampled leaves illustrating difference in phoma leaf spotting incidence due to fungicide application: a) untreated b) treated with triazole fungicide c) treated with novel fungicide. Cropping season 2013/2014.

## References


- Carter, H. E., *et al.* (2014). "Alterations in the predicted regulatory and coding regions of the sterol 14α-demethylase gene (CYP51) confer decreased azole sensitivity in the oilseed rape pathogen *Pyrenopeziza brassicae*." Molecular Plant Pathology.
- Eckert, M. R., *et al.* (2010). "Effects of fungicides on in vitro spore germination and mycelial growth of the phytopathogens *Leptosphaeria maculans* and *L. biglobosa* (Phoma stem canker of oilseed rape)." Pest Management Science **66**(4): 396-405
- Fitt, B. D. L., *et al.* (2008). "Strategies to prevent spread of *Leptosphaeria maculans* (phoma stem canker) onto oilseed rape crops in China; costs and benefits." Plant pathology **57**(4): 652-664.
- Huang, Y. J., *et al.* (2011). "Effects of fungicide on growth of *Leptosphaeria maculans* and *L. biglobosa* in relation to development of phoma stem canker on oilseed rape (Brassica napus)." Plant pathology **60**(4): 607-620.


University of Hertfordshire

Acknowledgements