University of Reading

Genetics behind the variability in sensitivity to the demethylation inhibitor (DMI) fungicides myclobutanil and tebuconazole in *Venturia inaequalis*

Thesis submitted in the fulfillment of the Degree of Doctor of Philosophy

School of Agriculture, Policy and Development

Laura Cordero Limon

March 2018

Declaration

I confirm that this is my own work and the use of all material from other sources has been properly and fully acknowledged

Laura Cordero Limon

March 2018

Acknowledgements

The work presented here would not have been possible without the cooperation and support of other people.

The research in this thesis was co-funded by NIAB EMR and the University of Reading, with financial support from Bayer Crop Science toward the cost of consumables. Their financial support was essential for the development of the work presented here.

The research in this study greatly benefitted from the help and advice of many people who lent their expertise and equipment. I would like to thank Les Crompton and Becky Morgan for allowing me to use the microplate reader in their laboratory necessary for the development of Chapter 3 and to Richard Harrison in NIAB EMR for his help and advice on how to set up my microplate experiment during my stay in NIAB EMR. I am grateful to Fay Newbery and Solomon Agyare for providing useful advice and information as well as laboratory help, and for introducing me to some new techniques. I would also like to remember here all other staff, postdoctoral researchers and PhD students in the University of Reading and NIAB EMR who ever lent me a hand and allowed me to move forward. I really appreciate the kindness and time you offered me.

A special thank you should go to Thomas Passey and Joyce Robinson, who gave the most assistance during the practical work included in this thesis. They both shared their knowledge and experience of years of working with *Venturia inaequalis*. Even more importantly, they provided great support during the experiments: Joyce was a big help during the fungicide tests and Tom with the molecular biology work. Apart from these, they also provided help throughout all the experimental work. Without them, this thesis would not have become a reality.

I would also like to thank my supervisors, Prof. Michael Shaw and Prof. Xiangming Xu, for their helpful advice and guidance over these past years. Also, for being understanding about the challenges of a PhD, providing the support I needed and allowing me to set the direction of my PhD during the final stages.

It is not only the practical support that is important to complete a thesis, but also the emotional support. I would like to thank my housemates in Great East, where I was staying during my time in NIAB EMR. I would like to specially thank Rong Fan, my unconditional friend during the PhD. Thank you for always being there reminding me that I was not alone and sharing the hardships of doing a PhD with me.

To my parents and family, thank you for supporting me all this time, for being my safety net through all this and reminding me that there is always a silver lining to look forward to.

To Joe, thank you for all the emotional support that has carried me through this PhD.

Abbreviations

The list of abbreviations presented below is also available as a fold-out at the end of this thesis.

ABC: ATP-Binding Cassette

AHDB: Agriculture and Horticulture Development Board

C/N: Carbon to Nitrogen ratio

CV: Coefficient of Variation

CYP51: Cytochrome P450, family 51

Cytb: Cytochrome b

DMI: Demethylation Inhibitor

ED_{50}: Concentration of fungicide at which growth is reduced to 50%; ED stands for effective dose

EUROSTAT: European Union Statistical Office

FAOSTAT: Food and Agriculture Organization Corporate Statistical Database

FRAC: Fungicide Resistance Action Committee

GWAS: Genome Wide Association Studies

ha: hectare

HSE: Health and Safety Executive

IMS: Industrial Methylated Spirit

log: Logarithm (considered as natural logarithm unless stated otherwise)

logED_{50}: Natural logarithm of the effective dose (ED_{50})

MBC: Methyl Benzimidazole Carbamate

MRL: Maximum Residue Limit

RG: Relative Growth (growth relative to the control treatment)

PCR: Polymerase Chain Reaction

PDA: Potato Dextrose Agar

PDB: Potato Dextrose Broth

ppm: parts per million

PWA: Plain Water Agar

Q-Q: Quantile-Quantile

QTL: Quantitative Trait Loci

SEM: Standard Error of the Mean

SNP: Single Nucleotide Polymorphism

w/v: weight per volume

Abstract

DMI (Demethylation Inhibitor) fungicides are the family of fungicides most commonly used for the post infection control of apple scab disease, which is produced by the fungus *Venturia inaequalis*. One of the most widely used DMI fungicides in the UK and many other countries is myclobutanil. Strains resistant to myclobutanil have been reported across the world, prompting a need to find new products effective against the disease and to improve the existing knowledge about the genetics behind the resistance and the evolution process.

Attempts to design a new microplate-based assay to test for fungicide resistance were unsuccessful.

A total of 40 isolates coming from two sources (a baseline orchard which had never been sprayed with fungicides and a collection of orchards with disease control problems thought to be a result from cases of fungicide resistance) were tested for resistance to fungicides myclobutanil and tebuconazole, a recently introduced DMI fungicide. Results confirm the trend of increased resistance to myclobutanil observed in other countries. Cross-resistance was discovered between the two chemicals, which lowers the prospects of tebuconazole to be used as a substitute to myclobutanil.

Sequencing of the target *CYP51A1* gene did not find any mutations linked to resistance in the gene sequence. An analysis of the progeny of a RxS cross for sensitivity to tebuconazole revealed quantitative control of the sensitivity, involving at least two genes, in the studied cross and possibly epistatic effects, reflected by the asymmetric distribution of the sensitivity in the progeny.

Computer modelling of the evolution of the resistance helped identify some factors that can influence the rate of evolution. Most of the factors tested had a small effect on the evolution (epistasis and inclusion of overwintered conidia in the primary inoculum). The biggest effects were for fungicide coverage and fungicides with a strong post-symptomatic antisporulant activity.

Table of contents

1. Introduction

1.1. Literature review

1.1.1. The apple crop

Known by the scientific name of *Malus x domestica* Borkh or the alternative *M. domestica* (Ferree and Warrington 2003), apples belong to the Rosaceae and, within that group, to the Maloideae (Brown and Maloney 2005).

The crop is best suited for temperate areas (Mazzola 2002; Ferree and Warrington 2003), with China and the United States ranked as the main apple producers in the world (FAOSTAT 2014). In the EU, Poland, Italy and France top the table of apple producers, where the UK stands in 7th place (EUROSTAT 2015). In the UK, production is concentrated in the south of the country (Collier and Else 2014).

During autumn and throughout the winter the trees phase into a dormant state that ends in spring with the beginning of the bud growth (Strand 1999). The transition between dormancy and the bud growth stage requires the tree to be subjected to a period of cool temperature, under seven degrees, for around 1200-1500 hours, with some varieties needing shorter periods than this (Strand 1999). This chilling period modifies the reaction of the bud to temperature (Jackson 2003) so that when temperatures increase again to a suitable level budbreak takes place. The buds give rise to either only leaves (vegetative buds) or both leaves and flowers (mixed buds) (Strand 1999). There is no consensus yet on all the factors that affect the transition from a vegetative bud to a mixed one (Rivero Casique 2015). Some of the factors that have been studied in this regard are light, temperature, C/N ratios, hormone levels or the number of leaves (Rivero Casique 2015).

In these mixed buds, flowers come out in white and pink colours and produce fruits with seeds sheltered in a cavity formed by carpels (Brown and Maloney 2005).

As with any crop, there are many diseases that affect apple, caused by pathogens which include bacteria, fungi and viruses (Ferree and Warrington 2003). Among these, fungal diseases constitute the largest group, including scab, powdery mildew, cankers, brown rots, collar rot, root rot and a range of summer fruit diseases, including fly speck, sooty blotch and different types of rot (Ferree and Warrington 2003; Rosenberger, Cox et al. 2012).

In the UK, the most important diseases are apple scab, powdery mildew and European canker. In young trees, crown rot and root rot, usually caused by *Phytophthora cactorum*, can also be a problem and result in tree death (Cross, Berrie et al. 2017). This outcome can also be observed at times in trees infected by the European canker pathogen (*Neonectria ditissima*), which also produces damage to branches and fruits (Cross, Berrie et al. 2017).

Powdery mildew (*Podosphaera leucotricha*) takes second place in order of importance in the UK (Xu, Gao et al. 2010). Apart from interfering with the growth of flowers, shoots and leaves, this disease can also produce defoliation, which has serious effects for tree yield, and reduces fruit quality (Cross, Berrie et al. 2017).

However, none of the above diseases is as damaging for apple production as apple scab, which can result in losses above 70% of the crop production (Cross, Berrie et al. 2017).

1.1.2. Scab as a disease

Apple scab is a disease of apple trees caused by the ascomycete fungus *Venturia inaequalis*. *Venturia inaequalis* is capable of infecting not only apples and crab apples, but also other species such as firethorn (*Pyracantha)*, hawthorn (*Crateagus*), loquat (*Eriobotrya japonica*) and mountain ash (*Sorbus spp.*) (Vaillancourt and Hartman 2000). However, isolates

specialised in colonising species other than apple are not able to cause scab disease on domesticated apple (Bowen, Mesarich et al. 2011). Previous studies (Gladieux, Zhang et al. 2008) have pinpointed the origin of the fungus, locating it in Central Asia, although these days the pathogen can be found all over the world. However, not all regions are affected to the same extent, with wet spring climates, as is the case in the UK, favouring more severe infections (Bowen, Mesarich et al. 2011).

Throughout the disease cycle, leaves and fruits are the main tissues infected, but the fungus is also capable of infecting others such as flowers, peduncles, new shoots, petioles and bud scales (Vaillancourt and Hartman 2000; Chapman 2011). The appearance of infections varies depending on the tissue and the timing of the infection.

On leaves, scab can be seen as marks which come in brown and green shades, and can be found both in the upper and lower side of the leaf (Carisse and Dewdney 2002). Extreme infection of leaves can lead to complete defoliation of trees, which, besides preventing photosynthesis, has an effect on tree yield by reducing tree defences against cold, insects and other pathogens (Vaillancourt and Hartman 2000).

Fruit infection symptoms may differ depending on the stage at which the fruit becomes infected. Late in the season, an infection would usually result in superficial lesions (Lucas, Campbell et al. 1992), while early infections are more likely to produce cracks, as the development of the callus tissue becomes stunted, and deformations, as the presence of the fungus interferes with the growth process (Carisse and Dewdney 2002). All fruit infections have serious consequences for the growers, since any lesion or deformation in the fruit could make fruit harder to market (Koller, Parker et al. 2005). Besides this aesthetic element, early fruit infections pose another danger in the form of cracks, which leave the fruit open to

infections by other pathogens (Turechek 2004). When the damage is only aesthetic in nature, fruits can still be eaten, as the fungus grows in the skin layer of the fruit between the cuticle and the epidermis, and its slow growth makes it easy to remove the infected area (Grierson 2014).

Leave and fruit infections are not the only type of infection to cause trouble. Infections of other tissues can also have an impact on production, such as infected pedicels, which can lead to fruit loss (Vaillancourt and Hartman 2000).

In addition to crop losses, another source of economic losses for the growers is the cost of managing diseases, which usually relies on investing on intensive spray programs (Wilcox 1992; Carisse and Dewdney 2002). In the UK, the cost of ca. 13 spray programs annually is roughly £300 per ha (Barbara, Roberts et al. 2008).

1.1.3. Epidemiology

Venturia inaequalis is an ascomycete fungus, producing both ascospores and conidiospores. Its epidemiology thus centres on these two types of spores. The former come from sexual reproduction, having undergone sexual recombination, and thus, likely differing from both parental isolates; the asexual conidospores are genetically identical to the isolate that produced them.

The disease cycle (Figure 1.1) starts in spring when wet weather (usually rainfall) promotes ascospore release (Vaillancourt and Hartman 2000). These ascospores are injected into the air following a rainfall event and then disseminated by wind (Vaillancourt and Hartman 2000), reaching susceptible tissue. Both moisture and temperature are important factors for

spore germination and infection (Vaillancourt and Hartman 2000; Carisse and Dewdney 2002).

The infection process starts with the ascospore attaching itself to the leaf by producing mucilage, and later making its way inside plant tissue, which is thought to involve a hydrolysis of the cuticle by fungal enzymes (Bowen, Mesarich et al. 2011). Inside the tissue, the mycelia develop in the subcuticular space, and after some time start developing conidia, at that point the plant cells start dying through a necrotic process accompanied by vacuolation (Bowen, Mesarich et al. 2011).

Ascospore infections happen only in a time window that stretches from the start of spring until the beginning of the next season (Bowen, Mesarich et al. 2011); by contrast, there could be potentially many cycles of conidiospore infection in the growing season (Aylor 1998; Vaillancourt and Hartman 2000), with spores carried by rain and, to a lesser extent, wind that end up colonising new tissue (Vaillancourt and Hartman 2000), which in turn will produce more conidiospores. As with ascospores, temperature and moisture are important for conidiospore production, germination and infection (Vaillancourt and Hartman 2000). When autumn comes, scab survives on the fallen leaves where sexual reproduction takes place (Carisse and Dewdney 2002). Eventually, a structure called a pseudothecium forms from which ascospores develop inside asci (Vaillancourt and Hartman 2000; Carisse and Dewdney 2002; Bowen, Mesarich et al. 2011), ready to give rise to a new disease cycle at the start of the next spring.

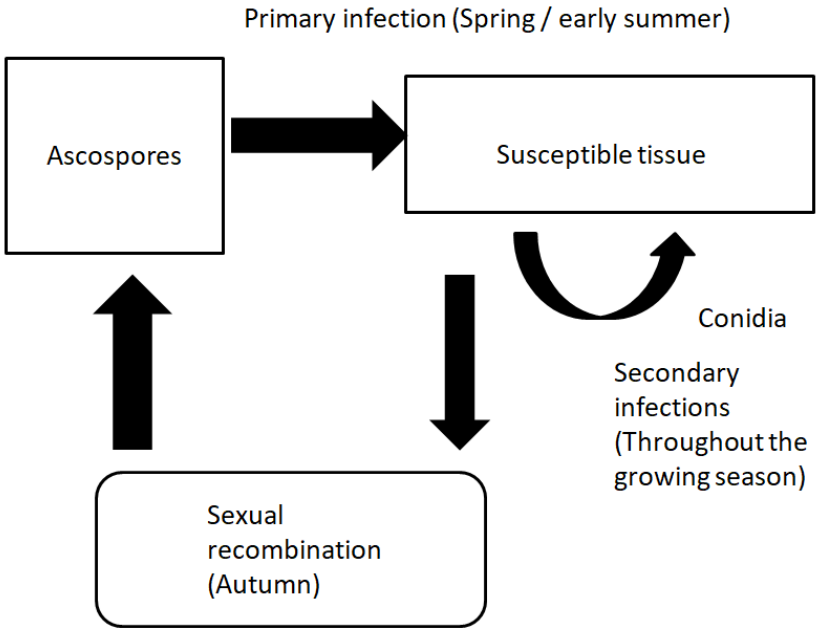


Figure 1.1. *Venturia inaequalis* disease cycle in apples.

1.1.4. Control

There are several types of disease control measures that can be employed in apple scab management: cultural techniques, introduction of resistant genes, biocontrol and chemical control. The first comprise a range of methods, mainly aimed at the ascospore stage of the disease cycle (Carisse and Dewdney 2002). Some of them seek to destroy the initial ascospore inoculum (Carisse and Dewdney 2002; Pfeufer and Ngugi 2012) by targeting the autumn fallen leaves where sexual reproduction takes place. Burning the leaves, destroying them, promoting their degradation by microorganism or earthworms are all examples of this category (Carisse and Dewdney 2002; Pfeufer and Ngugi 2012). The use of microorganisms to accelerate leaf degradation can be achieved through the use of urea sprays which have a positive effect on the growth of soil microorganisms (Adedeji 1986), apart from also having an inhibitory action on pseudothecium formation (Burchill, Hutton et al. 1965; Burchill 1968;

Carisse and Dewdney 2002). As mentioned before, wet tissue is important for an infection to develop, so another set of techniques tries to prevent infections by improving ventilation, either by frequent pruning or controlling the space between trees (Vaillancourt and Hartman 2000; Carisse and Dewdney 2002; Pfeufer and Ngugi 2012).

Genetic resistance is also exploited as a form of control (Carisse and Dewdney 2002; Bowen, Mesarich et al. 2011; Cusin, Revers et al. 2017). This resistance can come from genes that are introduced through breeding. The problem of breeding in resistance genes is that they often come accompanied with undesirable traits in fruit size, quality or production which, along with a consumer unwillingness to try new products, translate into unsatisfactory market performance (Carisse and Dewdney 2002; Cross, Berrie et al. 2017). In addition, the breeding process in apples is lengthy due to a long juvenile stage (Acquaah 2007; Gessler and Pertot 2012). This explains why most of the genetic resistance introduced is monogenic as the long process means it is quicker to introduce a single gene (Carisse and Dewdney 2002). Varieties with polygenic resistance do exist in nature though, and it is usually more difficult for pathogens to overcome this kind of resistance (Carisse and Dewdney 2002; Bowen, Mesarich et al. 2011). A number of genes that provide resistance have been identified, of which the one that has been used in breeding programs for the longest time is *Rvi6* (*Vf*) (Carisse and Dewdney 2002); unfortunately, resistance by *Rvi6* has already been overcome (Sansavini, Tartarini et al. 2000; Gessler and Pertot 2012). Other resistance genes are: *Vg* (*Rvi1*), *Vh2* (*Rvi2*), *Vh3* (*Rvi3*), *Vh4* (*Rvi4*), *Vm* (*Rvi5*), *Vfh* (*Rvi7*), *Vh8* (*Rvi8*), *Vdg* (*Rvi9*), *Va* (*Rvi10*), *Vbj* (*Rvi11*), *Vb* (*Rvi12*), *Vd* (*Rvi13*), *Vdr1* (*Rvi14*), *Vr2* (*Rvi15*), *Vmis* (*Rvi16*) and *Va1* (*Rvi17*) (Bus, Rikkerink et al. 2011).

Another control possibility is based on a form of partial resistance observed in mixed orchards (Carisse and Dewdney 2002; Barbara, Roberts et al. 2008; Passey, Shaw et al. 2016). There are two sides to this strategy. One of them takes into account how trees can have different degrees of resistance to scab, while the other is based on pathogens that are better adapted to infect a certain tree variety and unable to infect others as effectively (Carisse and Dewdney 2002). The idea in both instances is to grow more than one tree variety together, arranged in a manner that would slow down the spread of disease (Carisse and Dewdney 2002).

Biological control could be another option, although at the moment there are no commercial products available specifically against apple scab (Cross, Berrie et al. 2017). Right now, there are two organisms with some potential, *C. globosum* and *A.bombacina*, with a third, *Cladosporum* sp., that could be used during the sexual stage of the disease cycle (Cross, Berrie et al. 2017) .

Despite this variety of options, chemical control is still the preferred choice of many due to their greater efficacy and relatively cheap costs compared to some of the other control measures (Pfeufer and Ngugi 2012).

Fungicides can be classified into two categories according to their mode of action: protectant or curative (Jobin and Carisse 2007). Protectant chemicals are intended as prevention measures interfering with spore germination and infection (Koller, Parker et al. 2005; Cross, Berrie et al. 2017). They usually target more than one biological site and are often not able to get inside plant tissue (Koller, Parker et al. 2005). Curative fungicides act after infection has taken place, and their action is much more targeted to specific elements exclusive to the fungus biology, since they can cross the plant barriers (Koller, Parker et al.

2005). Their activities can include disrupting lesion emergence and inhibiting spore production (Koller, Parker et al. 2005). Some fungicides, such as the DMIs (Delye, Laigret et al. 1997; Koller, Parker et al. 2005), have both protectant and curative activities.

The use of fungicides does not mean that other control tactics cannot be used at the same time. In fact, tactics can be combined into strategies that may involve several types of control simultaneously or sequentially. Some of these strategies can be improved by the use of infection models. These models allow growers to adjust the timing of the control measure to maximise efficiency and reduce costs by predicting infection outbreaks and spraying in response to predicted outbreaks (Berrie and Xu 2003; Koller, Parker et al. 2005), which also reduces the amount of chemicals used (Vaillancourt and Hartman 2000). They base these predictions on weather conditions, such as leaf wetness and temperature (MacHardy and Gadoury 1989; Vaillancourt and Hartman 2000; Koller, Parker et al. 2005). If overhead irrigation is used, infections may be reduced by using model information, such as the numbers contained in the Mill's table, to make a judgement on the length of each irrigation period at each temperature (Swezey, Vossen et al. 2000). However, the most popular use of infection models is to improve the timing of fungicide sprays.

1.1.5. Spray programs

Fungicide programs to control apple scab usually start at bud-break (Figure 1.2) and finish when the grower decides there is no further danger of disease, at times extending all the way to harvest (Berrie and Xu 2003). Sprays are usually applied in 7-10 days intervals (Xu, Gao et al. 2010) or following scab warnings (Berrie and Xu 2003). A time window has to be left before the fruit is harvested; this fungicide-free period is known as the preharvest

interval (Table 1.1.). This is necessary to comply with legal requirements, which vary depending on the chemical, as each has a different degradation rate.

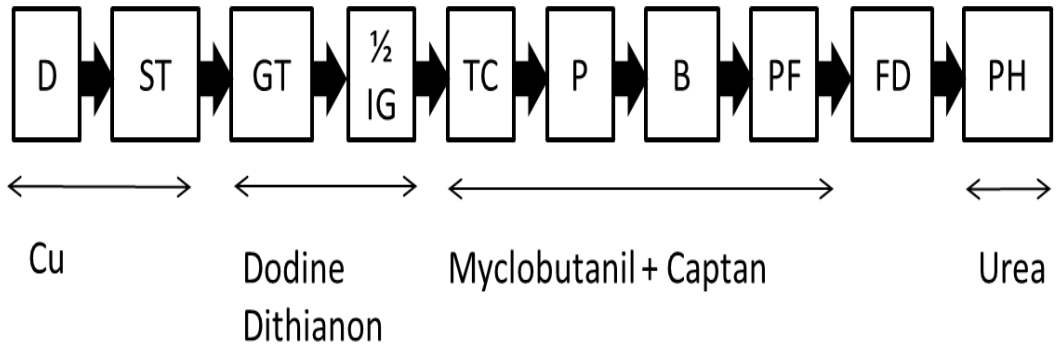


Figure 1.2. Example of a typical spray program to control apple scab constructed from information found in the Apple Best Practice Guide by AHDB Horticulture (Cross, Berrie et al. 2017). D: Dormancy, ST: Silver tip, GT: Green tip, ½ G: Half-inch green, TC: Tight Cluster, P: Pink, B: Bloom, PF: Petal Fall, FD: Fruit Development, PH: Postharvest.

Table 1.1. Example of fungicide characteristics and application parameters [1].

Product [2]	Active ingredient	Spray Interval (days)	Preharvest interval (days)	Maximum residue limit (mg/kg)	Degradation in soil (days) [3]
Systhane 20EW	Myclobutanil	7.0-14.0	14.0	0.6	560.0

[1] Information in this table was extracted from the Systhane 20EW (Dow AgroSciences) label, from the HSE MRL Database (HSE 2017) and the University of Hertfordshire Pesticide Properties Database (University of Hertfordshire 2017).

[2] Systhane is the model fungicide used in the experimental work in this thesis. Although it was in use for many years, it was to be withdrawn in 2016. However, the withdrawal was later reviewed and postponed to 2017.

[3] Degradation rate is given as the half-life of the chemical.

There are strategies that can be implemented to reduce residues in fruit. One consists of spraying only after a warning (Vaillancourt and Hartman 2000). Another tactic tries to avoid any spraying during fruit development and focus all control during dormancy and before petal fall (Berrie and Cross 2006). In this latter system spraying after dormancy is targeted at important points of the disease cycle and in response to warnings (Berrie and Cross 2006). After petal fall, growers resort back to cultural methods until harvest, and then return to fungicide use after fruit has been collected to help control ascospore inoculum (Berrie and Cross 2006).

There is a wide range of fungicides that can be used in spray programs (Table 1.2.); in the UK, the most popular fungicide family for the post-infection control of apple scab is the DMI family due to their also potent action against powdery mildew (Xu, Gao et al. 2010).

1.1.6. DMI fungicides

DMIs are a group of fungicides known as the demethylase inhibitors, including piperazines, pyridines, pyrimidines, imidazoles, triazoles and triazolinthiones (FRAC 2017). They target the protein 14-α demethylase, which is part of the ergosterol biosynthesis pathway (Pfeufer and Ngugi 2012). Its inhibition by DMIs renders isolates unable to make ergosterol, badly damaging the cell membrane (van den Brink, van Nistelrooy et al. 1996; Joseph-Horne and Holomon 1997).

The DMI family has a strong pre-symptom activity, preventing early infections from developing into lesions and significantly reducing conidiospore production (Schwabe, Jones et al. 1984; O'Leary and Sutton 1986; Vaillancourt and Hartman 2000). The benefits of post-symptom sprays are less clear, with some studies showing a reduction in conidiospore production or even germination, while others did not find any effect (O'Leary and Sutton 1986; Poblete and Latorre 2001).

Ascospore formation is also affected by DMI use. Both post-harvest applications and within season sprays have been reported to reduce the number of ascospores and pseudothecia produced (O'Leary and Sutton 1986; Cross, Berrie et al. 2017).

Table 1.2. Examples of fungicides that had been used in the past and those currently in use to control apple scab.

Fungicide Family [1]	Examples [2]	Activity [3]
Inorganic	**Copper, Sulfur**	Protectant
Dithiocarbamates	**Mancozeb**	Protectant
Quinones	**Dithianon**	Protectant
Phtalimide	**Captan**	Protectant
Guanidines	**Dodine**	Curative
MBCs	**Carbendazim, thiophanate-methyl,** benomyl, fuberidazole, thiabendazole	Curative
DMIs	**Fenarimol, myclobutanil, pyrifenox, fenbuconazole, penconazole, difenoconazole,** imazalil, oxpoconazole, pefurazoate, prochloraz, prochloraz-manganese, triflumizole, triforine, pyridinitrile, nuarimol, triarimol, azaconazole, bitertanol, bromuconazole, cyproconazole, diclobutrazol, diniconazole, epoxiconazole, etaconazole, fluotrimazole, fluquinconazole, flusilazole, flutriafol, hexaconazole, imibenconazole, ipconazole, metconazole, propioconazole, simeconazole, tetraconazole, triadimefon, triadimenol, triticonazole, uniconazole, prothioconazole	Curative

Fungicide Family [1]	Examples [2]	Activity [3]
Phenylpyrroles	**Fludioxonil**	Curative
QoI	**Kresoxim-methyl**, **trifloxystrobin**, **pyraclostrobin**	Curative
Anilinopyrimidines	**Pyrimethanil**, **cyprodinil**	Curative
SDHI	**Boscalid**, **penthiopyrad**, **fluxapyroxad**	Curative

[1] Inorganic fungicides are often used in organic production.

[2] Fungicides used for the control of apple scab appear in bold, and those which are currently approved for use in apple in the UK underlined (Locke, Berrie et al. 2011; HSE 2017).

[3] Fungicides that have curative activity are designated as curative, although a number of them also provide some protectant activity.

1.1.7. Fungicide resistance

Fungicides are unlikely to remain effective indefinitely. The amount of time a fungicide retains its efficacy is dictated by how quickly their intended targets develop resistance.

There are different ways to define resistance. Resistance can be viewed at different levels of biological organization from a cell to an isolate, and in terms of a whole population in the field (FRAC 2017). In an isolate, resistance would refer to changes following exposure to the chemical that increase the dose of chemical that the isolate can take without having its biological processes significantly disrupted (FRAC 2017). In a population, resistance is usually measured from field control, and therefore can be described as a state where control has become jeopardised by the increase of resistant isolates within the population (Koller, Wilcox et al. 1997).

Resistance may be qualitative or quantitative (Georgopoulos and Skylakakis 1986). The former refers to a resistance that is either present or absent in an individual isolate, such behaviour being due to a single gene (Georgopoulos and Skylakakis 1986; Brent and Hollomon 2007). The latter is observed when there is a continuous range of phenotypic response to a given fungicide (Georgopoulos and Skylakakis 1986; Brent and Hollomon 2007).

The development of fungicide resistance in a population is characterised by three, as described by van den Bosch, Paveley et al. (2011): the emergence stage, the selection stage and the adjustment stage. In the emergence stage, random mutations result in an isolate with greater resistance. This isolate, if it manages to survive, may lead up to a small population of resistant isolates that become an established part of the make-up of the whole

population, and thus resistance has effectively emerged. After this comes the selection phase in which the presence of the fungicide leads to further increase in the proportion of resistant isolates. It is the proportion of the resistant isolates in the population rather than total numbers that defines the extent of the resistance spread (van den Bosch, Paveley et al. 2011). This increase in resistant isolates gradually leads to losses in control efficacy. Once resistance poses a real problem, the time comes to try other control measures in an effort to fight back in what is known as the adjustment step.

Resistance development and spread are governed by two types of factors: those intrinsic to the pathogen and those coming from the fungicide (the selection pressure) (Georgopoulos and Skylakakis 1986; Hollomon 2015). The first are mostly dependent on the nature of the genetic alteration that conferred the resistance (Georgopoulos and Skylakakis 1986) and include fitness penalties associated to it, although other factors related to the biology of the pathogen also affect resistance evolution (Hollomon 2015). The second are the product of different factors, like the coverage of the spray, the efficacy of the fungicide, its degradation rate, the frequency of application (Skylakakis 1982; Georgopoulos and Skylakakis 1986; Chin 1987; Brent and Hollomon 2007) and the dose (Brent and Hollomon 2007; van den Bosch, Paveley et al. 2011). Other factors that may contribute to resistance development are more subtle. For example, wind may favour leaves holding different pathogen strains mixing on the ground, enriching the genetic diversity of the ascospores generated through sexual recombination (Gao, Berrie et al. 2009). Also, conidia that survive through the winter (Passey, Robinson et al. 2017) can act as a reservoir of resistance, since they would have already been put through and successfully endured fungicide sprays (Gao, Berrie et al. 2009).

In terms of fitness, when the resistance is mainly controlled by a single gene, isolates that experience no loss in fitness or very little loss are able to spread the resistance rapidly (Georgopoulos and Skylakakis 1986), while some authors believe that isolates with large fitness penalties, such as those resistant to kasugamycin and dicarboximides, will not last long after the fungicide is not being used (Georgopoulos and Skylakakis 1986). However, more recent studies contradict this point and suggest that the rate at which resistance is lost in the absence of a fungicide would be much slower than the rate at which the resistance evolved, even when there are fitness penalties of up to 50% (Shaw 1989; Shaw 2006; Mikaberidze and McDonald 2015). Fitness could behave differently in quantitative resistance. Georgopoulos and Skylakakis (1986) argue that the fact that more than one gene may need to go through mutation for the resistance to build up to a certain level means that fitness penalties would be more common. As a result, isolates that are very resistant would have a strong tendency to perform very poorly in the environment, so there would be more isolates around with a lesser level of resistance (Georgopoulos and Skylakakis 1986). Fitness, however, only affects the speed of evolution and cannot stop resistance from developing, as evidenced by the repetitive evolution of fungicide resistance to new chemicals (Chapman 2011). In fact, it seems that, in the field, resistant isolates with poor fitness are likely to acquire compensatory mutations that increase their fitness (Mikaberidze and McDonald 2015). This may explain why some researchers have not found any major fitness costs in field isolates of some pathogens. Chapman (2011) did not find any fitness costs in his *in vitro* tests of DMI-resistant isolates of *V. inaequalis*, and work on other pathogens and fungicide families seem to suggest that major fitness costs are not common (Deising, Reimann et al. 2008).

Dodine was introduced against apple scab in 1959, and after a decade cases of resistance had already arisen (Chapman 2011). After dodine came the MBCs, with episodes of resistance appearing during the 1970s (Chapman 2011). In the following decade (Chapman 2011), DMIs started to be used, with the first report of resistance happening in 1988 in an experimental orchard in Nova Scotia, Canada; and later, in 1997, a commercial orchard in Michigan (Errampalli 2004). Since then multiple studies of resistance have been carried out in countries all over the world (Table 1.3).

The issue of fungicide resistance has become of great concern, since there is only a few families of post-infection fungicides approved for apple scab which, as Chapman (2011) points out, would lead to a rise in cases of disease if they were to lose their efficacy, as there would not be any replacements available. This scarcity of products with different modes of action is a consequence of the development costs of fungicides and the extensive research needed to comply with strict legislation (Leadbeater 2014). For the industry, any costs in development have to be balanced by earnings from the fungicide sales (Leadbeater and Gisi 2010; Oliver and Hewitt 2014); for a new product it is estimated that the volume of the sales would need to generate around $1000 million to be able to cover development costs (Oliver and Hewitt 2014). Due to all the effort and great spending in the development of new fungicides, it is very important for the industry that products do not develop resistance quickly (Leadbeater and Gisi 2010), which underlines the importance of anti-resistance strategies.

Table 1.3. Sample of reports of reduced sensitivity across the world [1].

Country	Citation	Chemical	Production system
UK	(Gao, Berrie et al. 2009)	Myclobutanil	Test and commercial orchards
Germany	(Kunz, Deising et al. 1997)	Flusilazole	Test and commercial orchards
Austria	(Koller, Parker et al. 1991)	Fenarimol, triflumizole	Commercial
Serbia	(Stevic, Vuksa et al. 2010)	Flusilazole Difenoconazole	Test and commercial orchards
Chile	(Henriquez, Sarmiento et al. 2011)	Fenarimol, difenoconazole	Commercial
Uruguay	(Mondino, Casanova et al. 2015)	Difenoconazole	Commercial
US	(Chapman 2011)	Myclobutanil	Test and commercial orchards
	(Pfeufer and Ngugi 2012)	Myclobutanil, fenbuconazole, difenoconazole	Commercial
	(Villani, Biggs et al. 2015)	Myclobutanil	Test and commercial orchards (one orchard also resistant to difenoconazole)
Canada	(Jobin and Carisse 2007)	Myclobutanil	Commercial
New Zealand	(Beresford, Wright et al. 2012)	Myclobutanil, penconazole	Not specified

[1] Note that reduced sensitivity does not necessarily translate into field resistance.

1.1.8. Genetics of DMI resistance

Resistance to DMIs follows a gradual pattern in *V. inaequalis*, with a continuum of sensitivity levels instead of two categories (yes or no); this suggests that the resistance is probably polygenically controlled (Jobin and Carisse 2007).

There are a number of routes that could lead to resistance. Some of the mechanisms that are thought to be able to confer resistance involve mutations in the *CYP51A1* gene, overexpression of the gene, and transporters that work in the efflux of the antifungal compounds (Jobin and Carisse 2007; Pfeufer and Ngugi 2012).

The first mechanism would act by altering the conformation of the protein, and thus, interfering with binding of the fungicide; at times substrate binding may also be affected, requiring a compensatory mutation to balance this effect (Joseph-Horne and Holomon 1997). There have been reports in other pathogen species of mutations in the gene contributing to resistance to DMIs (Mullins, Parker et al. 2011; Cools, Hawkins et al. 2013). However, instances of this phenomenon have yet to be observed in scab (Schnabel and Jones 2001; Villani, Hulvey et al. 2016).

Overexpression of *CYP51A1* leading up to resistant isolates has previously been observed in apple scab (Schnabel and Jones 2001; Villani and Cox 2013). There are several molecular mechanisms that could potentially lead to increased expression: from mRNA with a longer life to increased transcription or the presence of other copies of the gene (Schnabel and Jones 2001). Research has confirmed the presence of insertions in the upstream region of the *CYP51A1* gene in *V. inaequalis* which correlate with fungicide resistance and generate overexpression (Schnabel and Jones 2001; Villani, Hulvey et al. 2016).

Some studies on transporters, such as the ABC family, have designated this group of transporters as candidates to confer fungicide resistance (de Waard, Andrade et al. 2006). These transporters could ensure that the fungicide would not reach toxic levels inside the cell, by promoting its exit back into the environment (de Waard, Andrade et al. 2006). Some laboratory studies have produced mutants that have their sensitivity increased by knocking out transporter genes, or decreased by overexpression of these gene (de Waard, Andrade et al. 2006). In *Botrytis cinerea*, the genes *BcatrB* and *BcatrD* have been shown to be linked with resistance in laboratory experiments, as have *MgAtr1* (*Mycosphaerella graminicola*) and *CDR1* and *CDR2* (*Candida albicans*) (de Waard, Andrade et al. 2006). However, research in field isolates of some pathogens has been unable to find evidence of any link between transport and resistance (de Waard, Andrade et al. 2006), while others have shown a correlation (Sang, Hulvey et al. 2015). As de Waard, Andrade et al. (2006) explain, this clearly implies that in the field there must be several mechanisms that confer resistance, with transport possibly just one among several options.

To this day, only a limited amount of data exists about the number of genes involved in fungicide resistance to DMIs. The depth of knowledge about this topic varies greatly with the pathogen species. In those species where several studies exist, the answers vary. While some QTLs have been identified in *Zymoseptoria tritici*, which include another protein in the pathway for ergosterol biosynthesis (Lendenmann, Croll et al. 2015), other species seem to have a major single gene control element where polygenic effects are only observed at times in the most resistant isolates (Dyer, Hansen et al. 2000). Past studies in *V. inaequalis* also indicated single gene control, with balanced 1:1 proportions in the progeny of several crosses exposed to the DMI fungicide fenarimol (Stanis and Jones 1985).

1.1.9. Studies on the evolution of fungicide resistance

It is important to know factors that contribute to the development of resistance in order to design effective strategies to slow down resistance progress.

Kable and Jeffrey (1980) introduced a model in which they followed the number of individuals remaining after a fungicide application. In this model, they considered the fungicide efficacy, making a distinction between the toxicity for the sensitive and resistant strains; and a measure of coverage, which they defined as the fraction of the population that evaded the fungicide. They followed how these two variables affected the numbers of surviving isolates after a number of sprays. The results of their work pointed to major effects for the efficacy of the fungicide, which depended on the magnitude of the difference of the fungicide effects on resistant and sensitive isolates; coverage, which they found to be more important for evolution than the efficacy of the fungicide; the initial size of the resistant subpopulation, when it was below 1% of the total population, it took a long time for the resistant strain to increase to 1%, but afterwards, the rate of evolution greatly increased. For the anti-resistance measures of mixture and alternation, they found that mixtures of fungicides with the same efficacy only delayed the build-up of resistance when coverage was incomplete; alternations, on the other hand, multiplied by two the time it took for the resistance to become dominant. It is important to mention that their model presented some limitations, as they failed to include some factors such as the biology and epidemiology of the pathogen (Kable and Jeffrey 1980).

In the same year, information appeared about a model by Varner (Skylakakis 1982) from du Pont. The new model also referred to number of survivors, but addressed aspects that had been neglected by Kable and Jeffrey (1980), such as the reproduction of the fungus.

Varner's model summarised this biological process through the number of reproductive cycles within a season and the number of spores produced by a parent isolate. This addition paid off when he noticed how reproduction had an effect on the rate of evolution. Pathogens with shorter latency, higher spore production and greater aggressiveness had a faster resistance development.

Skylakakis (1982) did a review of some of the models available at the time, after having submitted his own model in 1981 (Skylakakis 1981). In his review, he concluded that the factors that are more crucial to the development of the resistance include the efficacy of the fungicide, the coverage, the frequency of the resistant subpopulation before spraying and the magnitude of the difference in the sensitivity to the fungicide between the most resistant and the most sensitive isolate.

Chin (1987) came up with a model that considered the relative fitness of strains and coverage. He also followed the frequencies of the resistant and sensitive isolates. He came to some of the same conclusions of the authors before him, including the importance of coverage, initial frequencies of the two strains (resistant and sensitive) and reproductive aspects, but also underlined the importance of relative fitness.

All the early models focused mostly on single-gene resistance, but Shaw (1989) investigated the influence of polygenic resistance. Assuming a normal distribution of sensitivity and a linear correlation between sensitivity and fitness, he studied the effect of fungicide use on both asexual and sexual scenarios. In the asexual scenario, he concluded that the rate of evolution depends on the genetic diversity existent in the population, fitness and balancing effect of stabilising selection. He observed that dose did not really matter unless there is an interaction between dose and fitness. Sexual reproduction does not have much of an effect

either, except when heritability is greatly below 1 in which case the evolution rate was reduced.

Shaw (2000) developed another model that tackled an aspect of resistance evolution that had been overlooked in other models: variability of the dose. Isolates in the field rarely receive the actual dose applied due to many factors. In his model, Shaw observed that when the variability is great, the resistance progression is slower than when there is not much variability. When coverage is incomplete, though, there seems to be an optimum dose where the evolution rate is at its maximum, and when varying this dose the evolution rate decreases progressively. He also speculated that sexual reproduction would have a negative effect on the evolution rate, as it would separate positive effect mutations and generate isolates with no mutations. However, other authors (McDonald, Rice et al. 2016) have argued in favour of sexual reproduction increasing the rate of evolution, as it also makes it easier to discard negative effect mutations associated with a resistant-phenotype mutation. Furthermore, they point out that some of these negative-effect mutations would cease to be so if there are further changes in the genetic background after the exchange of genetic material during sexual reproduction.

van den Bosch, Paveley et al. (2011) analysed how different processes could affect the evolution of resistance. With a large dose in a qualitative resistance scenario, they noted that resistance would either evolve quicker or remain unchanged. They pointed out that fungicides may affect evolution in other ways than just selection, as they discussed the possibility of fungicides acting as a stress source that could influence mutation rate. They also debated the role of the susceptible tissue, and argued that there could be two steps in the evolution: one where there is an abundance of susceptible tissue at the start and

another later in the process where it is scarce. Another factor they considered important is the size of the whole population as, in a small population, although there would be less mutations happening, these mutants would have a higher chance to grow into a stable subpopulation.

Hobbelen, Paveley et al. (2011) developed a model and compared model predictions with field data. Hobbelen, Paveley et al. (2014) studied the much ignored emergence stage using similar model parameters to those in their 2011 model and differentiating the resistant from the sensitive isolates, taking into account the possibility of a fitness cost for the resistant isolates. They found that resistant mutations would not be established in the field before a fungicide started to be used for disease control. Once a fungicide was in use, positive factors that promoted emergence are high mutation rates and area of the crop. Negative factors for resistance emergence are high efficacy of the fungicide and fitness costs, measured as a penalty in infection rate in their study. Using mixtures of fungicides is able to stall the emergence of resistance, while changing doses above a certain threshold (50% of the label recommended dose) did not have a significant effect, although below this threshold increased dose sped up emergence.

From the above points, recurring elements important for selection in the results from different models are: an influence of coverage, the level of fungal toxicity of the chemical used, the initial frequencies of the sensitive and resistant subpopulation, reproduction factors such as number of spores, infection efficiency and latency, and the magnitude of the sensitivity range and the dose.

There are some points where researchers do not agree with each other. Even though both Hobbelen, Paveley et al. (2014) in the emergence study and Shaw (1989) in his polygenic

study reported small effects of dose on resistance development except when interacting with some other factor, Shaw (2000) did report a slowdown of the resistance when there is greater variability in the dose. This could be due to the other models using the mean dose in the tank mix rather than the actual dose received by an isolate. The effect of sexual reproduction is still unclear. Although Shaw (2000) reported possible negative effects due to sexual recombination breaking down the resistance in polygenic cases, his early polygenic model (Shaw 1989) actually only produced a slowdown in the resistance when heritability was incomplete; McDonald, Rice et al. (2016) argued for a positive effect of sexual reproduction on evolution as it would free resistant genes from negative mutations associated to them.

1.1.10. Cross-resistance

Apart from fungicide resistance, another concern for the farmer and industry is the existence of cross-resistance between chemicals, which refers to a loss of sensitivity to one fungicide resulting in a reduction of sensitivity to another fungicide (FRAC 2017). Although cross-resistance seems more common among fungicides that share the same target (FRAC 2017), cases of apparent cross-resistant isolates between different families of compounds have also been observed. In a study of 195 isolates of *V. inaequalis*, 12% were resistant to all of myclobutanil, kresoxim-methyl, dodine and thiophanate (Chapman 2011). In other reports, there seems to be a relationship between the resistance to dodine and fenarimol (Koller and Wilcox 1999; Koller and Wilcox 2000), although other studies found no correlation between the two (Stanis and Jones 1985). When isolates become resistant to two chemicals with different modes of action there are two possible explanations: either two independent mechanisms have evolved at the same time or a mechanism that is capable of dealing with

different kinds of compounds is responsible. In the second case, such a mechanism can be a multidrug resistance transporter working in the efflux of different chemicals (FRAC 2017).

The above explanations can also be applied to chemicals within the same family which, despite no apparent cross-resistance with another member of the family, may still develop resistance in orchards treated with another chemical without cross-resistance. Difenoconazole-myclobutanil could be such an example; although some studies have failed to detect cross-resistance to difenoconazole in myclobutanil resistant isolates, there was one case with some cross-resistance in a baseline orchard (Villani 2015).

As mentioned before, cross-resistance is mostly seen between fungicides with the same target site. Within the DMI family, cross-resistance has been reported between several fungicide pairs in apple scab, including myclobutanil-flusilazole, myclobutanil-fenarimol and myclobutanil-fenbuconazole (Koller, Wilcox et al. 1997; Jobin and Carisse 2007; Pfeufer and Ngugi 2012). Published information indicate that cross-resistance can be found not only between compounds with the same target belonging to the same chemical group, but also between those of different chemical groups such as the myclobutanil-fenarimol cross-resistance pair in *V. inaequalis*: myclobutanil is a triazole, while fenarimol is a pyrimidine (FRAC 2017). However, having the same target is not always indicative of cross-resistance. In other organisms, it has been observed that, within the same mode of action, some mutations produce cross-resistance between some fungicide pairs, but not to others (Cools, Hawkins et al. 2013).

The consequences of discovering cross-resistance between two chemicals in a pathosystem like that of *V. inaequalis* will probably not result in a chemical being discontinued, it is far more likely that growers will just be made aware of the dangers and discouraged from using

the two compounds too often within the same management program. In fact, general guidelines already take into account this danger of cross-resistance between fungicides with the same mode of action, and often recommend mixing or using fungicides sequentially with other families with no cross-resistance (FRAC 2017).

1.2. Objectives

Some of the fungicides more frequently used in the UK to control apple scab, such as myclobutanil, have experienced a loss efficacy since their introduction. Due to the time it takes to get new products ready for commercial use, which involves a series of tests to guarantee their safety in order to comply with current legislation, it is important to find substitutes to fungicides already in use, leaving enough time for them to get an approval. However, resistance has not spread as expected for myclobutanil, even though resistance to the chemical has been known for a long time. It looks like there could potentially be some mechanism that is extending the time that myclobutanil and similar products can still be used in the field.

Knowing the type of gene control involved in conferring resistance and identifying molecular mechanism behind the resistance would provide us not only with another tool to monitor resistance development, by searching relevant mutations, but also a better understanding of resistance evolution that would enable us to try to predict the behaviour of the apple scab pathosystem under fungicide treatment.

In order to answer these important issues, the objectives of this thesis are as follows: to determine the level of resistance to myclobutanil and tebuconazole, a DMI which has been recently introduced for use against apple scab, in a range of isolates coming from both

orchards with reported control problems and an untreated orchard, this section includes a description of the characteristics of the baseline populations and a look at cross-resistance between the two chemicals; to evaluate how important mutations in the *CYP51A1* gene sequence are to the overall resistance; to predict the number of genes involved in the resistance through classic genetic methods by phenotyping the progeny of a sensitive-resistant cross; and to analyse how the genetic background and other factors alter the evolution of resistance using an agent-based model.

2. General materials and methods

2.1. Isolating spores from lesions

2.1.1. Conidia

Conidia were isolated from leaf discs. Leaf discs were prepared from leaf samples by cutting circles around lesions with a cork borer, and then left to dry overnight. Individual leaf discs were placed in separate microcentrifuge tubes and later stored at -20 °C.

To isolate conidia, leaf discs were placed in a microcentrifuge tube with 1 ml deionised water, and conidia were released into the water by crushing with an eppendorf pestle followed by a few seconds of vortexing. A short spin in a centrifuge was used to make the leaf material settle in the bottom of the tube, and a volume of the supernatant was taken to evaluate spore concentration. Conidia were counted on a haemocytometer, and concentrations were adjusted to $8x10^4$ conidia/ml. Two dilutions were prepared out of the conidia suspension, one by a factor of ten and another by a factor of 20; and the undiluted suspension and the two dilutions were plated with a glass spreader on PWA (Plain Water Agar) prepared with Agar technical No. 3 (Oxoid; 1.2% w/v) amended with rifamycin (Sigma-Aldritch; 0.2 ppm). Plates were incubated at 20 °C overnight.

After incubation, individual germinating spores were picked with a needle under a dissecting microscope from areas of the plate where spores were abundant but well-spaced, and transferred to PDA plates (Oxoid; 39 g/L) amended with rifamycin (0.2 ppm).

2.1.2. Ascospores

For a given cross between two strains, segments were cut with a scalpel blade from the line where the two colonies meet, placed on a slide and crushed with the same scalpel blade. A few drops of sterile deionised water were deposited over the mycelia with a Pasteur pipette and a coverslip was set over the segment. A bladeless scalpel was used to further crush the mycelia. After determining that the ascospores had reached the appropriate stage of maturity under a compound microscope, spores were washed off with sterile water into bijous, which then were vigorously shaken and the suspension left to settle. A volume of 400 µl of the ascospore suspension was pipetted onto plates of PWA prepared with Agar Technical No. 3 (1.2% w/v) and spread with a glass spreader. The following day, plates were checked under a dissecting microscope for germinating spores, which were then cut with a sterilised needle and moved to a PDA plate (39 g/L) amended with rifamycin (0.2 ppm). Five spores were placed on each individual plate.

2.2. Subculturing isolates

Plugs of 4 mm were taken with a cork borer out of a colony and transferred with a needle to a plate with fresh media. For some experiments, more than one plug was moved onto the same plate to increase the quantity of fungal material. IMS 70% or ethanol 100% was used to sterilise all tools. Cultures were usually kept at 20 °C; however, plates produced during the fungicide tests were left to grow at room temperature, approximately 20 °C, due to the amount of storage space needed.

2.3. Fungicide tests

2.3.1. Preparing fungicide amended PDA media

PDA was prepared and autoclaved. Stock dilutions of 1 g/L of myclobutanil and tebuconazole were produced in a fumehood from commercial fungicides Systhane 20EW (200 g/L myclobutanil) from Dow AgroSciences and Folicur 25 EW (250 g/L tebuconazole) from Bayer Crop Science, using deionised water. Dilutions were taken to a flow cabinet where they were filtered with a 50 ml syringe (0.2 µm filter). For each chemical, subsequent dilutions at 300 mg/L, 90 mg/L, 18 mg/L, 6 mg/L, 2 mg/L and 0.8 mg/L were made. These dilutions were used to amend duran bottles containing pre-sterilised PDA medium (39 g/L). All PDA was prepared to the same final volume; since some evaporation was likely to occur during autoclaving and amendment volumes of chemical used would not exceed 2.2 ml, it was not considered practical to fill each bottle with a different volume as variations in final PDA concentrations would not be too great. Some bottles were left unamended to serve as a control treatment without fungicide. Four concentrations were prepared per chemical at 4 mg/L, 1 mg/L, 0.1 mg/L and 0.01 mg/L.

2.3.2. Fungicide tests: statistical analysis

Two perpendicular diameters were measured from each colony, using a ruler, three weeks after subculturing. The concentration at which growth is reduced to 50% (ED_{50}) was calculated through a logistic model, using the mean diameter of all colonies within a plate (one run of the experiment) as response variate; and concentrations, transformed by taking natural logarithm, as the explanatory variate. In order to allow the inclusion of the control (no fungicide concentration) in the analyses, which if left as 0 mg/L would result in an error

when taking logarithms, the control was replaced before transformation by a figure two orders of magnitude (0.0001) below that of the lowest fungicide concentration used in the test (0.01 mg/L). With only five observations per isolate, a very parsimonious function needs to be used to provide estimates of ED_{50}. Therefore, a two-parameter logistic distribution (Figure 2.1) was used where the slope parameter was fixed and the asymptote at high fungicide concentration is assumed to be 0 as growth is expected to be completely inhibited at some point. Different values of the slope parameter were tested (Figures 2.2, 2.3) and ED_{50} values obtained with each slope parameter were compared via a scatter plot matrix. After reviewing the results (Figures 2.4, 2.5, 2.6), a single suitable value of -1 for the slope parameter was chosen for all isolates, so that ED_{50} was not sensitive to variations in the slope. All statistical analyses undertaken were carried out using Genstat 16 (Payne, Murray et al. 2013).

$$y = A + \frac{C}{1 + e^{((-B)*(x-M))}}$$

Figure 2.1. Logistic equation used to calculate $logED_{50}$. A is the asymptote at high fungicide concentration, B is the slope of the curve, C is the value of the asymptote for no fungicide concentration, and M is the inflection point of the curve which equates $logED_{50}$. The form presented in this figure is a four-parameter logistic equation; it was transformed into a two-parameter logistic by assigning a value of 0 to A and fixing the slope (B) to a single value.

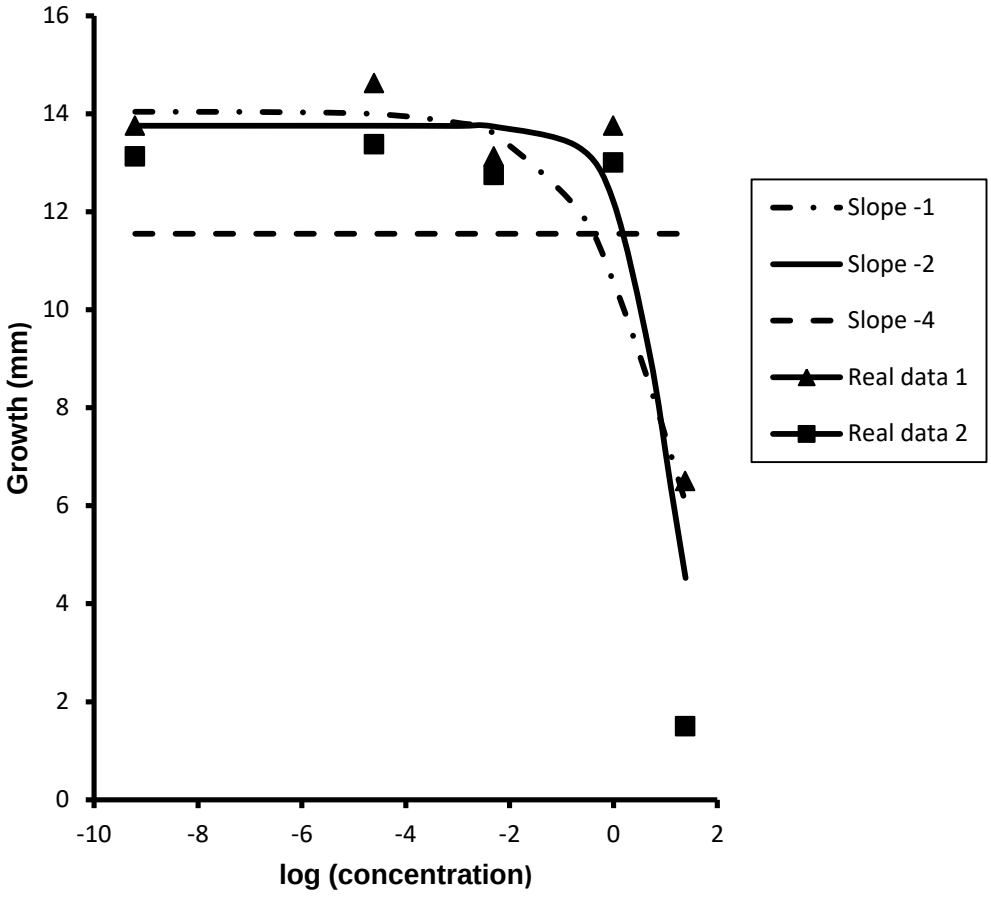


Figure 2.2. Example of the dataset for one isolate with curves fitted with different slope parameters. The real data points for the isolate are also present in the graph, with each concentration having two independent replicate data points (Real data 1 and Real data 2).

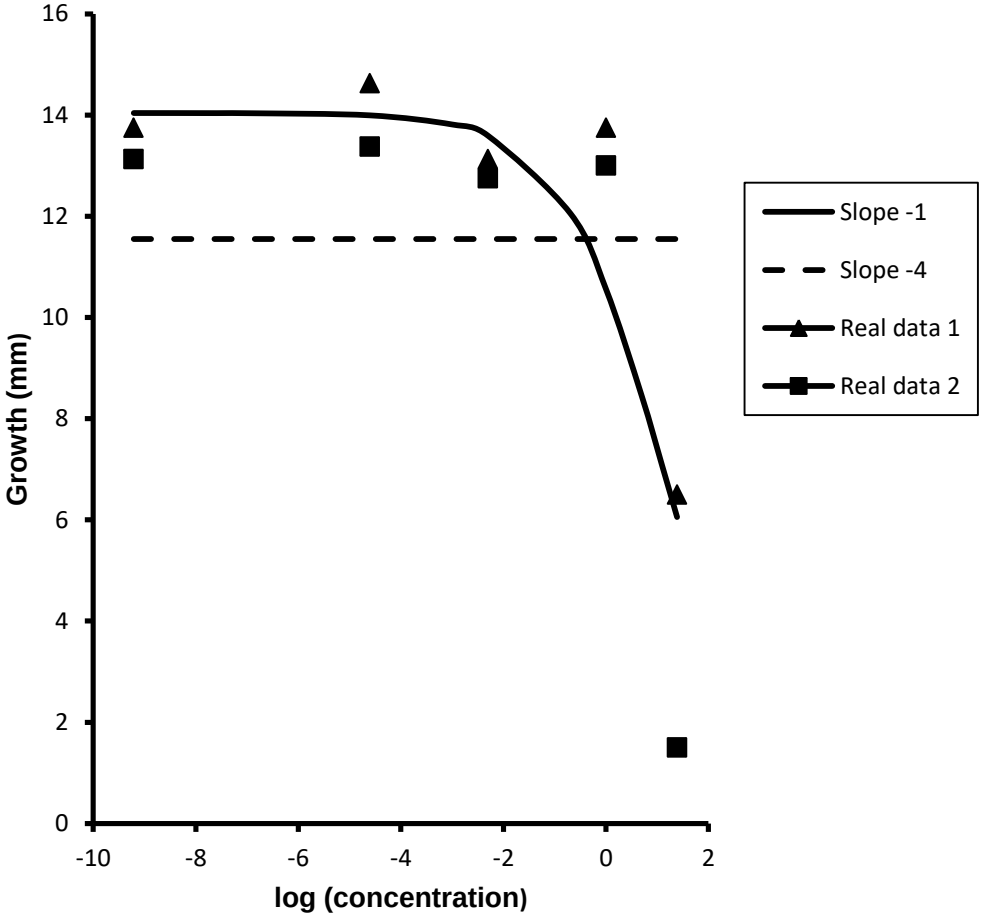


Figure 2.3. Example of isolate with different curve fits depending on the slope parameter selected. The real data points for the isolate are also present in the graph, with each concentration having two independent replicate data points (Real data 1 and Real data 2). For this specific isolate, it seems that a slope parameter of -2 fits the real data closer.

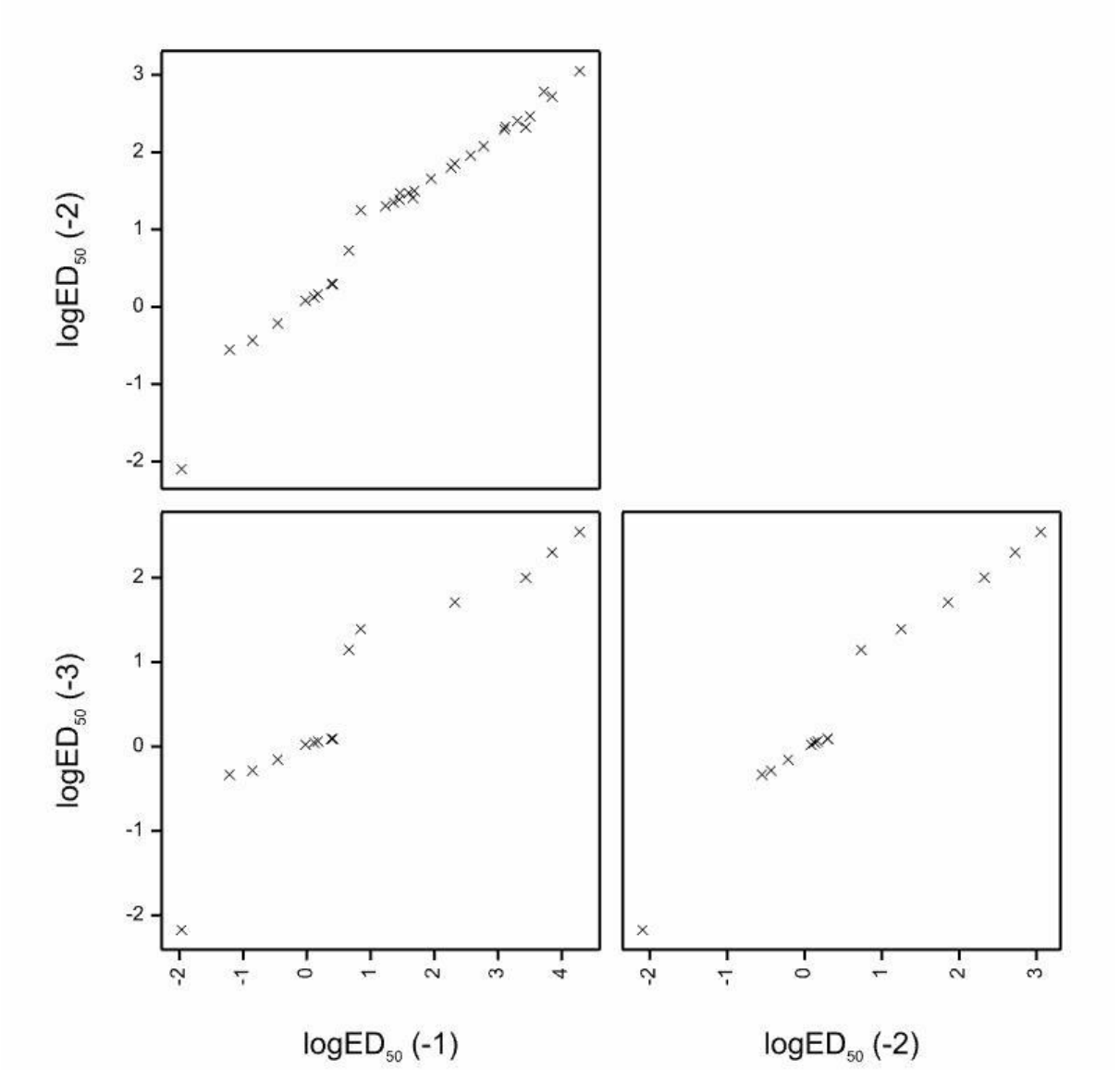


Figure 2.4. Scatter plot matrix of myclobutanil $logED_{50}$ values (Chapter 4) calculated with fixed logistic slope parameters of -1, -2 and -3 (slope parameters are indicated in parentheses in the figure). The values are rather insensitive to slope parameters between -1 and -2 ($r=0.9810$, $P < 0.001$).

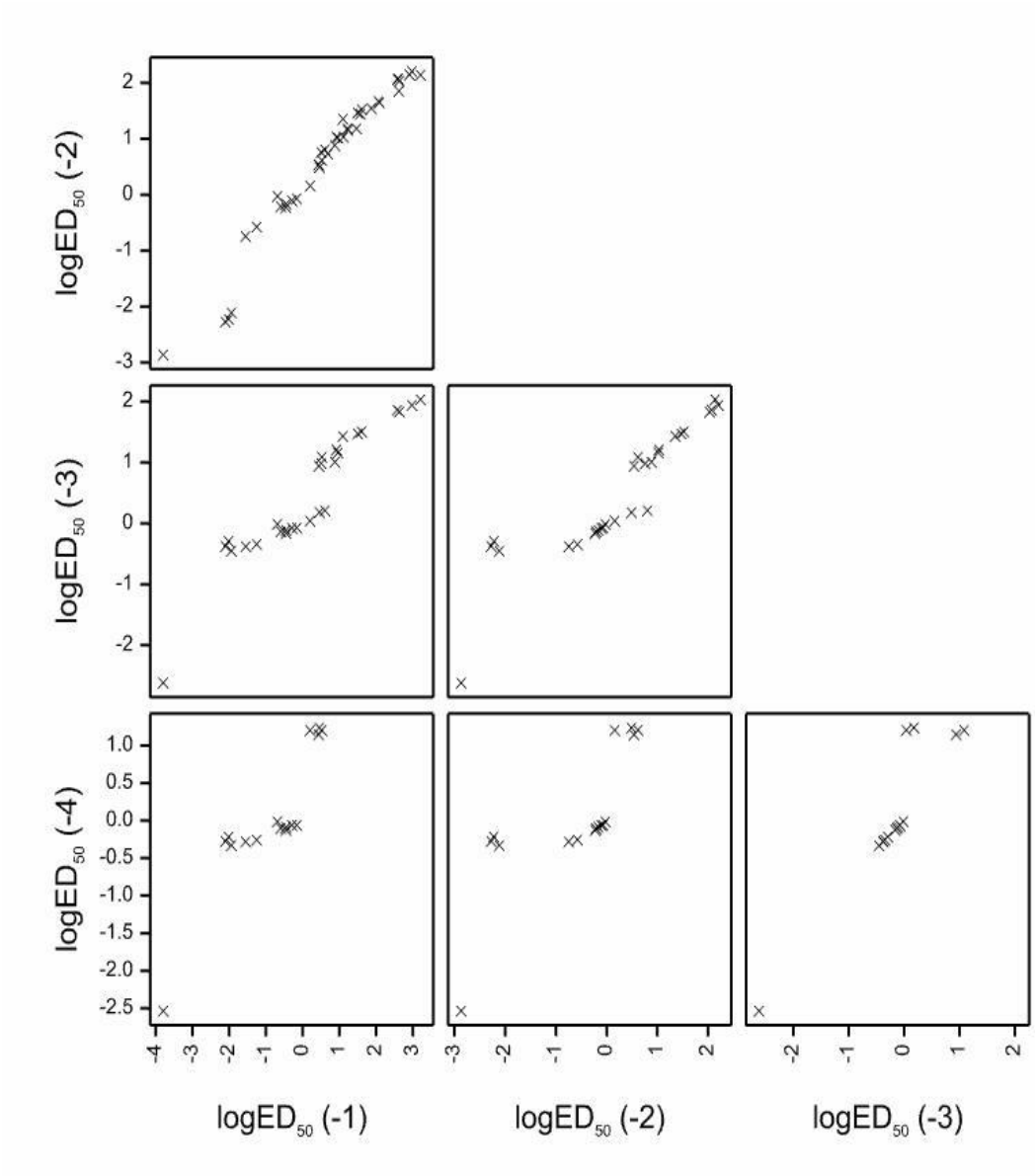


Figure 2.5. Scatter plot matrix of tebuconazole $logED_{50}$ values (Chapter 4) calculated with fixed logistic slope parameters of -1, -2, -3 and -4 (slope parameters are indicated in parentheses in the figure).

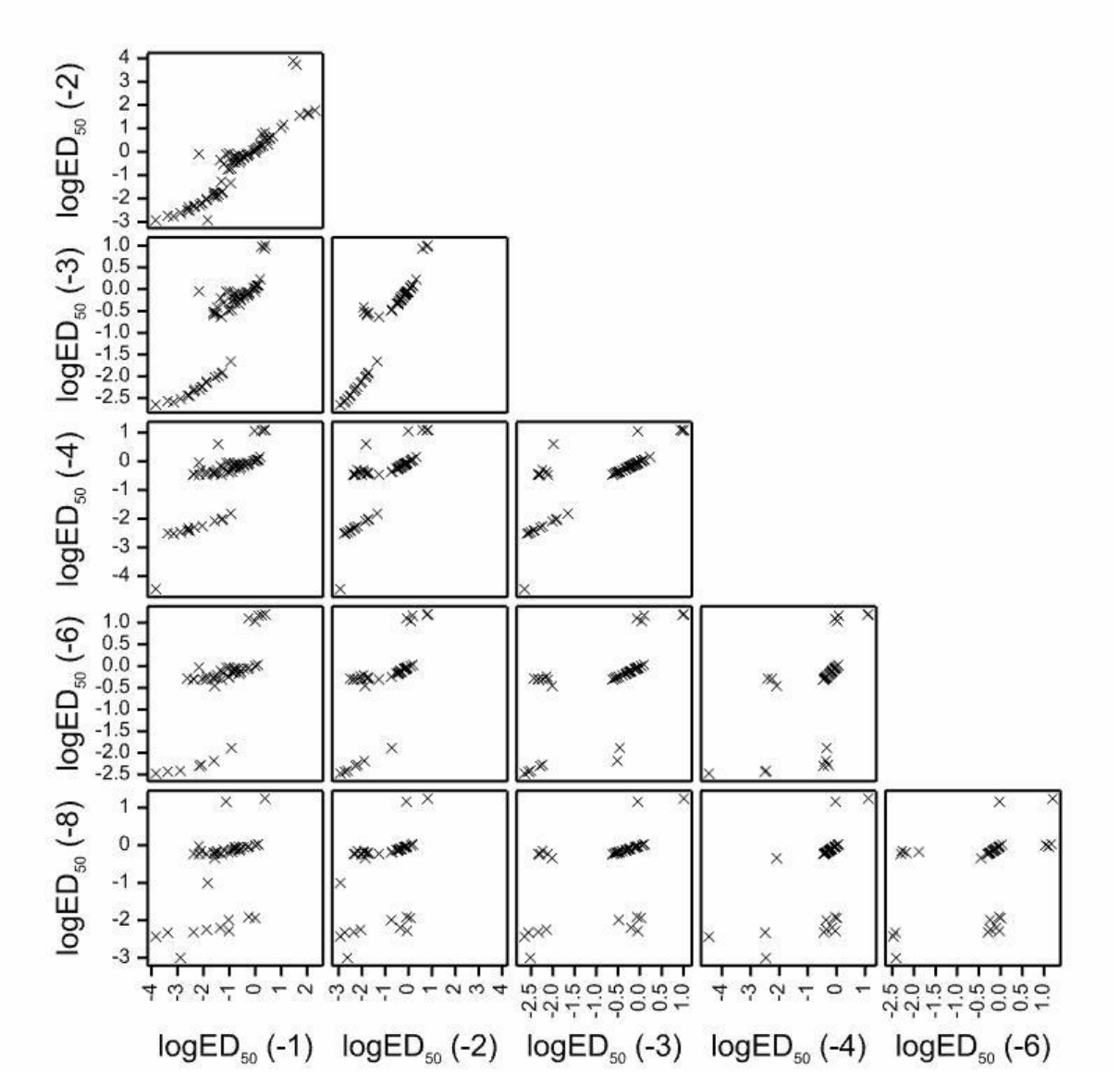


Figure 2.6. Scatter plot matrix of tebuconazole $logED_{50}$ values of the progeny of a resistant-sensitive cross (Chapter 6) calculated with fixed logistic slope parameters of -1, -2 , -3, -4, -6 and -8 (slope parameters are indicated in parentheses in the figure).

3. Development of a new method for testing fungicide resistance

3.1. Introduction

Tests based on doses of fungicide are one of the methods used to assess fungicide resistance (Xu, Gao et al. 2010). In apple scab, this has usually involved either measuring the colony diameters of single-spore isolates grown on fungicide-amended media or the length of the germ tube of spores from individual lesions that have been washed onto media containing a specific dose of a fungicide under consideration (Gao, Berrie et al. 2009). However, these approaches have the disadvantage of being time-consuming, or the possibility of mixing isolates with different genotypes, which can occur in the germ tube assay (Gao, Berrie et al. 2009).

Other assessment methods exist, some of which rely on analysing parameters describing different aspects of pathogen life cycles under fungicide treatments. These strategies go from monitoring effects on conidia production and differentiation to studying the amount of area infected (Kunz, Deising et al. 1997). A study employing a range of this type of tests found good correspondence between these tests; however, compared to laboratory assays, the sensitivity values estimated with one of the *in vivo* techniques were between 24 to 64 times higher (Kunz, Deising et al. 1997).

Looking for a laboratory method that was less time-consuming than the traditional *in vitro* techniques, some researchers have opted for a different strategy : growing the isolates in liquid media in a microplate format (Raposo, Colgan et al. 1995; Silveira Gomes, Bibiano et al. 2014; Frac, Gryta et al. 2016). The microplate allows for the testing of many isolates simultaneously, reducing the amount of storage space required and speeding up the

assessment process. This method employs absorbance of certain wavelengths as an indicator of growth.

The microplate technique has been used successfully with a variety of fungicides and pathogens. Raposo, Colgan et al. (1995) used it to test the sensitivity of *B. cinerea* conidia to iprodione and a mixture of carbendazim and diethofencarb. The method allowed obtaining results approximately one day earlier than with the *in vitro* measurements of colony diameter. Both Pelaez Montoya, Vasquez David et al. (2006) and Silveira Gomes, Bibiano et al. (2014) tried the method on *M. fijiensis* using mycelial fragments. They tested a variety of fungicides from different families, including DMIs propiconazole and tebuconazole, and were able to cut the time necessary to get results by around 7-8 days, with colony diameter measurements carried out at around 15 days. FRAC (2017) has recognised a number of fungicide sensitivity tests that employ microplates for pathogens such as *B. cinerea* (using spore or mycelial suspensions), *F. graminearum* (using a spore suspension to test DMIs tebuconazole and prothioconazole), *Microdochium nivale* (using a spore suspension to test DMIs tebuconazole and prothioconazole), *Phytophthora infestans* (using sporangia), *Oculimacula* spp. (using spore suspension or mycelial fragments, the latter with prothioconazole) and *Septoria tritici* (spore suspension, and testing some DMIs such as prochloraz, epoxiconazole, metconazole, tebuconazole and prothioconazole).

Due to the advantages mentioned before over the traditional methods, it was attempted to develop a microplate fungicide test for use in *V. inaequalis*. When designing a new protocol, various factors have to be considered to ensure accurate results. For spectrophotometry these factors include wavelength, medium or type of inoculum.

For the wavelength, it is important to consider things such as metabolite secretion and pigmentation (Granade, Hehmann et al. 1985). Pigments may vary depending on the media used having an impact on absorption of the culture (Myers, Curtis et al. 2013). One thing that can be done to reduce possible changes in absorption due to shifts in pigment profile is to choose wavelengths at which the pigment does not have a high absorption (Myers, Curtis et al. 2013).

The effect of the medium on absorption can often be reduced by choosing an appropriate blank. Blanks can range from water to growth medium to the culture liquid environment (Myers, Curtis et al. 2013).

For the inoculum, the choice is usually between using a conidial or mycelial suspension (FRAC 2017). The latter usually involves a step in which mycelia are fragmented to avoid clump formations that could interfere with absorbance readings (Granade, Hehmann et al. 1985). Sometimes the deciding factor could be something as simple as the ability of the fungus to produce conidiospores, for species with poor production the mycelial option is usually better (Silveira Gomes, Bibiano et al. 2014).

Despite their advantages, microplate-based methods have their own problems. One is the possibility of wells getting contaminated, which at times can be difficult to detect (Raposo, Colgan et al. 1995). It seems that this effect may not be as worrying for species that have a fast growth rate (Raposo, Colgan et al. 1995). There are means to prevent this problem from occurring: something as simple as sealing the plate or using a microplate lid can help keep sterility (Jones, Michael et al. 2012).

Another issue occurs with fungicides that turn into vapour easily. With such chemicals, special care should be taken, as concentrations in wells may change (Raposo, Colgan et al. 1995).

In experiments over a long period of time, loss of volume due to evaporation is another problem, particularly along the wells closest to the perimeter of the plate (Lundholt, Scudder et al. 2003). One way such problem can be handled is to just avoid the outer wells and only use the rest (Lundholt, Scudder et al. 2003).

3.2. Objectives

To adapt the microplate technique for use in apple scab. This entailed the following:

- To check the ability of scab to grow in liquid media

- To optimise parameters such as type of liquid media, wavelength and initial inoculum

- To evaluate the ability of isolates to grow in fungicide-amended liquid media and determine how reliable the method is to measure growth over time.

3.3. Materials and methods

3.3.1. Testing *Venturia inaequalis* ability to grow in liquid media

Venturia inaequalis isolates were grown in flasks and later microplate wells. Two kinds of liquid media were tested (PDB and Sabouraud), wavelengths tried ranged from 340 to 620 nm (340 nm, 405 nm, 450 nm, 492 nm and 620 nm) and, in the microplate tests, mycelia were added in a fragmented form both as an undiluted suspension and in serial dilutions.

3.3.1.1. Flask assay

In the flask experiment, each type of media (PDB or Sabouraud) was tested in four flasks, each containing 20 ml volume. Fungal material from one scab isolate was scraped with a sterilised loop and introduced into the flasks. Flasks were maintained under constant shaking at 104 rpm on a Stuart orbital shaker SSL (Camlab). After two weeks, the same volume (200 µl) of each flask was pipetted into the wells of a microplate and two rows containing only medium (one with PDB and the other with Sabouraud) were added as blanks. Measurements of absorbance at the wavelengths specified above were taken with a Multiskan Ascent plate reader (Thermo Labsystems). Two readings were taken per wavelength to evaluate equipment errors, and plates were shaken at 60 rpm for 10 seconds inside the plate reader before measuring.

3.3.1.2. Mycelial fragmentation test

Confusing and inconsistent absorbance results, including negative absorbance values, in the flask assay led me to try a different approach. Due to published research pointing to a tendency of hyphae to form clumps (Meletiadis, Mouton et al. 2001), a decision was taken to develop a suspension of mycelial fragments as the starting inoculum (Granade, Hehmann et al. 1985; Pelaez Montoya, Vasquez David et al. 2006; Silveira Gomes, Bibiano et al. 2014) to guarantee a uniform suspension and prevent clump formation.

Some mycelia was placed in an eppendorf with 1 ml sterile water and processed in the following ways: using a tissue lyser (TissueLyser II, Qiagen) at different speeds (13 s^{-1}, 10 s^{-1}, 6 s^{-1}, 3 s^{-1}) during 5 seconds or a Genius vortex at settings 4 and 6 (maximum speed) for the

same amount of time. Resulting mycelial suspension was studied under a microscope using cotton blue to evaluate its degree of fragmentation.

3.3.1.3. Microplate assay

Scraped mycelia from cultures were distributed into 1.5 ml eppendorfs containing some sterile water, and fragmented by vortexing at maximum speed for 5 seconds. After a quick spin in a centrifuge, letting the centrifuge reach maximum speed (10000 rpm) and then stopping it, to drag all non-fragmented mycelia to the bottom of the eppendorfs, the supernatant of all tubes was collected into a single test tube. Serial dilutions of three further steps, each with a 1:5 dilution ratio, were prepared out of this initial suspension and added to the wells of a microplate where the media (PDB and Sabouraud) had been previously dispensed. The initial undiluted suspension was also added. Three plates were produced in this manner. Of these: one plate was measured immediately after preparation, the other two were left in a Stuart orbital shaker SSL (Camlab) at 120 rpm, and then analysed in a Multiskan Ascent plate reader (Thermo Labsystems), with one of them being measured after a week and the last one after two weeks. As with the flasks assay, two readings were taken at each wavelength, and plates were shaken inside the plate reader before measuring.

3.3.2. Testing for fungicide resistance in microplates

After choosing the medium, wavelength and dilution that allowed for a better absorption, experiments were repeated in fungicide amended media both by taking measurements at a set time – two weeks after the preparation of a plate - or by measuring continuously over a period of time. The set time experiment included a blank of media (PDB), control with no fungicide and five concentrations (0.016 mg/L, 0.08 mg/L, 0.4 mg/L, 2 mg/L and 10 mg/L).

The continuous measurements experiment (Figure 3.1) had a blank for each concentration of fungicide, a control and four concentrations of fungicide adjusted to better encompass the sensitivity range observed in the results obtained from the set time experiment. These new concentrations went from 4 mg/L to 0.01 mg/L (4 mg/L, 1 mg/L, 0.1 mg/L and 0.01 mg/L). Two isolates were tested in both types of experiments. Initial concentration of fungal material in the original suspension was not checked in the set time experiment. This was rectified during the continuous test where the concentration of fragments was controlled via use of a haemocytometer to ensure it was the same for both isolates, estimating the number of fragments and adjusting the concentration to around $8x10^4$ fragments/ml.

Absorbance readings were taken with a Multiskan Ascent plate reader (Thermo Labsystems) in the set time experiment, at the same range of wavelengths used for the liquid media tests, and optical density readings were taken with a Fluostar Galaxy (BMG labtechnologies) plate reader for the continuous experiment. For the latter, a wavelength of 595 nm was used, as that was the only filter available for the equipment.

	A	B	C	D	E	F	G	H
1	PDB	PDB	PDB	PDB	PDB	PDB	PDB	PDB
2	PDB	PDB	PDB	PDB	PDB	PDB	PDB	PDB
3	PDB	Control 1 R1	Myc 0.01 R1	Myc 0.1 R1	Myc 1 R1	Myc 4 R1	Control 2 R1	PDB
4	PDB	Control 1 R1	Teb 0.01 R1	Teb 0.1 R1	Teb 1 R1	Teb 4 R1	Control 2 R1	PDB
5	PDB	Control 1 R2	Myc 0.01 R2	Myc 0.1 R2	Myc 1 R2	Myc 4 R2	Control 2 R2	PDB
6	PDB	Control 1 (Blank)	Teb 4 Blank	Teb 1 Blank	Teb 0.1 Blank	Teb 0.01 Blank	Control 2 (Blank)	PDB
7	PDB	Control 1 R3	Myc 4 R3	Myc 1 R3	Myc 0.1 R3	Myc 0.01 R3	Control 2 R3	PDB
8	PDB	Control 1 R2	Teb 4 R2	Teb 1 R2	Teb 0.1 R2	Teb 0.01 R2	Control 2 R2	PDB
9	PDB	Control 1 (Blank)	Myc 4 Blank	Myc 1 Blank	Myc 0.1 Blank	Myc 0.01 Blank	Control 2 (Blank)	PDB
10	PDB	Control 1 R3	Teb 4 R3	Teb 1 R3	Teb 0.1 R3	Teb 0.01 R3	Control 2 R3	PDB
11	PDB	PDB	PDB	PDB	PDB	PDB	PDB	PDB
12	PDB	PDB	PDB	PDB	PDB	PDB	PDB	PDB

Figure 3.1. Layout of the microplate for the continuous measurements fungicide test. Each cell represents a well. The outer wells contained only medium to counter the edge effect. The rest of the cells contained either medium (control) or medium amended with

myclobutanil (Myc) or tebuconazole (Teb). For those wells containing fungicide, the concentration present in that well is indicated by the number after the fungicide identifier. Replicates are indicated with an R and the number of the replicate; there were three replicates per fungicide, each containing four fungicide concentrations and two control wells without fungicide (Control 1 and Control 2). All wells contained *V. inaequalis* mycelia save for those marked as Blank and the outer wells.

3.3.3. Data handling

Absorbance and optical density values were corrected by subtracting the appropriate blank in all experiments. Genstat 16 (Payne, Murray et al. 2013) was used to analyse all data. Analysis of variance was performed on the liquid growth data to study the effects of the different factors (wavelength, dilution, medium), and the resulting tables of means are reported in the form of scatter plots. Fifth order effects and fourth order effects were excluded from the analysis of variance to increase the numbers of degrees of freedom for error. The dilution and medium that gave the highest changes in absorption over time were chosen for use in the fungicide tests. Plots illustrating the evolution of the corrected optical density over time (continuous experiment) were also obtained.

3.4. Results

3.4.1. Testing *Venturia inaequalis* ability to grow in liquid media

The flask tests yielded inconsistent results. This could be due to mycelia forming clumps of hyphae concentrated in a small region of the flask, small black circles were visible floating in the flasks which could have been *Venturia inaequalis*. However, this was not verified at the

time. Due to the reduced space occupied by this black matter in comparison to the whole volume inside the flask, it is possible that samples taken for measuring contained mostly mycelium-free medium, resulting in uninformative results.

Results were more encouraging in the microplate tests where mycelia were more clearly visible (Figure 3.2).

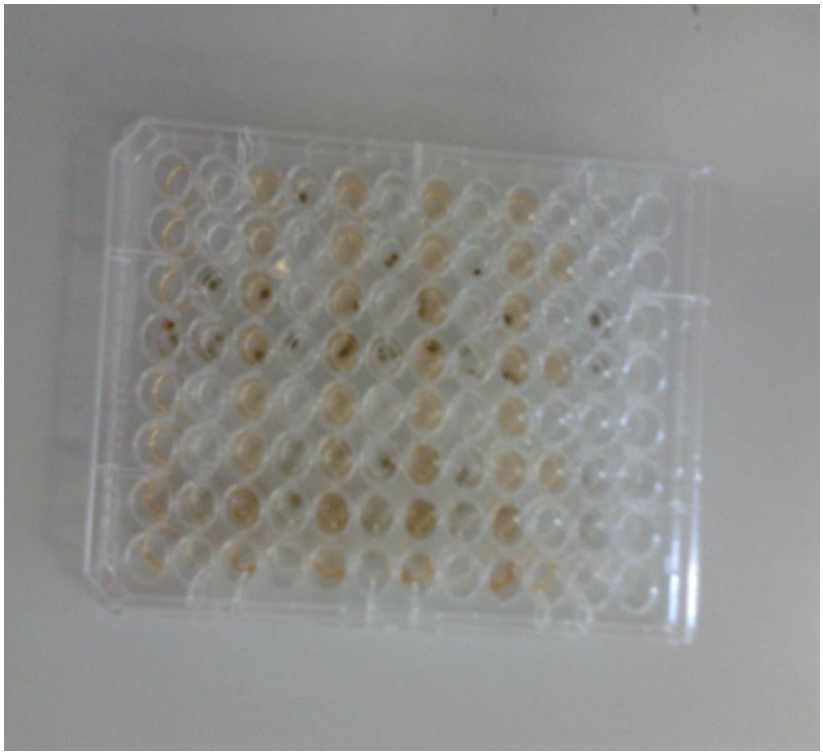


Figure 3.2. Microplate with wells filled with growth medium (PDB or Sabouraud) and inoculated with *V. inaequalis* mycelial fragments.

The parameters that seemed to promote fungal growth in the microplate were longer wavelengths (Figure 3.3) and to use the first dilution in the series tested (Figure 3.4) – the mycelial suspension diluted at 1:5. The medium that provided the best results was PDB (Figure 3.5), with a percentage increase of 2781% against 67% for Sabouraud.

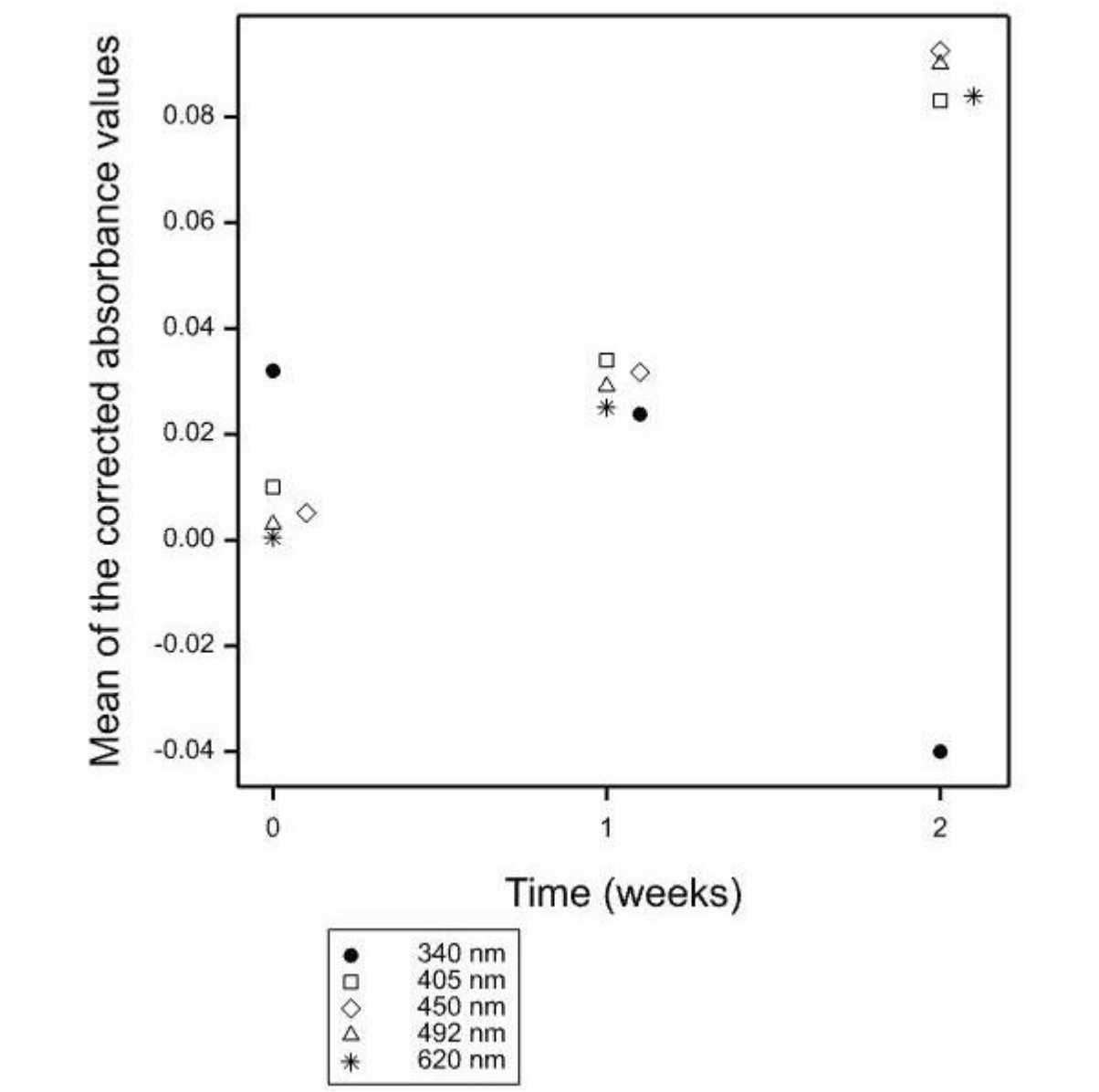


Figure 3.3. Mean of the corrected absorbance values of microplate wells of growth medium inoculated with *Venturia inaequalis* mycelial fragments in relation to wavelength of the illumination source and time since inoculation (SEM= 0.025). Superimposed values have been separated along the x axis.

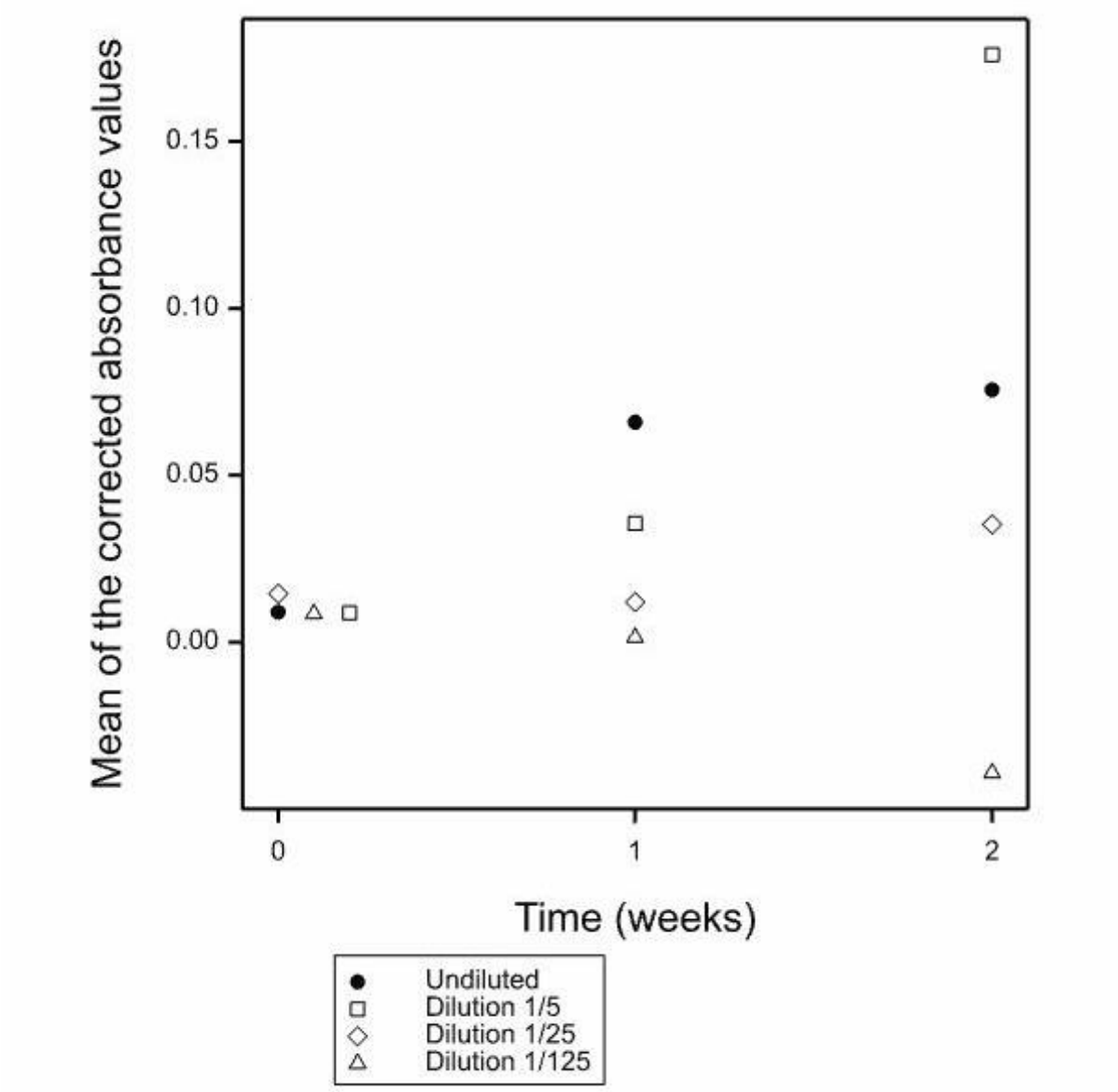


Figure 3.4. Mean of the corrected absorbance values of microplate wells of growth medium inoculated with *Venturia inaequalis* mycelial fragments in relation to dilution factor, (1/5, 1/25 and 1/125) of the inoculum (expressed relative to the undiluted suspension) and time since inoculation (SEM=0.025). Superimposed values have been separated along the x axis.

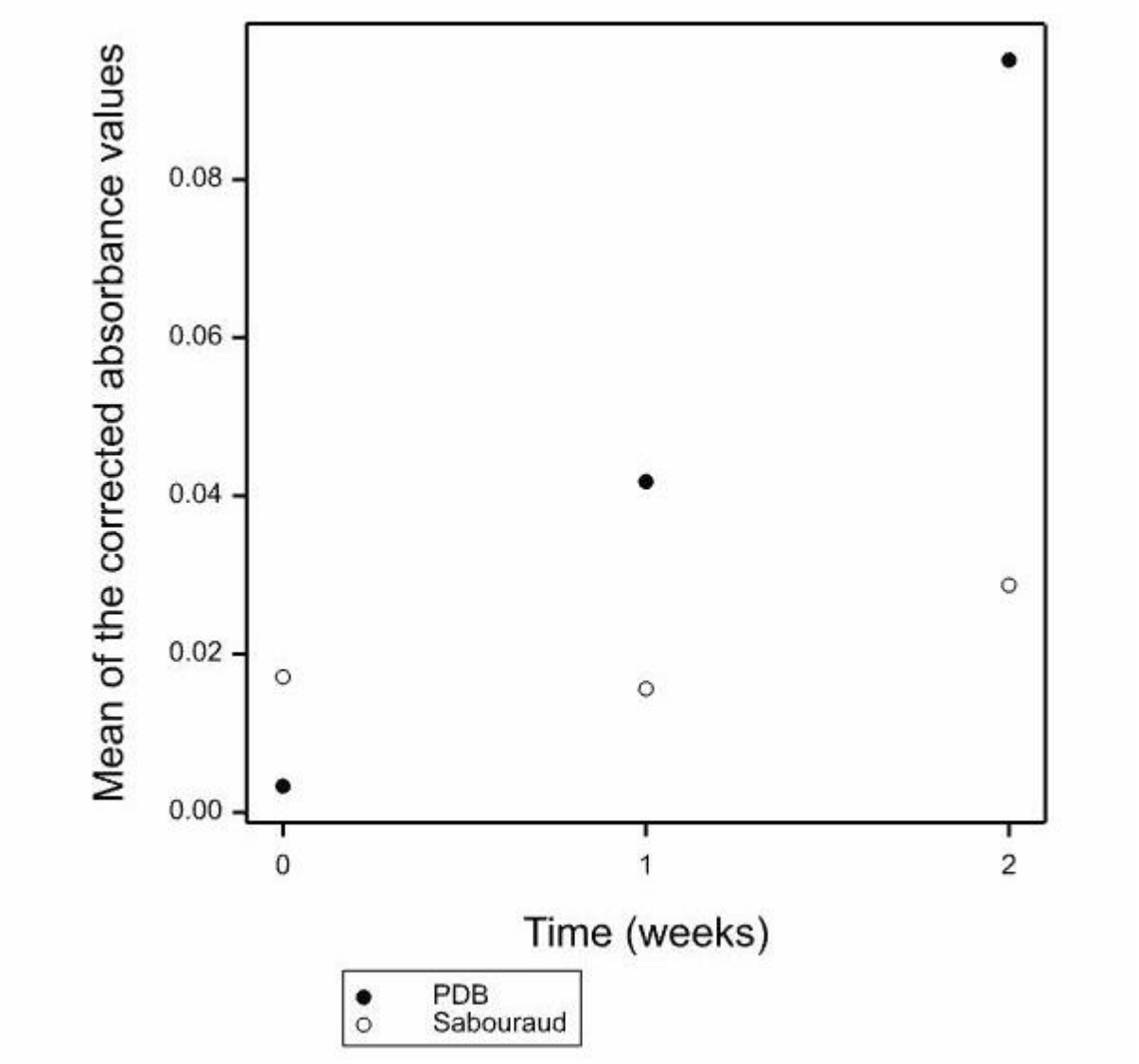


Figure 3.5. Mean of the corrected absorbance values of microplate wells of growth medium inoculated with *Venturia inaequalis* mycelial fragments in relation to type of growth medium and time since inoculation (SEM=0.024).

3.4.2. Testing for fungicide resistance in microplates

The results from the continuous experiments were generally inconsistent between replicates, and not much can be inferred from the data.

For the myclobutanil-amended tests, corrected optical density (after subtracting the blank) was generally negative and seemed to decline with time (Figure 3.6); for the tebuconazole-amended tests (Figures 3.7A, 3.7B), optical density did not change much over the course of the experiment across the range of fungicide concentrations.

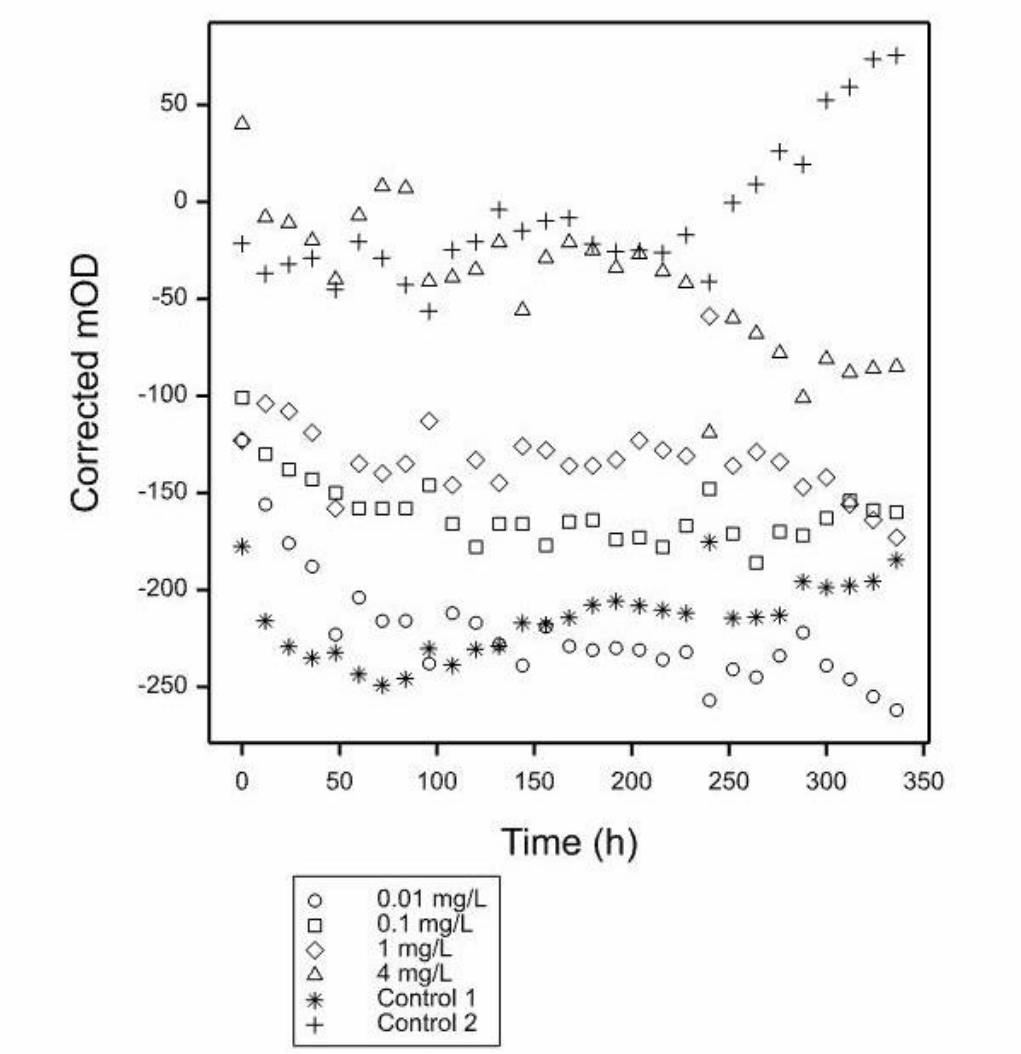
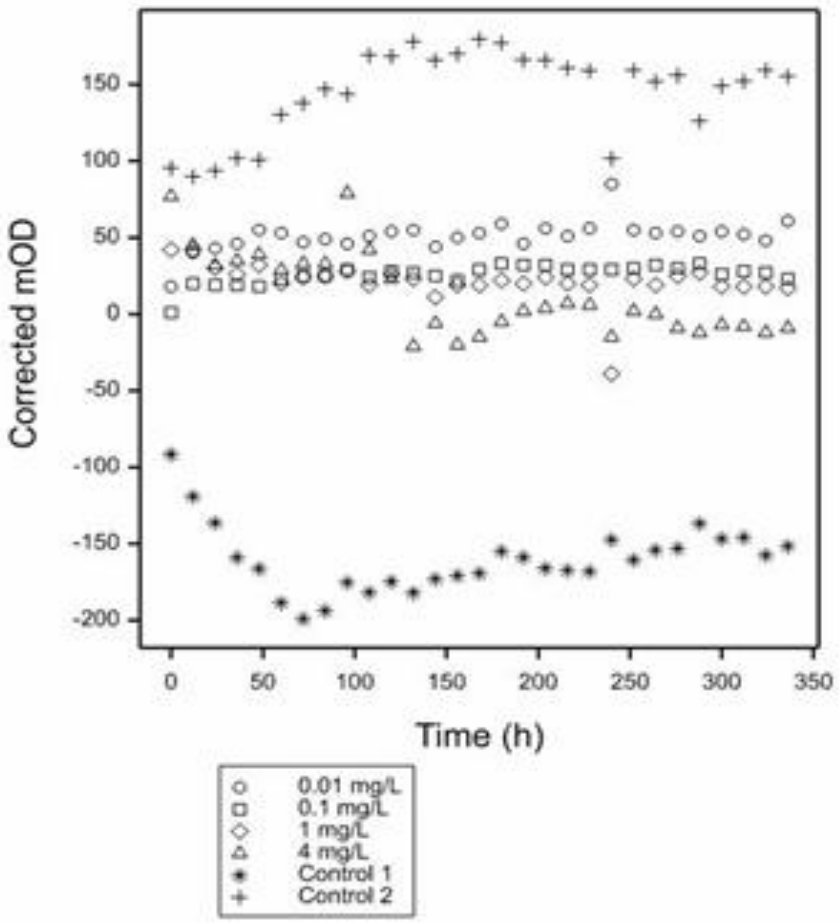
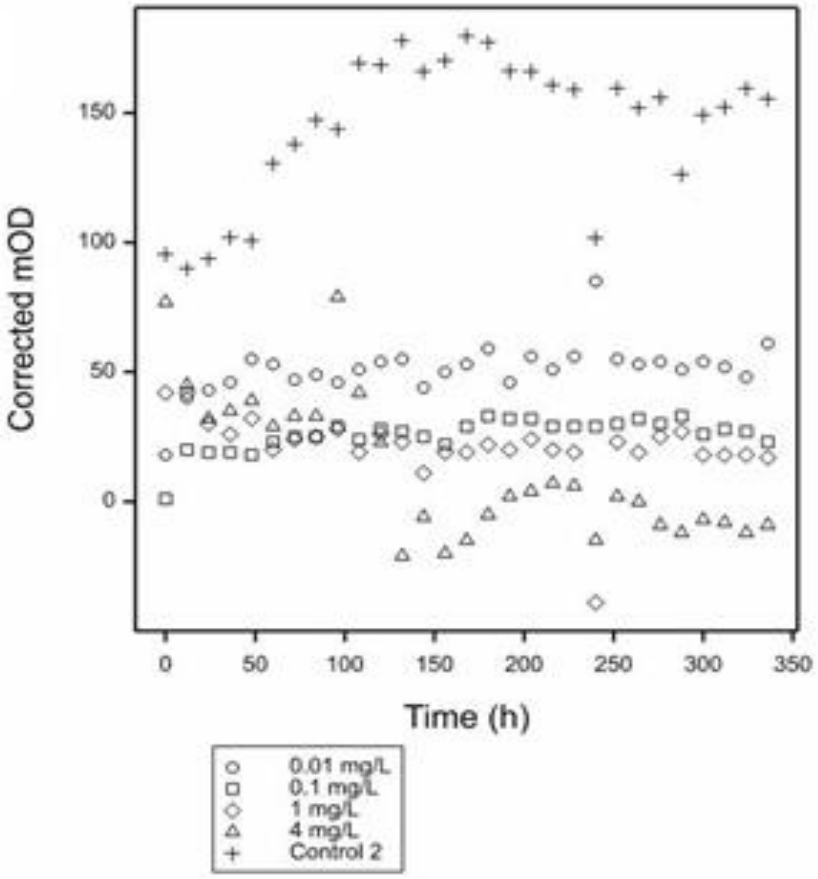


Figure 3.6. Change in optical density, measured as milli-optical density (mOD), over time in wells containing *V. inaequalis* mycelia grown in both PDB media amended with myclobutanil (0.01 mg/L, 0.1 mg/L, 1 mg/L, 4 mg/L) and two control wells containing only medium (PDB).

A


Corrected mOD
150
100
50
0
-50
-100
-150
-200
0
50
100
150
200
250
300
350
Time (h)
0.01 mg/L
0.1 mg/L
1 mg/L
4 mg/L
Control 1
Control 2

B


Corrected mOD
150
100
50
0
0
50
100
150
200
250
300
350
Time (h)
0.01 mg/L
0.1 mg/L
1 mg/L
4 mg/L
Control 2

Figures 3.7A and 3.7B. Change in optical density, measured as milli-optical density (mOD), over time in wells containing *V. inaequalis* mycelia grown in both PDB media amended with tebuconazole (0.01 mg/L, 0.1 mg/L, 1 mg/L, 4 mg/L) and two control wells containing only medium (PDB). Figure 3.7A includes the results from the two control wells, while Figure 3.7B includes only one of the control wells.

3.5. Discussion

The results presented here indicate the microplate technique, at least in the way it was developed in these experiments, would not work to determine the sensitivity of *V. inaequalis* isolates to fungicides. If any changes such as switching the type of inoculum to spores would make a difference, it is something that could be tested in the future. In the current study the use of fragmented mycelia was chosen as a rapid method and because the sporulation ability of *Venturia inaequalis* is rather variable (Kirkham 1957), with some isolates having poor spore production *in vitro*.

Visual assessment of the liquid growth experiments confirmed that *V. inaequalis* is able to grow in liquid media. Longer wavelengths seem better than short wavelengths to track growth dynamics in microplates. It maybe that at shorter wavelengths there is greater interference from metabolites secreted or the melanin and melanoprotein pigments (Hignett, Carder et al. 1977) found in *V. inaequalis*.

Despite the fact that monitoring methods involving use of microplates work well and have been approved for a number of other pathogens (FRAC 2017), in this case the microplate tests resulted in very inconsistent behaviours.

The negative values of the corrected optical density which declined over time in the myclobutanil data are especially disconcerting, as they imply the presence of the fungi is making those wells absorb less than the corresponding blank. Three possibilities that could explain the decrease with time observed in the data are degradation of the fungicide, contamination of the fungicide and absorption of the fungicide into the cells. The first two do not seem likely as both effects would have also been present in the corresponding blank and taken into account when subtracting the blank data. The third, absorption of the fungicide into the cell could explain the observed pattern.

The control data do not help in clarifying the data in the rest of the plate. The controls in both fungicides showed a location effect, in which the controls on one side of the plate gave negative corrected optical density, while the controls on the opposite side of the plate generally gave positive corrected optical density (Figures 3.1, 3.6, 3.7A, 3.7B). This position-related effect could be due to reading errors by the equipment that were position-related or maybe a greater evaporation in wells on the side of the plate with negative optical density. Other explanations could also be possible.

In the myclobutanil replicate in Figure 3.6 there might have been a contamination problem in control two as increased optical density starts rising much later (at around 250 h) than in the same control in the tebuconazole replicate presented here (Figures 3.7A, 3.7B).

Optical density of tebuconazole-amended wells with the fungi was positive in two out of three replicates. But in the positive optical density replicates, there was no change in optical density over time in most fungicide concentrations. In these replicates, control 2 (Figures 3.7A, 3.7B) presented a rise of optical density stopping later on, maybe indicating saturation of the well. It may be that all tested tebuconazole concentrations were able to stop fungal

growth, as optical density is stationary in the fungicide-amended wells in contrast to the growth observed in control 2, but the negative optical density of control 1 (Figure 3.7A) makes it difficult to confirm if this might have been the case.

The above results, in combination with time constraints, meant this approach had to be abandoned and the subsequent fungicide tests in Chapter 4 and 6 had to be carried out employing the traditional and commonly used method of measuring colony diameters explained in the next chapter.

4. Evolution of myclobutanil resistance and prospects for tebuconazole

4.1. Introduction

Growers have become dependent on the use of fungicides as an effective method of disease control, but this continued reliance on the use of fungicides has led to resistant isolates emerging (Chapter 1, Table 1.3). As there are only a limited number of products available for control of apple scab, the spread of fungicide resistance could potentially leave growers without a sufficient number of products for scab control due to a lack of alternatives (Chapman 2011). As a result, it is imperative to gain a deeper understanding of the dynamics and genetic control of fungicide resistance in order to make a more effective and sustainable use of available fungicides in the market to preserve their efficacy for a longer period of time.

Myclobutanil is a fungicide from the DMI family with a long history of use in controlling apple scab. It is frequently used in the UK since it can also effectively control apple powdery mildew (Xu, Gao et al. 2010). It is better known as a component of the commercial fungicide Systhane. Although Systhane was withdrawn from the market in 2017 (HSE 2017), it is still a useful fungicide model to study fungicide resistance, as other DMI products are still used in commercial agriculture, including in the control of apple scab; e.g. tebuconazole, sold under the name of Folicur.

Reduced sensitivity to myclobutanil has been reported across the world (Chapter 1, Table 1.3). Past work on myclobutanil in the UK reported ED_{50} values for isolates going up to 5.2 mg/L, based on *in vitro* assessment of hyphal length, and mean ED_{50} values for an untreated

orchard of 0.34 mg/L, determined through hyphal length measurements, or 0.2 mg/L, determined through colony diameter measurements (Xu, Gao et al. 2010). In a New Zealand study, the sensitivity level to myclobutanil went up to 11.35 mg/L, calculated through measurements of colony diameters of single-spore isolates (Beresford, Wright et al. 2012). In Pennsylvania, around 66% out of 644 isolates had a RG (Relative Growth) value of 75% of the control or higher (Pfeufer and Ngugi 2012); 63% out of 141 orchard populations in North East United States were considered to be resistant to the fungicide after comparing their sensitivity distributions to the distributions of both a baseline orchard and a resistant model orchard via a K-S two sample test (Villani, Biggs et al. 2015). In Ontario, an orchard reported a mean sensitivity that was 7.8 times higher than the mean of the baseline population (Errampalli 2004).

As mentioned above, having new fungicides, whether they might be completely novel compounds or others already in use for other pathogens, is crucial to maintain good control of the pathogen in the long run. However, when a new plant protection product is introduced, there are a number of variables that need to be considered. For one, it is necessary to test the new fungicide efficacy against the disease. Another factor to be considered is the possibility of cross-resistance between the new product and others used against the pathogen. Cross-resistance, if existent, would restrict the use of the product in that crop.

4.2. Objectives

Tebuconazole is a DMI fungicide recently introduced for use on apple scab in the UK. For this new fungicide to do well in the market, it should provide growers with good control of scab and have no cross-resistance with other DMIs. To this end, this chapter characterises the

efficacy of tebuconazole against *Venturia inaequalis* and assesses the existence of its cross-resistance with myclobutanil. The distribution of the ED_{50} values of isolates from an unsprayed population is a good indicator of the efficacy of a particular fungicide, as well as providing us with a baseline against which to assess resistance evolution in sprayed orchards. Comparisons of ED_{50} values within the same group of isolates to two different fungicides help to infer the relationship, if any, between the sensitivities to the two chemicals.

Data obtained in this chapter were also used to select appropriate resistant isolates to study the genetics of DMI resistance, with results presented in Chapter 5 and Chapter 6.

4.3. Materials and methods

4.3.1. Experimental design

A total of 40 isolates were tested for fungicide resistance to myclobutanil (Systhane 20EW, Dow AgroSciences) and tebuconazole (Folicur 25 EW, Bayer Crop Science). Those isolates belonged to one of the following categories: baseline isolates (20 isolates) and problem isolates (20 isolates).

The baseline isolates were used to determine the baseline level of sensitivity, and additionally, to identify isolates suitable to carry out the experiments described in Chapter 5 and 6 of this thesis. This latter purpose also applied to the problem isolates. In Chapter 5, it was important to have a range of sensitivities to establish that any mutation found in the *CYP51A1* gene was linked to fungicide resistance; and in Chapter 6, a sensitive and resistant parent with as much difference as possible between their sensitivity levels was necessary to design crosses to study gene control.

Each group of isolates was obtained from different sources. The baseline isolates came from trees from a non-commercial orchard in west England that has never been treated with fungicides. The majority of problem isolates were provided by consultants who collected scabbed leaves from orchards with known problems of scab control. Four of the problem isolates were collected from potted trees in the NIAB EMR site.

Single-spore isolates were obtained from leaf discs (Chapter 2). For each isolate, its level of sensitivity was tested by subculturing on PDA media amended with the following concentrations of the fungicide: 4 mg/L, 1 mg/L, 0.1 mg/L and 0.01 mg/L (Chapter 2). A treatment without fungicide was used as control. For each combination of isolate and concentration, there was one plate with four to three 4 mm plugs of mycelia, the number of plugs depended on the quantity of material available for that isolate at the time of experimentation. In some cases, due to lack of fungal material, only two plugs were used.

On each day when subcultures were made, isolates from both baseline and problem orchard categories were used in a similar proportion to minimise the effect of batch variability on comparisons between the two groups of isolates.

The whole experiment was repeated once for each isolate.

4.3.2. Data Handling

Genstat 16 (Payne, Murray et al. 2013) was used to analyse all data. For each isolate a scatter plot was obtained to show the pattern of growth under the studied range of fungicide concentrations. Q-Q plots, where the experimental distribution of the $logED_{50}$ is compared to the expected distribution if the data followed a theoretical distribution (in this case, a normal distribution), and histograms were used to characterise the baseline

sensitivity. For each isolate, a value for their $logED_{50}$ was estimated through a logistic model (Chapter 2).

In the case of myclobutanil, there were eight isolates where the logistic model fitted gave abnormally large values of ED_{50}, which have been included in the analyses with values above 4 mg/l to compare with the tebuconazole data. The values assigned to these irregular isolates were roughly estimated from the shape of their growth scatter plots. Errors could not be calculated for the myclobutanil $logED_{50}$ data of the eight irregular isolates. A high upper error was assigned to these isolates above the highest standard error that could be calculated. A different approach was used for the lower error. From the growth curve of the eight isolates, which does not show any decline at the highest concentration tested, it is likely estimations of the $logED_{50}$ values of these isolates are underestimates of the true ED_{50} rather than overestimates. Therefore, any lower error bars for these isolates should not be below the highest concentration tested. To reflect this, the value assigned to the lower error bars of these isolates was calculated using the mean of the standard errors of all other isolates.

The distributions of the $logED_{50}$ values of the baseline isolates and the problem isolates were compared through a Kolmogorov Smirnov test.

To study cross-resistance, the log-transformed values of ED_{50} for both chemicals were plotted against each other in a linear regression.

4.4. Results

4.4.1. Characteristics of the populations

Isolates showed a variety of growth patterns, with some, including the eight isolates where the model did not fit, having a different response from the typical dose curve that drops at higher concentrations (Figures 4.1, 4.2).

Isolate 09/034-11

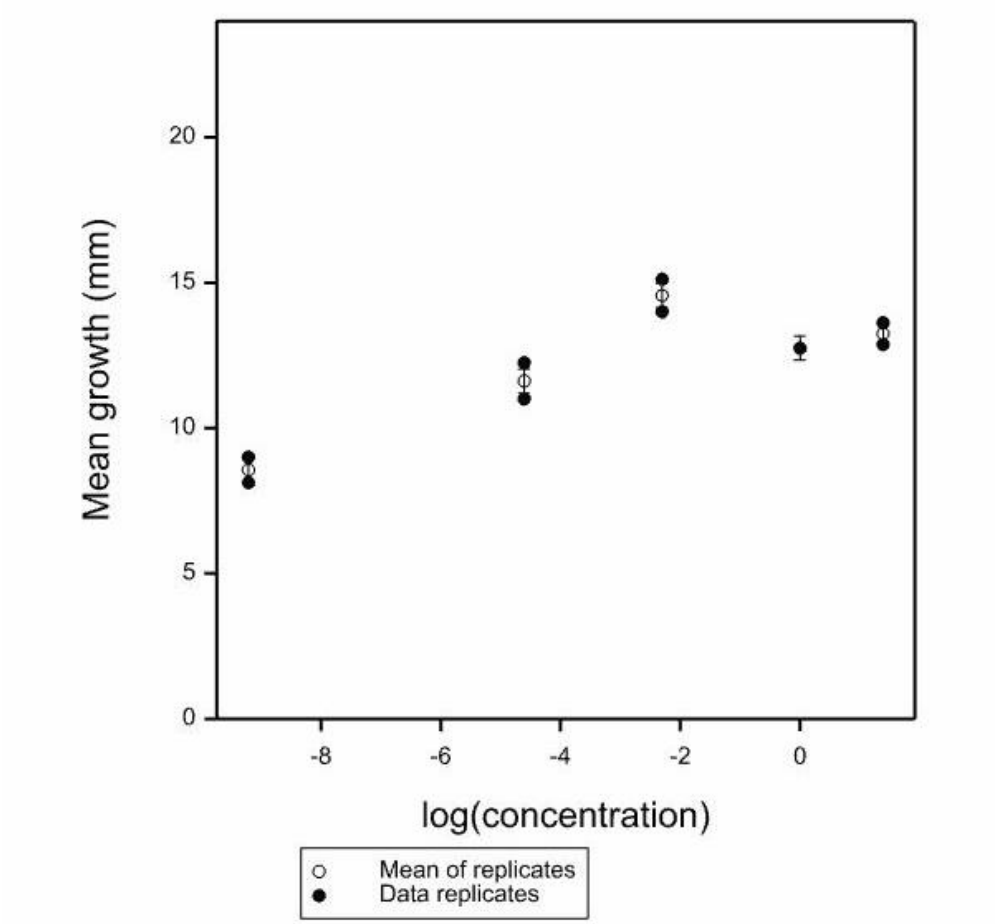


Figure 4.1. Case study: resistant isolate with increased growth in the presence of myclobutanil. Error bars, calculated as 2SEM of the mean radial growth on a plate averaged over all plates, are centered on the mean of the two replicate points.

Isolate 09/34-4

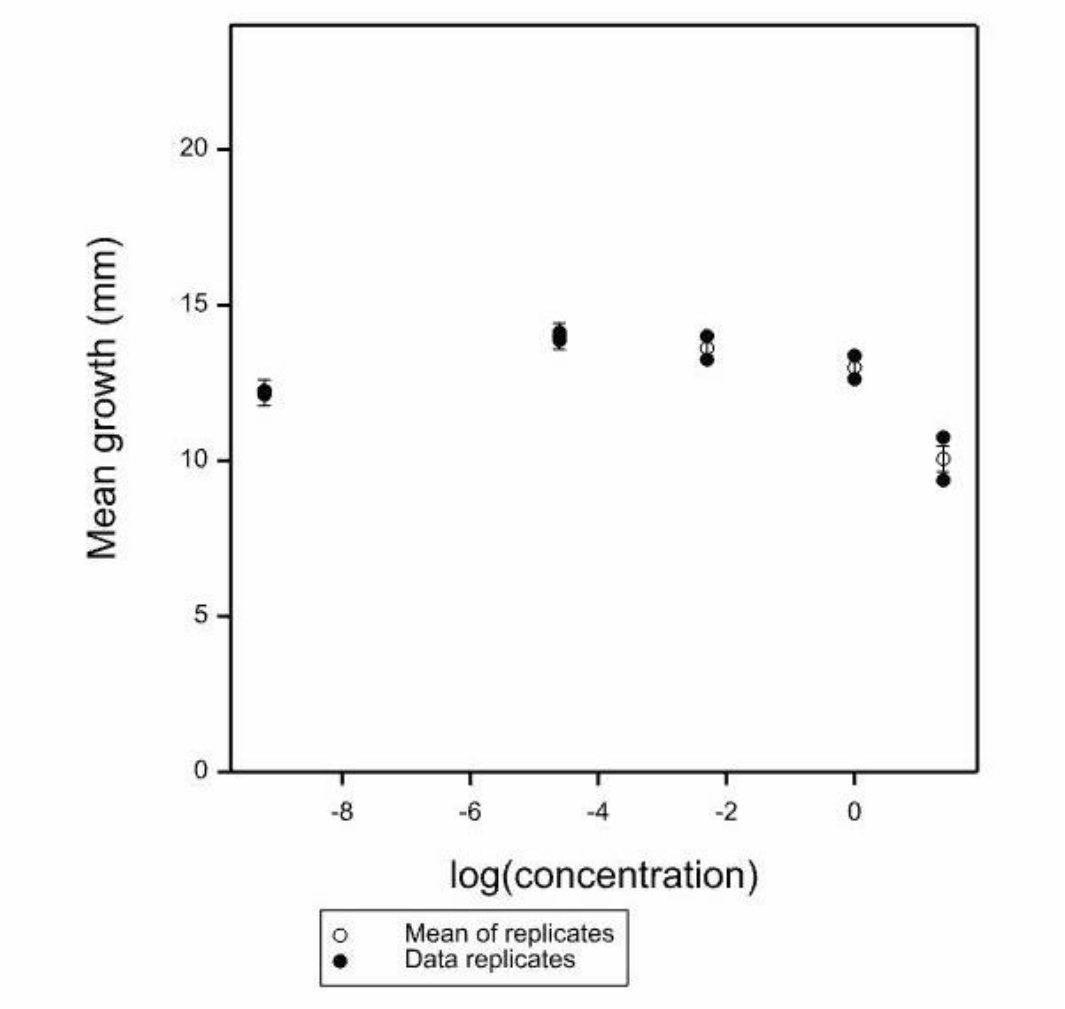


Figure 4.2. Case study: Isolate with a slight initial growth spurt at low concentrations of myclobutanil. Error bars, calculated as 2SEM of the mean radial growth on a plate averaged over all plates, are centered on the mean of the two replicate points.

Sensitivities for myclobutanil go from 0.14mg/L to 46.94 mg/L for the baseline orchard (average ED_{50}= 6.46 mg/L); in the problem orchards, the sensitivity range went from 4.30 mg/L to around 72.39 mg/L. For tebuconazole, the sensitivity goes from 0.022 mg/L to 2.99 mg/L in the baseline population (average ED_{50}=1.03 mg/L), and from 1.68 mg/L to 24.75 mg/L for the problem orchards. The logED_{50} values of the baseline orchard showed

statistically significant differences (p-value<0.001) with the $logED_{50}$ values of the problem orchards for both the myclobutanil and the tebuconazole datasets.

4.4.2. Baseline sensitivity distribution

The histogram of $logED_{50}$ values for myclobutanil (Figure 4.3) and tebuconazole (Figure 4.4) fit well to a normal distribution. Q-Q plots further confirm the proximity of the $logED_{50}$ data to a normal distribution (Figures 4.5, 4.6), and thus the sensitivity of the baseline population would closely follow a lognormal distribution.

4.4.3. Cross-resistance

There is a strong correlation (Figure 4.7) between the $logED_{50}$ values of the two chemicals ($r=0.9063$; $P < 0.001$). The eight resistant isolates for which a myclobutanil ED_{50} could not be accurately determined were included in the cross-resistance regression with values above 4 mg/l, as described before.

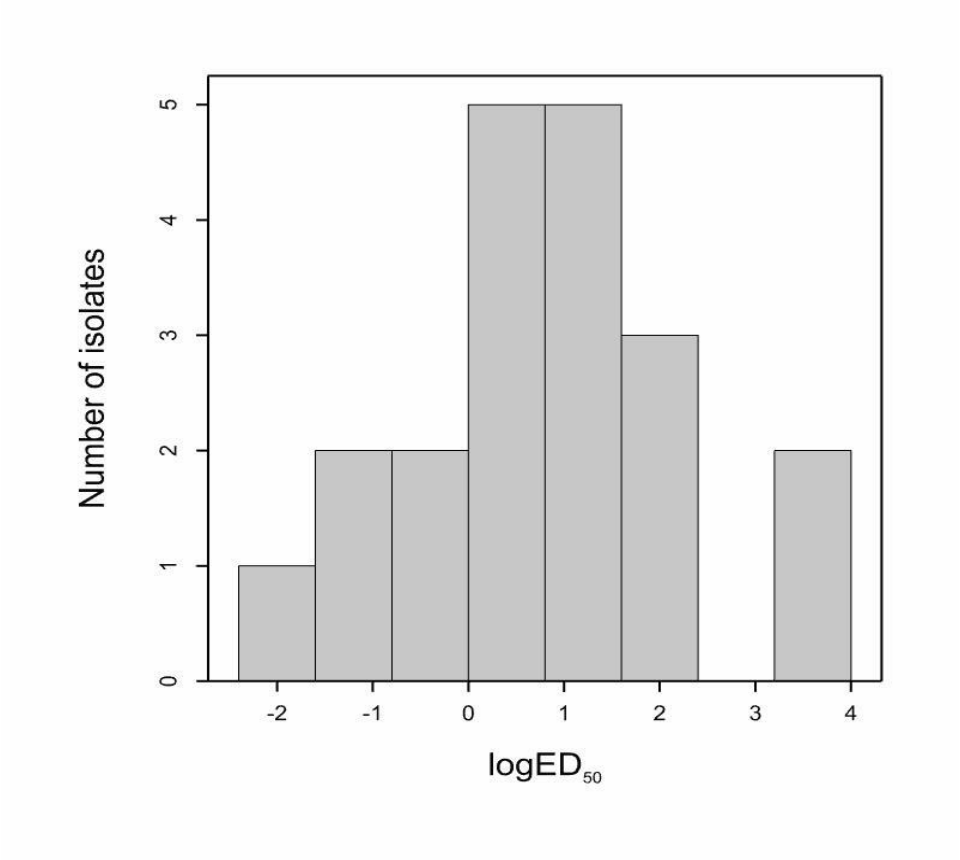


Figure 4.3. Distribution of myclobutanil $logED_{50}$ estimated from radial growth rate on PDA in a baseline population never exposed to DMI fungicides.

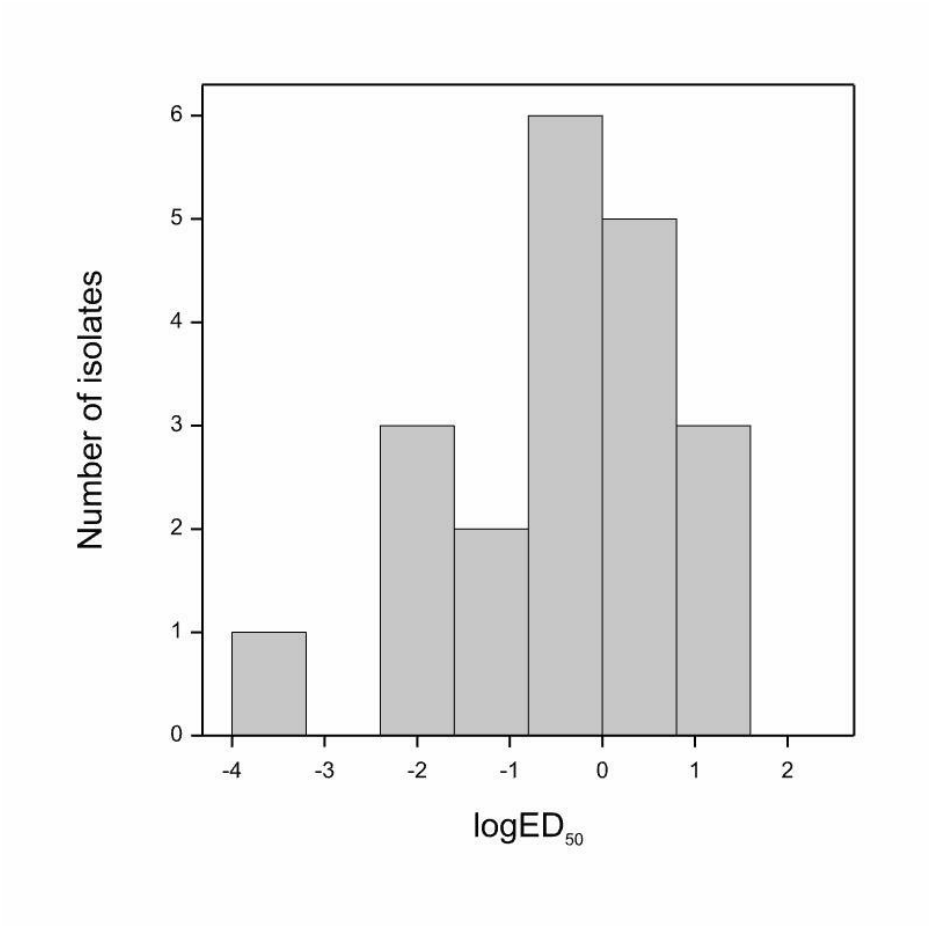


Figure 4.4. Distribution of tebuconazole $logED_{50}$ estimated from radial growth rate on PDA in a baseline population never exposed to DMI fungicides.

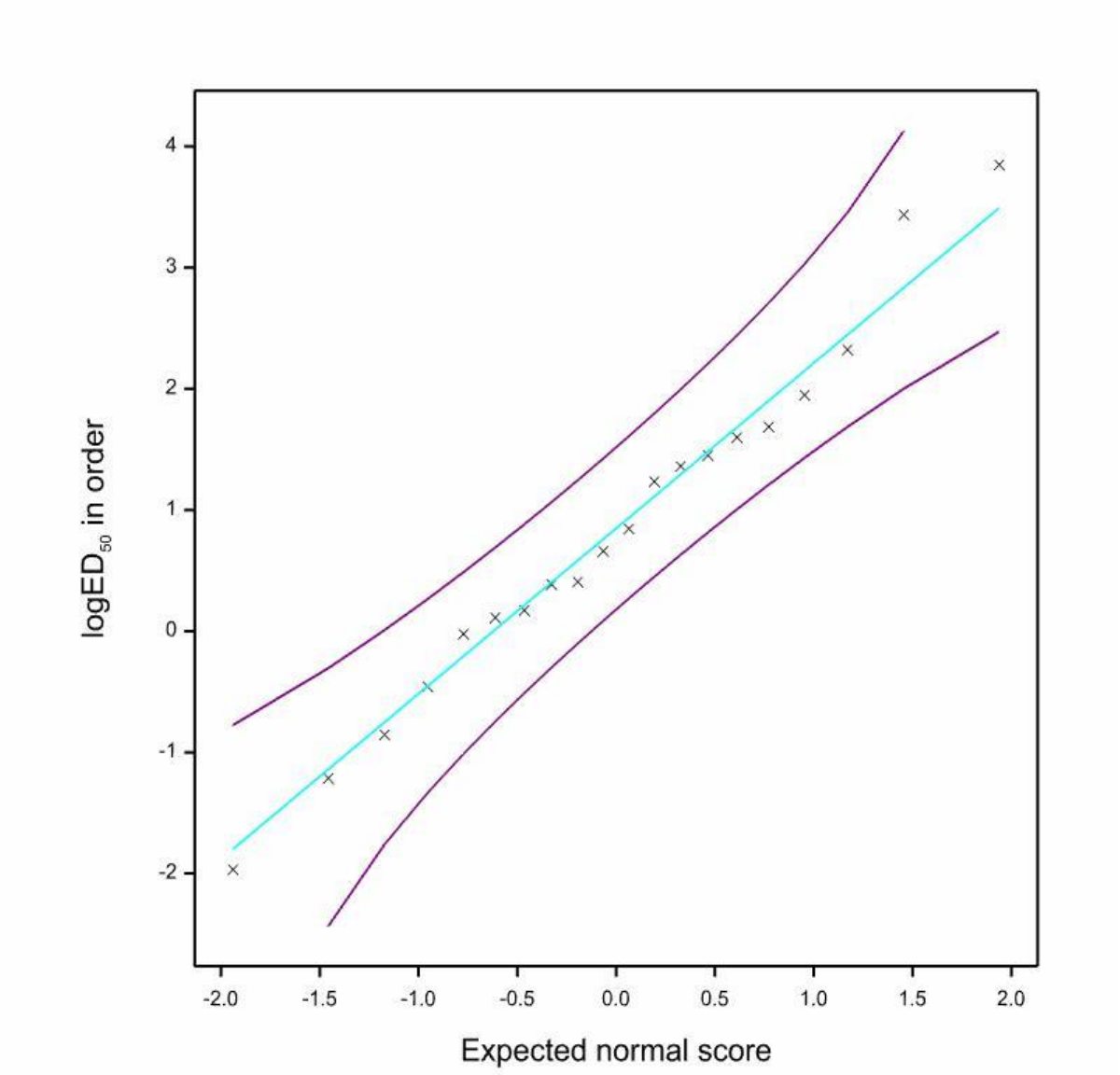


Figure 4.5. Q-Q plot (in which a normal distribution will appear as a straight line) of the $logED_{50}$ values of myclobutanil. Purple lines represent a 95% confidence interval, a true normal sample would fall within this interval 95% of the time. The blue line shows the correlation between the distribution of the experimental data and the distribution expected if the data was normally distributed.

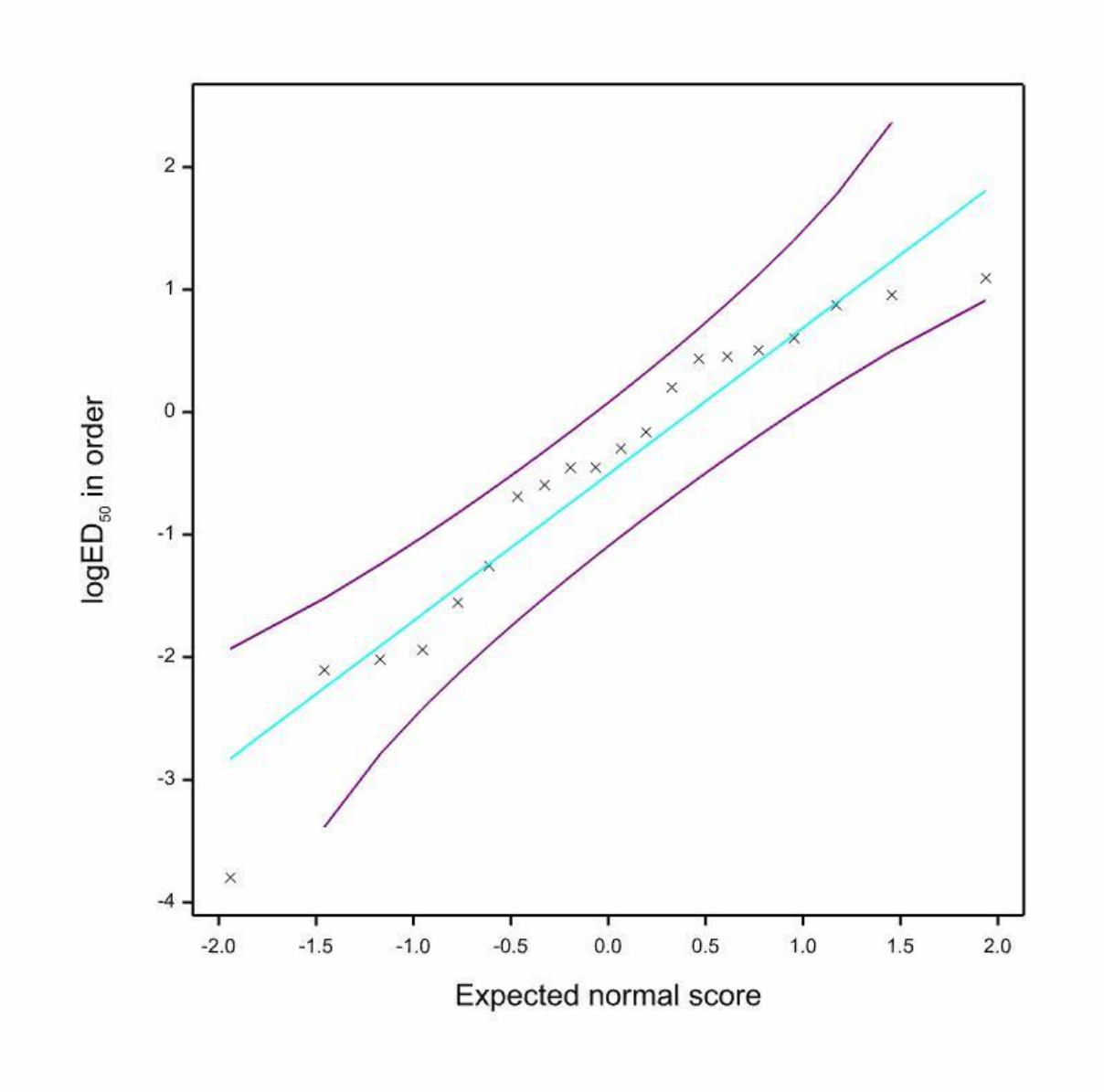


Figure 4.6. Q-Q plot (in which a normal distribution will appear as a straight line) of the $logED_{50}$ values of tebuconazole. Purple lines represent a 95% confidence interval, a true normal sample would fall within this interval 95% of the time. The blue line shows the correlation between the distribution of the experimental data and the distribution expected if the data was normally distributed.

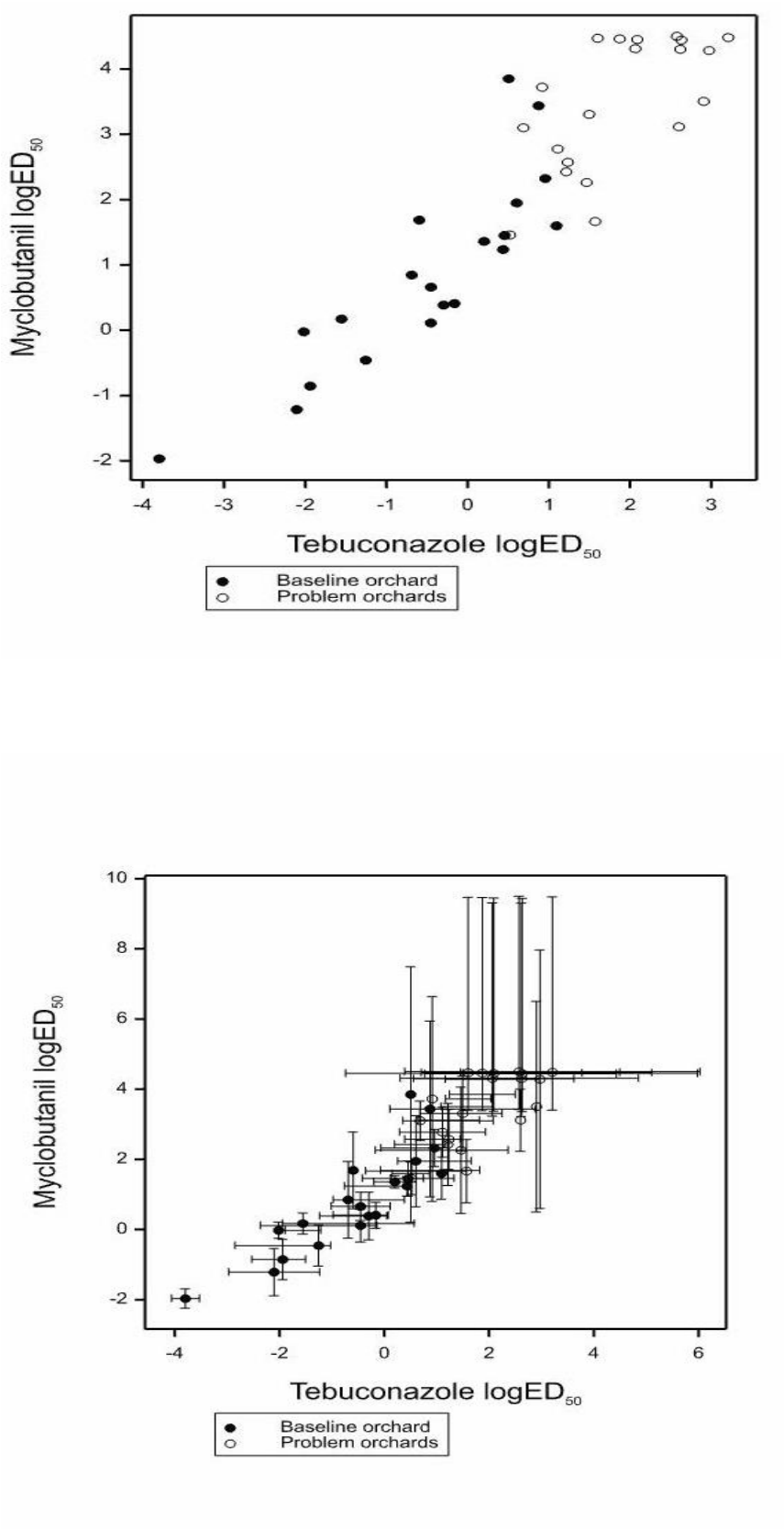


Figure 4.7. Cross-resistance between myclobutanil and tebuconazole in isolates from one baseline orchard and several problem orchards. Error bars were calculated from the

standard error of each logED_{50} estimate (2* Standard error), with a different approach for the eight irregular myclobutanil isolates as described in the Materials and methods section.

4.5. Discussion

The baseline values for myclobutanil in the current study are greater than those previously reported in the UK with the single-spore *in vitro* assessment, which included a mean baseline sensitivity for myclobutanil of 0.24 mg/L (Xu, Gao et al. 2010). Some of this variation could be due to the chance inclusion of two isolates in the current sample with very high resistance, with an ED_{50} greater than 30 mg/L, pulling up the mean sensitivity. Regarding the baseline sensitivity for tebuconazole, a study in Germany (Kunz, Deising et al. 1997) found a baseline mean ED_{50} for tebuconazole (1.22 mg/L) similar to the one reported in the current study (1.03 mg/L).

It is important to mention that the study in this chapter used a different methodology from either of the above. Kunz, Deising et al. (1997) used *in vivo* methods which have been said to result in greater ED_{50} values than the ones derived from *in vitro* methods. In addition, the *in vivo* method they used involved the use of spore suspensions from lesions found on leaves, and posterior evaluation of spore differentiation in lesions from leaves inoculated with these spore suspensions; this means that the lesions examined may have contained a range of genotypes, resulting in the higher ED_{50s}. Differences in methods resulting in different estimations of ED_{50} have also been observed between *in vitro* methods. Xu, Gao et al. (2010) reported smaller values of ED_{50} obtained with hyphal length measurements than with colony diameter measurements, a product of colonies having greater biomass, which makes them more resistant than the germination tubes. The study by Xu, Gao et al. (2010) where they

measured the diameters of single spore colonies diverged from the current study in its statistical approach. While their response variate was growth of the colony relative to the control (RG), which is used in many other *in vitro* studies (Koller and Wilcox 1999; Pfeufer and Ngugi 2012; Beresford, Wright et al. 2013; Mondino, Casanova et al. 2015), the statistical analysis carried out here used the actual mean growth at each concentration of chemical as response. The decision to use growth rather than relative growth has its basis on a desire to avoid decreasing the accuracy of the results, as dividing the growth by that of the control introduces the error associated with the control into every measurement and loses a degree of freedom in the error estimate, while the use of raw means only involves the error in each mean, and retains five points for estimating the dose-response, instead of four. This would affect the error of the ED_{50} estimates, although the estimates themselves should not be affected. It must be noted that differences like those listed here derived from either the experimental approach or the statistical approach have to be taken into account in order to compare the results in the current study with those of previous studies.

In the current study, baseline values for myclobutanil reached greater ED_{50} values than tebuconazole, indicating a greater absolute efficacy of the latter (Figures 4.3, 4.4). From another study that estimated mean baseline sensitivity for different DMI chemicals, the following rank order of toxicity to *V. inaequalis*, from higher toxicity to less toxicity, could be surmised: difenoconazole, flusilazole, fenarimol, tebuconazole, pyrifenox (Kunz, Deising et al. 1997).

In regard to the distribution of the baseline sensitivity, both chemicals (myclobutanil and tebuconazole) fit a lognormal distribution well. Other authors have also found lognormal distributions to myclobutanil in baseline populations (Errampalli 2004). Baseline sensitivity

distributions of DMIs fenarimol, flusilazole and difenoconazole also followed a lognormal distribution in other studies (Koller, Parker et al. 1991; Carisse and Pelletier 1994; Mondino, Casanova et al. 2015). Xu, Gao et al. (2010) observed ED_{50} distributions that were strongly skewed to the right for fenbuconazole and myclobutanil, possibly indicating an approximately lognormal distribution. It is important to note that some of the studies cited only used one dose rather than a range in the sensitivity tests (Koller, Parker et al. 1991; Carisse and Pelletier 1994; Mondino, Casanova et al. 2015). Using a single discriminatory dose does not pose much risk in a bimodal distribution scenario (qualitative resistance) where the cut-off point can be moved without affecting results as long as it is kept between the two modes; in other types of distribution, like a normal or lognormal, the cut-off point is greatly going to determine how many isolates are reported as resistant. Taking a normal distribution as an example (since the sensitivity data fits a lognormal distribution, the log-transformed sensitivity data would fit a normal distribution), when the cut-off point is in the peak of the distribution moving it one standard deviation does not have the same effect as doing the same when the cut-off point is toward the end of the distribution, which is usually the case. In the latter case, since there is less data toward the end of the normal distribution, the number of isolates reported as resistant would be substantially less. Also cut-off points can be potentially abused, as it is possible to set them to over report or underreport resistance. It is worth mentioning that cut-off points can be empirically validated to prevent this.

Moving to the individual datasets for each isolate, while most isolates exhibited a typical dose response curve that fell at higher concentrations, a few had abnormal sensitivity responses. Irregular behaviours included increased growth in the presence of the fungicide

(Figures 4.1, 4.2). It would be interesting to try to find which mechanisms could be behind such behaviour. It is important to note that the current study employed radial growth and an increase in diameter on the fungicide-amended plates does not necessarily imply that the isolates were benefitting from the presence of the fungicide. In toxicology, there is a well-known phenomenon in which a growth spurt occurs at low concentrations of a chemical, this phenomenon is referred to as hormesis (Flores and Garzon 2013). It could be that isolates are having a hormesis-like response trying to send out new hyphae in an attempt to escape the fungicide. For an isolate that grows more than the control at all fungicide concentrations (Figure 4.1), the same could be happening, note that in the isolate in Figure 4.1 the growth spurt is less intense at higher concentrations than at lower doses which could be pointing to the efforts of the isolate to escape being impaired by a stronger toxic effect at higher doses. However, these are only theories and molecular analyses would have to be conducted to test them.

From a grower point of view, the main question would be how all these findings translate in terms of behaviour in the field. The sensitivity levels derived from the data in this chapter, a maximum of 72 mg/L for myclobutanil, are still far from the concentrations sprayed that go from 92 mg/L, recommended spraying dose during full leaf if using seven days intervals (Systhane 20EW label, Dow AgroSciences), to 450 mg/L, recommended spraying dose for early season using 14 days intervals (Systhane 20EW label, Dow AgroSciences). Even though these doses seem high when compared to the results presented here or, more markedly, those from previous studies, the findings should be viewed in light of conditions in the field. It is unlikely for an isolate to receive such elevated doses, as spray deposits will change concentration in the environment (Shaw 2000; Xu, Wu et al. 2006) due to mobility of the

fungicide outside plant tissue, mobility inside plant tissue and degradation of the fungicide Laboratory trials seem to confirm that the actual doses on the leaf surfaces are much lower than those sprayed. In an unpublished report to AHDB (Xiangming Xu, personal communication 2017, NIAB EMR, Kent UK) it was pointed out how spray concentrations on the leaves can be greatly reduced after rain events. In the same report, it was estimated, based on analysis of the active substance on leaf samples, that 1 cm^2 of leaf surface contained concentrations on the day of spraying (at a dose of 450 mg/L) that went up to 25 mg/L (for myclobutanil), the most resistant isolates in the current study had an ED_{50} that reached 72 mg/L and the maximum ED_{50} for tebuconazole almost reached the 25 mg/L mark, so these isolates would be theoretically able to withstand these leaf doses. However, the level of sensitivity is just one of the factors to consider in terms of practical field resistance. Another important factor would be the fitness of the resistant strains as that would determine whether the isolate would be able to establish a resistant population. Even if the resistant isolates in the current study have ED_{50} values similar to or above the concentrations on leaf surfaces, if they are not competitive under field conditions, they may not be able increase their numbers to pose a problem for the orchard.

The isolates in problem orchards in this study all came from orchards where a loss of control had been observed. In fact, the data used in the elaboration of this chapter points to a very clear separation between the $logED_{50}$ values of the baseline isolates and the problem orchard isolates (Figure 4.7), with the latter generally having greater $logED_{50}$ values. Although other factors may have contributed to the loss of control in these orchards, the presence of resistant isolates in them was certain to have contributed. Thus, it is worrying that the level of resistance around the world is on the increase (Chapter 1, Table 1.3).

It was already mentioned previously in this chapter the necessity of finding new chemicals to replace those that have suffered a loss of efficacy (Chapman 2011). Tebuconazole was a possibility, but, from the cross-resistance graphs presented here (Figure 4.7), it can be gathered that tebuconazole would not be a good replacement for myclobutanil, as their $logED_{50}$ data correlates linearly; this is more evident in the baseline isolates, although that could be due to the eight isolates with a $logED_{50}$ that could not be reliably calculated. For those eight isolates, the estimated myclobutanil $logED_{50}$ values seem to be lower limits; therefore, the information from these values is only valid in terms of the direction of the cross-resistance relationship. The findings about cross-resistance in this study mean new chemicals or other existing DMIs used in other crops would need to be tested to find a suitable replacement to use in orchards with high resistance to myclobutanil. One of the main alternatives currently employed is difenoconazole which did not seem to have cross-resistance in a population of myclobutanil resistant isolates; however, a baseline population did have some cross-resistance (Villani, Biggs et al. 2015). There are a number of factors that could be behind these differing patterns of cross-resistance in orchards with different backgrounds. Some of these are: different resistance mechanisms evolving in each orchard, environmental factors (Xu, Gao et al. 2010) and different control measures employed in each orchard (Villani, Biggs et al. 2015). Even if there is no apparent cross-resistance between myclobutanil and difenoconazole in some orchards, the presence of cross-resistance in one of the studied orchards (Villani, Biggs et al. 2015) and reports of resistance to difenoconazole in some countries (Mondino, Casanova et al. 2015) points to the necessity to continue to monitor the evolution of resistance to difenoconazole. At the same time, the agriculture industry should get ahead of risks of losses of efficacy with more research on other candidates, including chemicals with new modes of action.

5. Determining the importance of *CYP51A1* for DMI resistance

5.1. Introduction

There is still much to explore regarding the genetics and mechanisms of pathogen resistance to DMI fungicides. Although there have been a number of mechanisms identified as likely or confirmed to operate, this does not mean that all these alternatives are equally important, or common, in all pathogens. Some well-known mechanisms involve mutations or overexpression of the *CYP51A1* gene, which encodes the target of DMI fungicides, or transporters in the cell membrane (Jobin and Carisse 2007; Pfeufer and Ngugi 2012).

Overexpression of *CYP51A1* has already been identified as a very likely candidate, with several studies that correlate upstream insertions with resistance in *V. inaequalis* (Schnabel and Jones 2001; Villani, Hulvey et al. 2016). Some research points to the possibility of resistance to different chemicals working by different mechanisms. Villani, Hulvey et al. (2016) reported a case of overexpression associated with resistance to difenoconazole, but in myclobutanil, although relative expression was higher in resistant isolates than in sensitive isolates, the difference was not statistically significant. It might also be that strains belonging to different regions or backgrounds end up selecting for different mechanisms. For example, although Villani, Hulvey et al. (2016) could not find a link between resistance to myclobutanil and overexpression in isolates from New York, Maine and West Virginia, Schnabel and Jones (2001) in Michigan did obtain data to support such a connection.

Transporters also seem to be able to generate resistance, as suggested by laboratory mutants of *V. inaequalis* and other pathogen species (Vijaya Palani and Lalithakumari 1999; de Waard, Andrade et al. 2006). Field studies in some pathogens did not find any isolates

that employed this mechanism (de Waard, Andrade et al. 2006), but other species are shown to have such a connection in the field (Sang, Hulvey et al. 2015).

Mutations in the target demethylase protein that confer resistance against DMIs have been observed in other species such as *Mycosphaerella graminicola (=Zymoseptoria tritici)* (Mullins, Parker et al. 2011), but thus far, there have been no reports of similar mutations in *V. inaequalis*, and mutations that provide other species with resistance have not been found in this pathogen (Schnabel and Jones 2001). Whether there could be other mutations with similar effects is a possibility that cannot be ruled out. After all, *CYP51* sequences have shown great variability between species, and amino acids essential for activity in some species - such as Gly at position 310 - are replaced by other amino acids in other pathogens (Joseph-Horne and Holomon 1997). This indicates that substitutions which cause fungicide resistance in some organisms may not be found in others.

5.2. Objective

To sequence the *CYP51A1* gene region of isolates with differing sensitivities against mycobutanil in order to identify mutations linked to resistance.

5.3. Materials and methods

5.3.1. Experimental design

Venturia inaequalis isolates with varying levels of sensitivity against myclobutanil were chosen, using their ED_{50} values as criteria. These isolates came from orchards where resistance problems had been reported and from an orchard that had never been sprayed; a total of 29 isolates were sequenced (Table 5.1). First, the upstream region was amplified

with primers AJ455 and AJ414. Unfortunately these primers failed for most isolates (Table 5.2).

Table 5.1. List of isolates selected for sequencing and their sensitivity levels.

Isolate [1]	Orchard type	Myclobutanil [2] $logED_{50}$	Tebuconazole $logED_{50}$	Sequencing outcome
09/034-13	Problem	> 4	3.21	Failed
09/034-7		> 4	2.63	Successful
09/030i		> 4	2.62	Successful
09/034-10		> 4	2.58	Failed
09/034-11		> 4	2.09	Failed
09/37-1		> 4	2.06	Successful
09/034-2		> 4	1.87	Successful
09/037-2		> 4	1.60	Successful
09/034-5		4.28	2.97	Successful
C3		3.50	2.91	Successful
G4		3.3	1.5	Failed
09/034-6		3.11	2.60	Successful
G1		2.77	1.11	Successful
09/34-4		2.57	1.23	Successful
Spartan 1		2.42	1.21	Successful
09/039		2.26	1.46	Failed
AF2	Baseline	3.85	0.5	Failed
AF8		2.32	0.96	Failed
AF17		1.95	0.6	Failed
AF12		1.6	1.09	Failed
AF16		1.36	0.20	Failed
AF31		1.23	0.43	Failed
AF25		0.66	-0.45	Failed

Isolate [1]	Orchard type	Myclobutanil [2] $logED_{50}$	Tebuconazole $logED_{50}$	Sequencing outcome
AF3	Baseline	0.41	-0.16	Successful
AF10		0.38	-0.29	Failed
AF4		0.11	-0.45	Failed
AF24		-0.46	-1.25	Successful
AF15		-0.85	-1.94	Successful
AF28		-1.21	-2.10	Successful

[1] Isolates in bold were those in which the region upstream the gene was successfully amplified.

[2] Within each orchard type, $logED_{50}$ values are ordered from more resistant to myclobutanil (top) to less resistant (bottom).

Table 5.2. Primer combinations tested for use in amplification.

Region	Primer combination	Success rate [1] (isolates amplified/ total isolates tested)
Upstream region and part of the 5' region of the gene	AJ455 - AJ414	3/29
5' half of the gene	829F - 1764R	15/29
3' half of gene	1559F - 2625R	15/29

[1] Success rate with published primers AJ455 and AJ414 (Schnabel and Jones 2001) was particularly low with the current collection of isolates; however, further tests with six additional isolates from a different source (not included) were successful. As success rates were also not good for the other primers, it is likely that there was some problem with the DNA (Thomas Passey, personal communication 2017, NIAB EMR, Kent UK).

Table 5.3. Primers used for the amplification and sequencing of the *CYP51A1* gene.

Gene position	Primer	Primer sequence
5’	829F	AGACGAGCAACACCACACTT
	1764R	TTGACGCCCTCTGCTTTTCT
3’	1559F	CGCTCACTACAAGGCAAGGA
	2625R	CTAGCCCCGCGAGAAATCAT

Primers to amplify and sequence the *CYP51A1* gene were designed with the Primer3 tool in Geneious version 14 (Kearse, Moir et al. 2012) (Table 5.3). The gene was subdivided into two halves: one fragment of 936 bp, including the 5’ end, and the other of 1067 bp, including the 3’ end. Each half was sequenced from both ends to ensure as much good quality coverage as possible. Positions of primers within the gene are detailed in Figure 5.1.

Figure 5.1. *CYP51A1* gene region sequence (Genbank Accession No. AF227920). Forward primers are highlighted in blue and reverse primers in pink. The start and stop codons appear encased within a rectangle and introns are underlined (Schnabel and Jones 2001).

5.3.2. Sequencing *CYP51A1*

DNA extractions were carried out with DNeasy Plant mini kit (Qiagen), using freeze-dried mycelia samples homogenised with a MM2 oscillating mill (Retsch). After extraction, DNA concentrations were measured with Nanodrop 1000 spectrophotometer (Thermo Scientific), and concentrations of all samples were adjusted to 1 ng/µl. PCR reactions were carried out in a volume of 100 µl, and included Buffer 10x 10 µl (final concentration 1x), $MgCl_2$ 8 µl (final concentration 2 mM), dNTPs 8 µl (final concentration 0.2 mM), forward and reverse primers 10 µl (final concentration 0. 2 µM), Taq (Moltaq Basic, VH Bio Ltd) 0.4 µl (final concentration 0.02 units/ µl), 41.6 µl water (Sigma-Aldrich) and 16 µl DNA template (final concentration 0.16 ng/µl).

PCR conditions were: 95 °C 3 minutes; followed by 35 cycles of 95 °C for 30 seconds, 52 °C for 30 seconds and 72 °C for 1 minute; after the cycles an additional extension time of 5 minutes at 72 °C continued. PCRs were performed in a Biorad T100 Thermal Cycler. After PCR, electrophoresis gels at 1% were used with TAE 1X buffer to check for the presence of desired bands. The gel was stained with GelRed Nucleic Acid Gel Stain 10000X (Biotium) and visualised with Biorad GelDoc XR+.

PCR products were purified through use of GeneClean Turbo Kit (MP Biomedicals). DNA concentrations were adjusted to around 35-40 ng/µl either by dilution or concentration by a vacuum concentrator (Speedvac SC110, Savant). Samples were sent to GATC (Cologne, Germany) for sequencing through the LightRun Sanger Sequencing service. Most samples were prepared following the requirements of the service, but for those that were low on concentration even after attempts to concentrate them, the following volumes were used: 7.5 µl purified PCR template and 2.5 µl primer (primer stock concentration 10 µM).

5.3.3. Data analysis

Sequences were aligned with Geneious version 14 (Kearse, Moir et al. 2012). Mutations were searched by comparing sequences with a reference gene sequence from Schnabel and Jones (2001).

5.4. Results

5.4.1. Upstream region

There were only three isolates (AF10, AF15 and AF25) where the upstream region could be successfully amplified (Tables 5.1, 5.2).

Isolates AF10 and AF15 yielded fragments of approximately 1500 bp and isolate AF25 yielded a fragment of approximately 900 bp. These fragments were all larger than the 725 bp obtained by Schnabel and Jones (2001) using the same primers with an isolate with no apparent upstream insertions; therefore, it seems likely that all three isolates (AF10, AF15 and AF25) had insertions upstream the gene. However, neither of these isolates was resistant to myclobutanil or tebuconazole (Table 5.1).

5.4.2. *CYP51A1* sequence

A total of 15 isolates with varying levels of sensitivity were sequenced with primers designed from a reference sequence provided by Schnabel and Jones (2001), a further 14 isolates failed to amplify (Table 5.1). On the whole, sequenced isolates showed good correspondence with the reference (Figure 5.2). Although sequence quality tended to drop at the start and end of each sequence, the approach of sequencing from both ends of each half usually compensated for this effect so that bases differing from the reference located in

low quality areas could be contrasted against the complementary sequence for confirmation or rejection.

Most isolates showed no differences from the reference sequence; only two sensitive isolates from the baseline orchard showed the same silent mutation towards the end of the gene: G/A 1665.

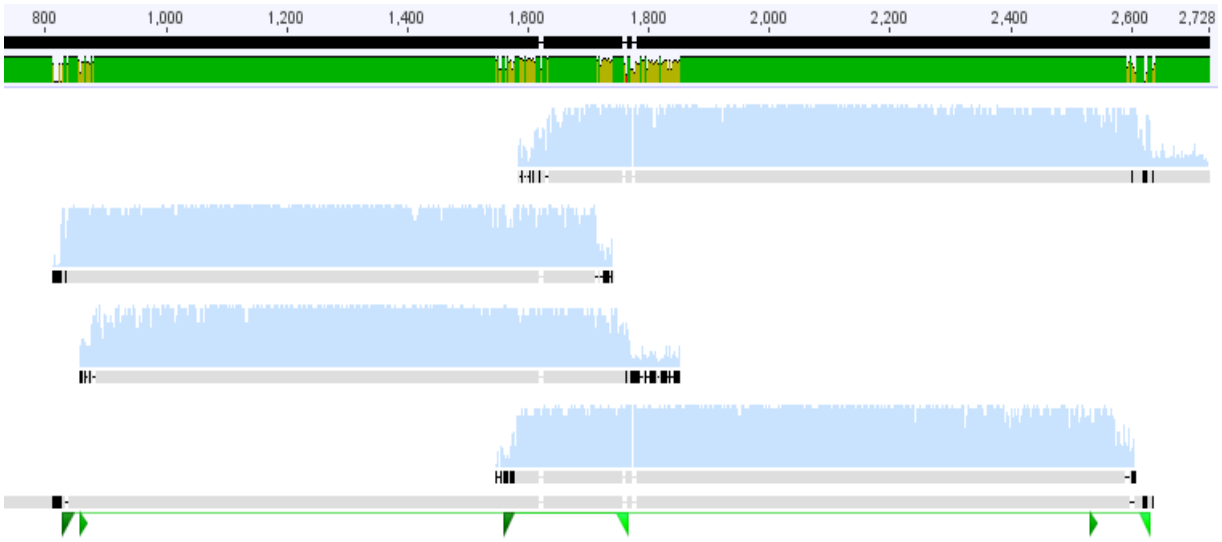


Figure 5.2. Alignment of the four sequences corresponding to the two halves of the gene as seen in Genious 14 (Kearse, Moir et al. 2012) . From the top: 3' end sequenced with primer 1559F, 5' end sequenced with primer 1764R, 5' end sequenced with primer 829F and 3' end sequenced with primer 2625R. Above in green are areas with high consensus with the reference sequence.

5.4.3. Mutations

Only one mutation was discovered in two isolates from the unsprayed orchard (Figure 5.3), AF15 and AF24. Both of these isolates had the G/A 1665 mutation. However, the change from an AAG codon to AAA does not affect the amino acid sequence, which will still have a lysine residue at that position.

This data coupled with those from experiments from other authors (Schnabel and Jones 2001; Villani, Hulvey et al. 2016) suggest that mutations in the *CYP51A1* sequence are not a common resistance mechanism in field isolates of *V. inaequalis*.

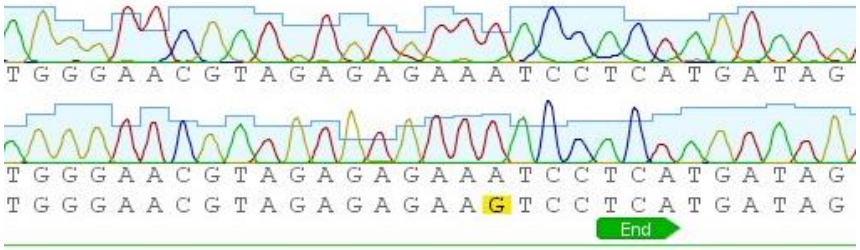


Figure 5.3. Position of the G/A 1665 mutation, with the wild-type reference in yellow - image taken with Geneious 14 (Kearse, Moir et al. 2012). Sequences from top to bottom: AF15 3' end sequenced from primer 1559F, AF15 3' end sequenced from primer 2625R, reference sequence from Schnabel and Jones (2001) (GenBank Accession Nos. AF227920, AF262756 and AF262757).

5.5. Discussion

This study is not the first of its kind to find no mutations in the gene sequence of *CYP51A1* gene, other researchers also failed to discover any alterations in the sequence of 15 and three isolates respectively (Schnabel and Jones 2001; Villani, Hulvey et al. 2016). This absence of changes strongly contradicts findings with other pathogens such as *Mycosphaerella graminicola* where a variety of alleles of *CYP51* conferring resistance has been observed (Mullins, Parker et al. 2011). There is a certain flexibility in the amino acid sequence of CYP51 across species, and this may account for this effect (Joseph-Horne and Holomon 1997). Some alterations are shared between some organisms, but others seem to be completely exclusive to a particular pathogen (Cools, Hawkins et al. 2013). As mentioned before, amino acids that are necessary for the activity or functionality of CYP51 in one species do not always have the same role in others. Examples of changes in the role of amino acids between species have been observed in resistance to other fungicide groups. In a case of fungicide resistance to QoI fungicides in *P. teres* and members of *Puccinia*, a substitution (G143A) in the fungicide target *cyt b* involved in resistance in other organisms was not present due to this amino acid being important for the removal of an adjacent intron, as it is part of an intron recognition signal, meaning that the mutation was not viable in these species as it interfered with the splicing process (Stammler, Miessner et al. 2013). A similar mechanism could be operating in *Venturia* with some amino acids susceptible to replacement in other fungi having an additional task that makes changing them detrimental in some way. Therefore, some modifications observed in other fungi could possibly result in non-functional proteins or proteins with reduced activity which may reduce the ability of an isolate to survive. In addition, for a resistance mutation to happen, it is sometimes necessary

for another change to happen within the protein that enables the mutation that generates resistance (Cools, Hawkins et al. 2013); therefore, it might not be the resistance mutation that has a deleterious effect but this other previous necessary change within the protein.

Whatever the real explanation, *CYP51A1* still seems to have a contribution to DMI resistance through its upstream region. Schnabel and Jones (2001) found three types of insertions upstream *CYP51A1*: one of them, of 553 bp, was located at position -64 and the other two, found at -319 bp, had a length of 225 and 500 bp. The insertion at -64 was found only in resistant isolates (nine out of 11 isolates in one orchard, with two other orchards having one resistant isolate with the insertion), and analyses indicated the possible existence of a promoter sequence within this insertion. However, the effect of this insertion on resistance has been challenged after Cox, Russo et al. (2008) found sensitive isolates with the insertion.

Originally, the plan had been to include the upstream region of the gene in the current study, using published primers AJ455 and AJ414, which had been one of the primers sets used to detect the insertions described above (Schnabel and Jones 2001). However, most of the isolates in the current study failed to amplify with this set of primers (Table 5.2), and a lack of sufficient upstream sequence in the GeneBank database to design good quality primers in the upstream area in combination with time constraints resulted in this approach being abandoned. The three isolates that successfully amplified in the current study (Table 5.1) may have contained insertions upstream the gene. When amplified with primers AJ455 and AJ414 two isolates (AF10 and AF15) yielded fragments of approximately 1500 bp and one isolate (AF25) of approximately 900 bp. Schnabel and Jones (2001) using the same primers obtained fragments of 725 bp, 949 bp, 1225 bp and 1278 bp; in the last three they found the insertions described above. Since the fragments obtained in this study are all

bigger than 725 bp, the fragment without insertions in Schnabel *et al.*, it seems likely that their greater length is due to the presence of insertions. However, all these isolates came from the unsprayed orchard and had $logED_{50}$ values for myclobutanil below 0.7 (Table 5.1), so any changes upstream the sequence for these three isolates do not correlate with higher resistance.

Other insertions have been found apart from those described by Schnabel and Jones (2001). Villani, Hulvey et al. (2016) found the same two insertions of approximately 225 bp (224 bp) and 500 bp (499 bp), Schnabel and Jones (2001) found at -319, although they reported them at -323. They also found one other insertion at -321 of 193 bp, and another isolate in their study had a region deleted and in its place it had an insertion which had a long section identical to part of the 499 bp fragment at -323. However, as Schnabel and Jones (2001) before them, they could not find any correlation between resistance and the fragments at -323 bp. The novel insertions at -321 and the one that replaced the deleted area had no links either, and this also held true for an insertion at -65 bp that had a promoter within its sequence. In the end, it was much farther upstream where they finally came upon an element correlating with resistance. The element, EL 3,1,2, was made up of different types of repeats found in a fragment at -379 bp, and contained binding sites for transcription factors. There were three types of repetitive short fragments - 42 bp, 67 bp and 60 bp – within EL 3,1,2 that were also found in other places in the sequence, either alone or forming part of a tandem repeat series. Even though EL 3,1,2 was associated with resistance, it was not an essential prerequisite as some resistant isolates did not have this element (Villani, Hulvey et al. 2016), adding to the hypothesis that there is more than one mechanism for resistance in *Venturia inaequalis*.

6. Gene control of DMI resistance

6.1. Introduction

The mechanisms involved in fungicide resistance in *V. inaequalis* are not entirely known. Candidates that could play a role in resistance development are transport proteins and changes in the expression or structure of the target demethylase protein (Jobin and Carisse 2007; Pfeufer and Ngugi 2012).

In apple scab, laboratory mutants have confirmed the possibility of transport systems involved in fungicide efflux playing a role (Vijaya Palani and Lalithakumari 1999; de Waard, Andrade et al. 2006). Studies in field isolates have found correlation with overexpression of the *CYP51A1* gene, although not all field isolates with resistance displayed this mechanism (Schnabel and Jones 2001; Villani, Hulvey et al. 2016), so there must be other pathways of resistance in the field.

Having a deeper understanding of the genetics of the resistance would improve our ability to predict how quickly the resistance would spread. One of the factors that affect how quickly resistance develops is the number of genes involved in the resistance. With more than one gene, resistance tends to evolve more slowly (Brent and Hollomon 2007).

Studies conducted in several species indicate that resistance mechanisms vary between different pathogens. For example, while some QTL studies support a quantitative resistance pattern in species like *Zymoseptoria tritici* (Lendenmann, D. et al. 2015), other crosses in other species, such as *Tapesia yallundae*, seemed to suggest that the resistance in those species was largely controlled by one gene, with only a minor contribution by other genes observed in some of the isolates with higher resistance (Dyer, Hansen et al. 2000). Carter,

Fraaije et al. (2014) found in some resistant isolates of *Pyrenopeziza brassicae* another gene with a major effect apart from *CYP15*, which was also found to have a major effect in this pathogen. And in *Zymoseptoria tritici*, Mavroeidi (2004) found epistatic effects (non additive) in a cross, with some progeny being more resistant than the resistant parent or more sensitive than the sensitive parent, with many more cases of the latter. This sensitivity pattern in the progeny suggests control by more than one gene and presence of epistatic effects, which is indicated by the asymmetry of the distribution.

A previous study in *V. inaequalis* pointed to a single-gene form of control. Stanis and Jones (1985) observed a 1:1 pattern in nine crosses involving resistant-sensitive pairs, resistant-resistant pairs and sensitive-sensitive pairs. However, only one discriminatory dose was used, and isolates were considered resistant or sensitive depending on whether they were able to grow with the fungicide present. This assessment method does not take into consideration the continuum of phenotypes found in the field, restricting the classification to only growth or no growth, which is a very broad classification given the existing diversity, and necessarily cannot reveal control by multiple loci. As such, a study based on more doses would better reflect the gradual shift of fungicide sensitivity observed in the field and be a more comprehensive method to study gene control.

There are different methods to study gene control from those that depend on crosses to others based on sequencing or gene expression. Crosses can be used to determine the type of gene control through the distribution of phenotypes in the progeny and can also help identify important genomic regions, known as quantitative trait loci (QTL), when combined with genotyping with molecular markers. This method does not require knowledge of genome sequences, only to have a high enough number of the progeny to be able to

differentiate between phenotypes and a range of markers distributed across the genome. Genome sequencing can also help through comparisons with other species where mutations or changes associated with resistance are known, or by comparison of genomes from resistant isolates with genomes from sensitive phenotypes in search of differences. Another approach that uses whole genomes are the GWAS (Genome Wide Association Studies) that use genomic sequence and SNP arrays to find SNPs linked with a given trait (Norrgard 2008; Witte 2010). When a gene or gene-family is known to contribute to resistance in another species, such as *CYP15*, these genes can be sequenced in search of alterations in the studied species; however, concentrating in a group of genes that had been found to be important in another pathogen may risk missing other genes that are also involved. Gene expression can also be used to identify important genes through methods such as microarrays, although these only detect genes responding to the fungicide which does not imply it is important for resistance. Methods relying on gene expression cannot pick up changes involved in resistance where the structure of a protein changes, but not its expression pattern, such as an amino acid substitution that interferes with binding.

6.2. Objectives

This chapter seeks to determine the number of genes involved in resistance to DMI fungicides in *V. inaequalis* by phenotyping the progeny of a cross between a sensitive and a resistant isolate, and then using classical genetic methods to estimate the number of genes involved.

6.3. Materials and methods

6.3.1. Experimental design

Six of the most sensitive isolates (AF28, AF24, AF10, AF3, AF15 and AF4) and six of the most resistant isolates (09/030i, C3, 09/037-2, Spartan 1, 09/034-4 and G1) were chosen from the 40 isolates tested in Chapter 4, using ED_{50} values to evaluate their sensitivity level. Crosses were conducted following the design presented in Table 6.1. All crosses were replicated once.

Table 6.1. Crossing design.

Sensitive Parent [1]	Resistant Parents [2]	Crosses
AF28	09/030i	AF28 x 09/030i
	C3	AF28 x C3
	09/037-2	AF28 x 09/037-2
	Spartan 1	AF28 x Spartan 1
	09/034-4	AF28 x 09/034-4
	G1	AF28 x G1
AF24	09/030i	AF24 x 09/030i
	C3	AF24 x C3
	09/037-2	AF24 x 09/037-2
	Spartan 1	AF24 x Spartan 1
	09/034-4	AF24 x 09/34-4
	G1	AF24 x G1
AF10	09/030i	AF10 x 09/030i
	C3	AF10 x C3
	09/037-2	AF10 x 09/037-2
	Spartan 1	AF10 x Spartan 1
	09/034-4	AF10 x 09/034-4

Sensitive Parent [1]	Resistant Parents [2]	Crosses
AF10	G1	AF10 x G1
AF3	09/030i	AF3 x 09/030i
	C3	AF3 x C3
	09/037-2	AF3 x 09/037-2
	Spartan 1	AF3 x Spartan 1
	09/034-4	AF3 x 09/034-4
	G1	AF3 x G1
AF15	09/030i	AF15 x 09/030i
	C3	AF15 x C3
	09/037-2	AF15 x 09/037-2
	Spartan 1	AF15 x Spartan 1
	09/034-4	AF15 x 09/034-4
	G1	AF15 x G1
AF4	09/030i	AF4 x 09/030i
	C3	AF4 x C3
	09/037-2	AF4 x 09/037-2
	Spartan 1	AF4 x Spartan 1
	09/034-4	AF4 x 09/034-4
	G1	AF4 x G1

[1] Sensitive parents had ED_{50} values for tebuconazole of: 0.85 mg/L (AF3), 0.74 mg/L (AF10), 0.64 mg/L (AF4), 0.12 mg/L (AF28), 0.28 mg/L (AF24) and 0.14 mg/L (AF15)

[2] Resistant parents had ED_{50} values for tebuconazole of: 18.3 mg/L (C3), 13.72 mg/L (09/030i), 4.97 mg/L (09/037-2), 3.36 mg/L (Spartan 1), 3.43 mg/L (09/034-4) and 3.03 mg/L (G1).

6.3.2. Experimental procedure

6.3.2.1. Preparing Apple Leaf Malt Extract Agar

Senescent leaves were collected in autumn (10th November); 50 g of leaves were weighed and boiled for 15 minutes in 1000 ml of deionised water. The decoction was then filtered through muslin and water was added to top up to 2000 ml. After this, 2 g of Malt Extract (Oxoid) and 6.8 g of agar (Agar technical no. 3, Oxoid) were added to five duran bottles, followed by 400 ml of the decoction. The media was then autoclaved and plates were poured.

6.3.2.2. Crossing isolates and picking spores

Mycelium plugs (4 mm) were taken from each of the two isolates for each cross and placed together in a petri dish, leaving around 2 cm distance between them.

They were left to grow at room temperature for five weeks at approximately 20 °C, and then transferred to cold store at 4 °C, where they were kept for six months. After this period, crosses were transferred to 20 °C to induce ascospore release. From the successful crosses, one sensitive-resistant cross was chosen (AF28 x Spartan 1), using the sensitivity levels of the parents, low level of sensitivity for the resistant parent (ED_{50}=3.36 mg/L) and high level of sensitivity for the sensitive parent (ED_{50}=0.12 mg/L) as criteria. Two hundred ascospores were then picked following the protocol detailed in Chapter 2.

6.3.2.3. Testing isolates for fungicide resistance

A total of 150 ascospores were maintained in PDA media amended with Rifamycin (0.2 ppm; Sigma-Aldrich) for testing. Of those, only 81 spores were tested due to losses of plates to

contamination. Parental isolates were also tested to determine loss of sensitivity after being continuously subcultured for a long period. Tebuconazole was selected as the chemical for the test, and four fungicide concentrations (4 mg/l, 1 mg/l, 0.1 mg/l and 0.01 mg/l) and a control without the fungicide were tested. Each concentration was replicated once and two plugs were used per plate. After three weeks, two perpendicular diameters were measured per plug.

6.3.3. Data Handling

A cumulative frequency plot of the $logED_{50}$, including both the ascospore progeny and the parental isolates, was obtained to determine the type of gene control. A histogram and a Q-Q plot of the $logED_{50}$ values, the latter compares an experimental distribution to the distribution expected if the experimental data followed a certain distribution type (in this case, a normal distribution), were also obtained. All statistical analyses were carried out with Genstat 16 (Payne, Murray et al. 2013).

6.3.4. Estimating number of genes

To estimate the number of genes involved in the resistance, graphs of the expected cumulative distribution of the progeny under one, two and three additive gene control were plotted. The expected distributions were constructed by recalculating the cumulative relative frequency of the $logED_{50}$ of each spore through a series of equations.

Each point in the new distributions is computed taking into account that for one locus there would be two phenotypes, with two loci three phenotypes and with three loci four phenotypes. However, in reality all spores within a given phenotype group would not have the same $logED_{50}$ due to errors between replicates, giving rise to a continuous distribution.

So around each phenotype there would be a normal distribution of points with a standard deviation equal to the error between replicates. The mean of the distribution for each phenotype would be the mean $logED_{50}$ of that phenotype; in the calculations below, this mean is expressed relative to the mean of the whole $logED_{50}$ distribution, which includes all the phenotypes for a given number of loci. For example, for one locus the mean of the sensitive and resistant phenotypes would be at a distance of ½ from the mean of the whole distribution, so the mean of the resistant phenotype can be expressed as $\mu + \frac{1}{2}d$, with μ the mean of the entire distribution, and the mean of the sensitive phenotype would be $\mu - \frac{1}{2}d$.

Depending on the number of loci, the cumulative distribution is therefore calculated through the following equations which give the probability density at each $logED_{50}$ (x):

One locus:

$$\frac{1}{2}\left(N\left(\mu - \frac{1}{2}d, s\right) + N\left(\mu + \frac{1}{2}d, s\right)\right)(x)$$

Two loci:

$$\frac{1}{4}\left(N\left(\mu - \frac{1}{2}d, s\right)\right) + \frac{1}{2}(N(\mu, s)) + \frac{1}{4}\left(N\left(\mu - \frac{1}{2}d, s\right)\right)(x)$$

Three loci:

$$\frac{1}{8}\left(N\left(\mu - \left(3 * \frac{1}{6}\right)d, s\right)\right) + \frac{3}{8}\left(N\left(\mu - \frac{1}{6}d, s\right)\right) + \frac{1}{8}\left(N\left(\mu + \left(3 * \frac{1}{6}\right)d, s\right)\right) + \frac{3}{8}\left(N\left(\mu + \frac{1}{6}d, s\right)\right)(x)$$

The probability for a given phenotype are ½ for both sensitive and resistant phenotypes (one locus); ¼ for the sensitive phenotype, ½ for the intermediate phenotype and ¼ for the resistant phenotype (two loci) and 1/8 for the resistant and sensitive phenotypes and 3/8 for the two possible intermediate phenotypes (three loci). The N stands for a normal distribution where the mean is the mean of each phenotype and the standard deviation is a measure of the error between replicates. As mentioned above, means for each phenotype are calculated relative to the mean of the whole sensitivity distribution (μ). The mean of the actual distribution of the progeny ($logED_{50}$) was used as μ for this purpose. The d parameter represents the distance of a given phenotype from the mean. For the standard deviation, s comes from the root square of the variance of the standard errors of the $logED_{50}$ estimates.

The value of d can be obtained through the following equations:

One locus:

$$d = \sqrt{4\ (\sigma_{tot}^2 - \sigma_{rep}^2)}$$

Two loci:

$$d = \sqrt{8\ (\sigma_{tot}^2 - \sigma_{rep}^2)}$$

Three loci:

$$d = \sqrt{12\ (\sigma_{tot}^2 - \sigma_{rep}^2)}$$

Where σ_{tot}^2 is the variance of the $logED_{50}$ values and σ_{rep}^2 has been taken as the variance of the standard errors of the $logED_{50}$ estimates.

The expected cumulative relative frequencies for the different gene scenarios were then plotted against the $logED_{50}$ values (the values estimated from the progeny data). The new distributions were compared to the actual cumulative distribution from the progeny data through a one-sample Kolmogorov-Smirnov test to determine if the progeny data adjusted well to any of the gene scenarios.

6.4. Results

6.4.1. Distribution of sensitivities to DMI fungicide tebuconazole

The graph of the cumulative distribution of the sensitivity levels of 81 spores, which also includes the two parental isolates (Figure 6.1), shows a continuous distribution with no apparent discontinuities allowing a division into distinct classes, indicating a case of polygenic control of the character in the progeny of the AF28 x Spartan 1 cross. This was further supported by the Q-Q plot (Figure 6.2) and histogram (Figure 6.3). Interestingly, 8.64% of isolates were more resistant than the resistant parent, Spartan 1, and around 37% were more sensitive than the sensitive parent, AF28 (Figure 6.1). In a single-gene scenario, the extremes marked by the parental isolates should only be exceeded by the error estimate for the $logED_{50}$, and therefore there would be fewer isolates with $logED_{50}$ values beyond the parents. Parental isolates presented higher ED_{50} values after being tested again, probably due to variability between the tests, which were 4.32 mg/L for the resistant parent (Spartan 1) and 0.28 mg/L (AF28), and are the parent values that appear in Figure 6.1.

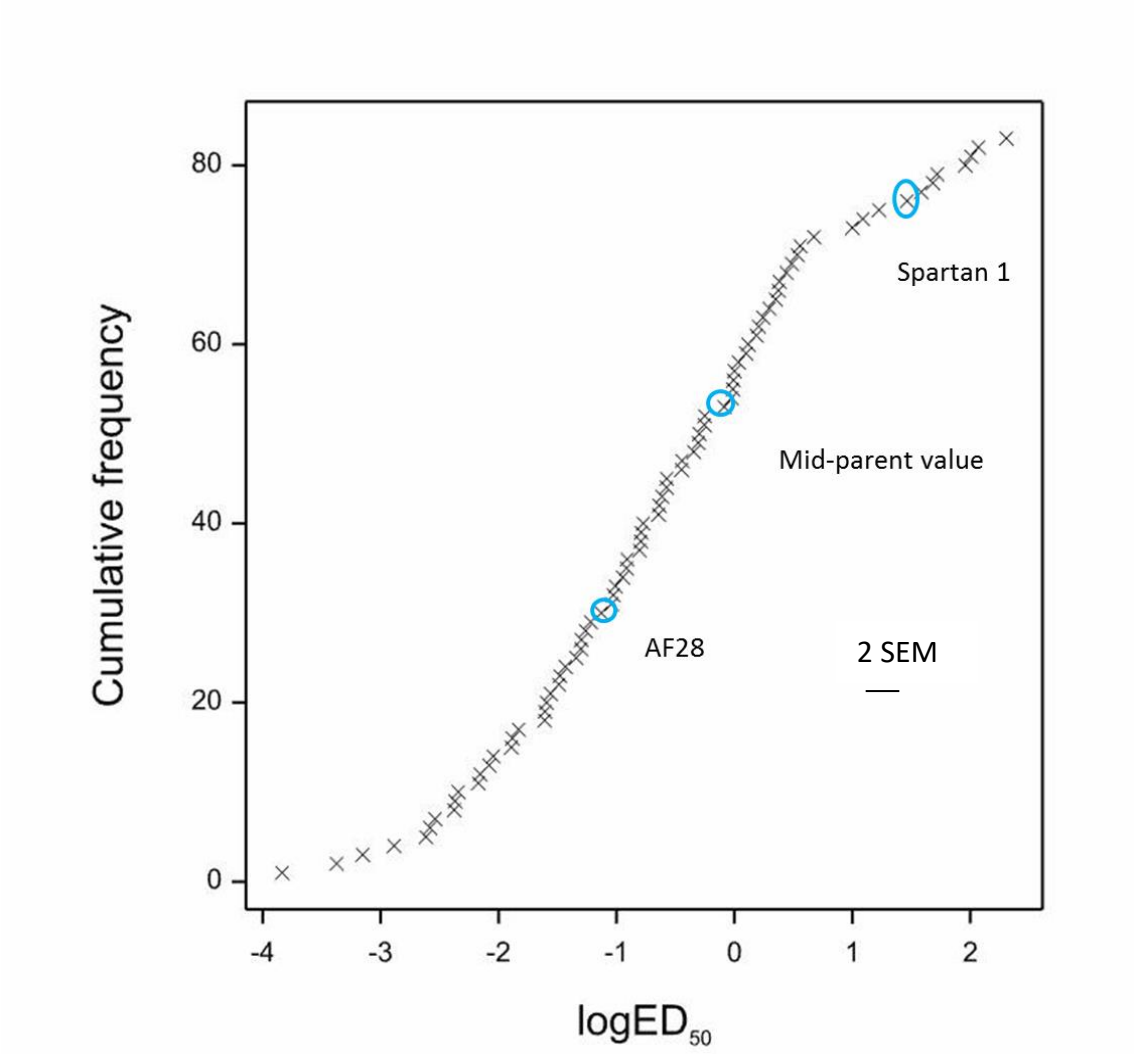


Figure 6.1. Cumulative distribution of the sensitivity levels of the AF28 x Spartan 1 progeny. The sensitivity levels of the sensitive parent, AF28, the resistant parent, Spartan 1, and the mid-point of sensitivity between parents have been marked with a circle. A floating error bar indicates 2SEM (standard error of the mean).

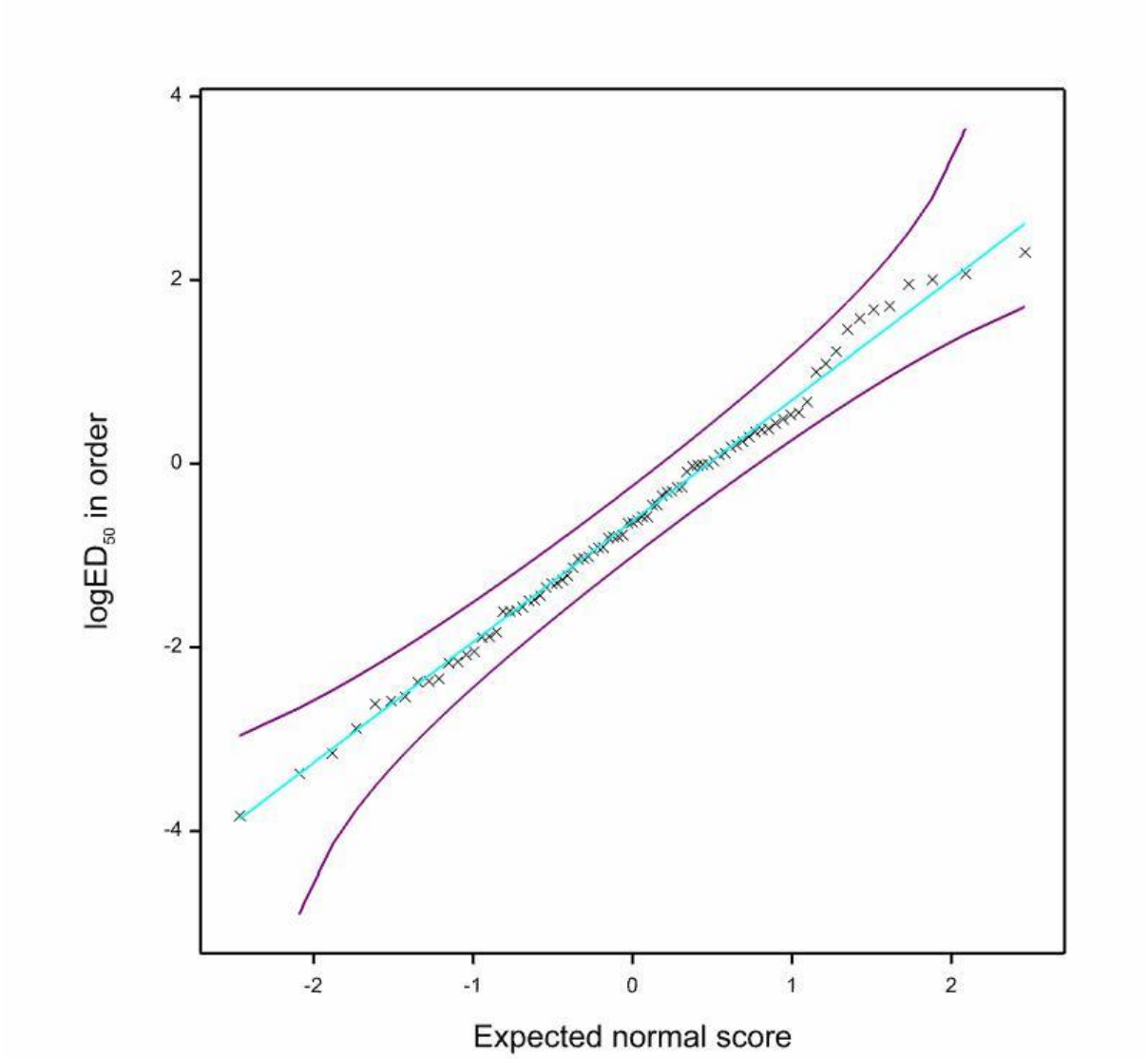


Figure 6.2. Q-Q plot of the $logED_{50}$ values of the progeny of the AF28 x Spartan 1 cross. The straight line indicates a normal distribution of the $logED_{50}$ data. The purple lines define a 95% confidence interval, a true normal sample would fall within this interval 95% of the time.

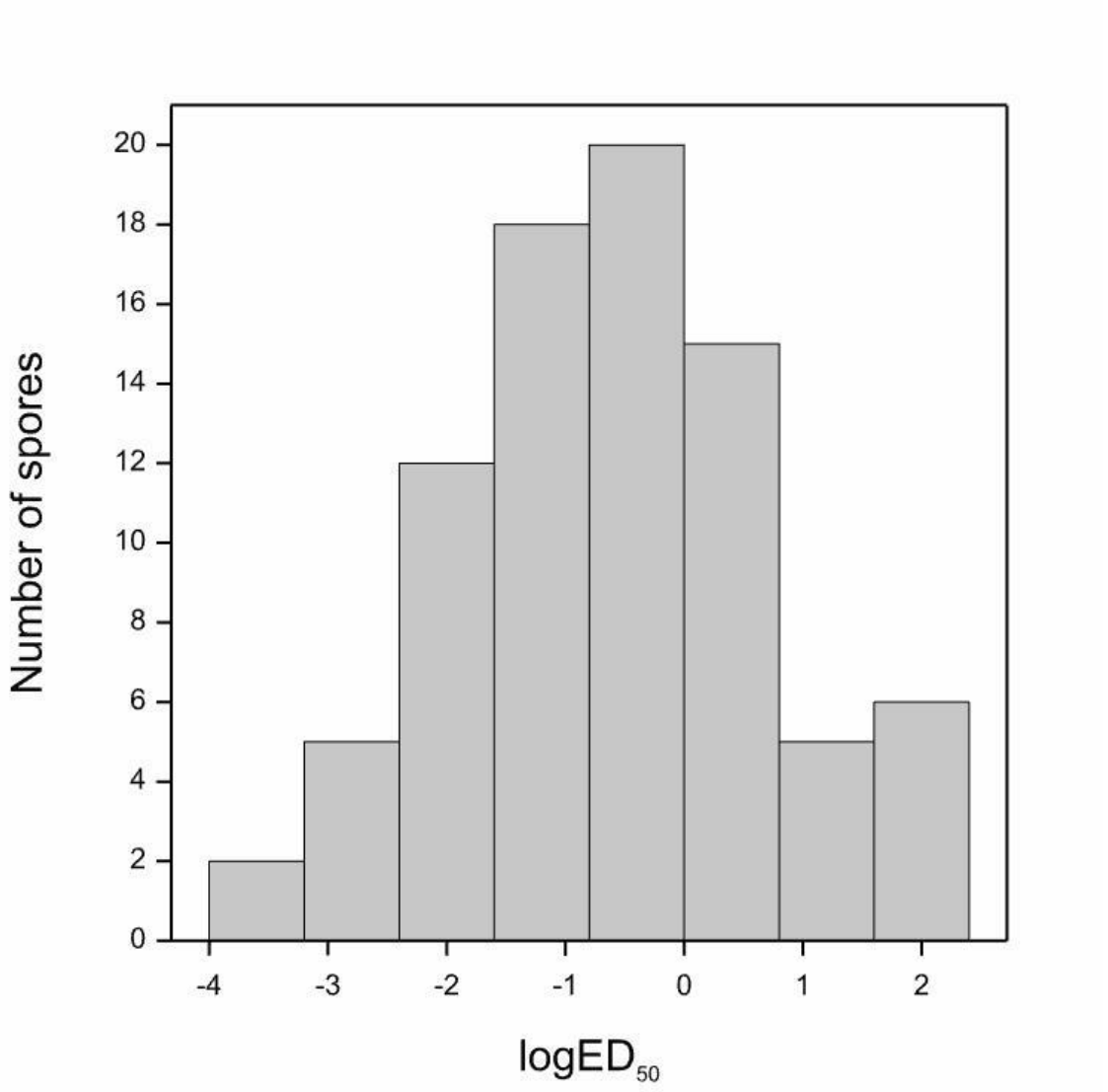


Figure 6.3. Distribution of the $logED_{50}$ data of the AF28 x Spartan 1 progeny. The bell-shaped distribution of the data is approximately that of a normal distribution.

6.4.2. Estimating number of genes

If all genes had major effects, only acted additively and contributed equally to the sensitivity, the shapes of the cumulative distribution graph that would have been expected, keeping the same sensitivity range and number of ascospores as those in this study, are detailed in

Figures 6.4 to 6.6. The shape of the cumulative distribution of the ascospore progeny in this study clearly does not resemble those for one gene; a one-sample Kolmogorov-Smirnov test found that the distribution in the progeny data did not fit the distribution under the one-gene scenario ($p<0.01$) . The two-gene and three-gene scenario are possible ($p>0.01$).

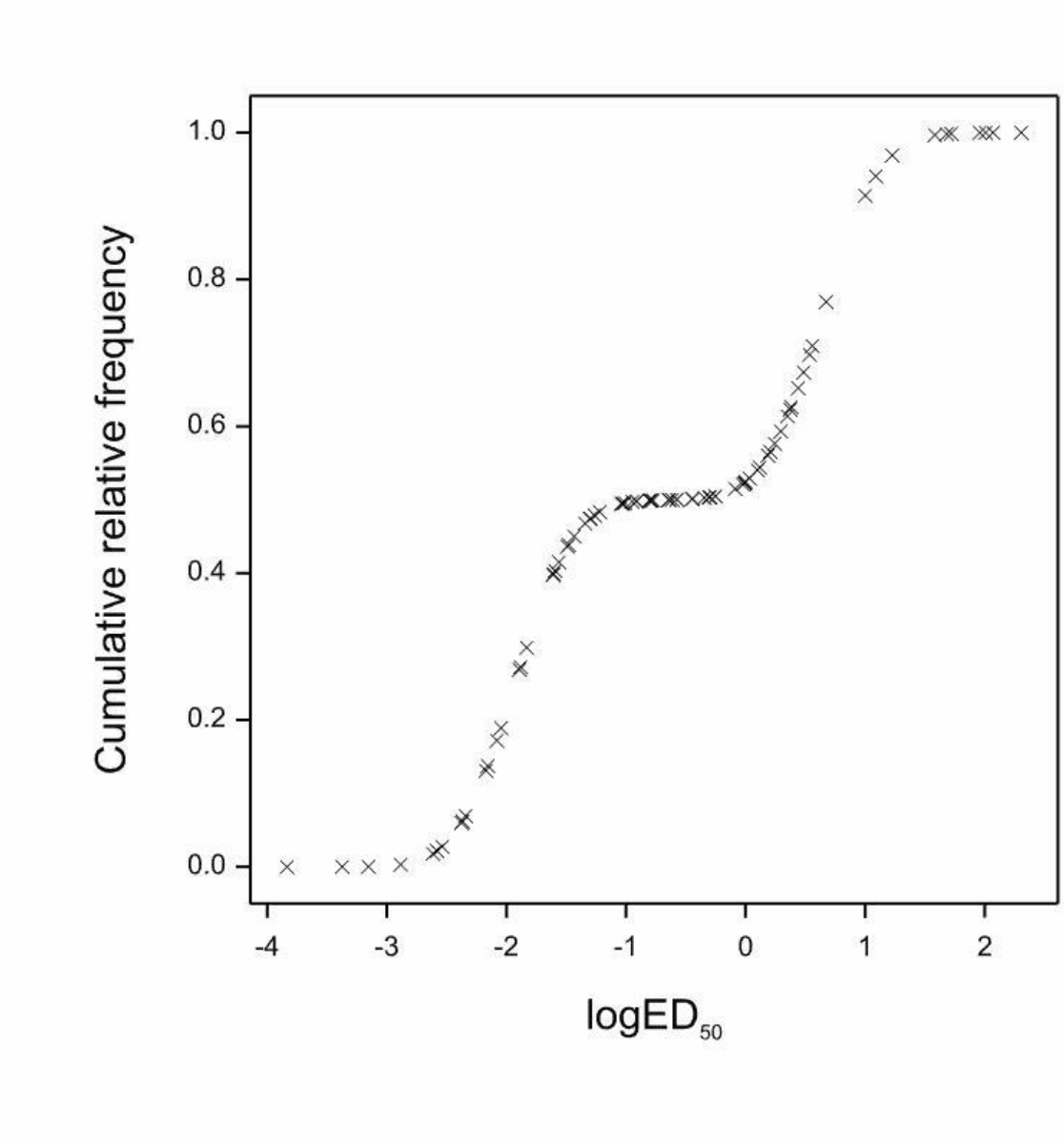


Figure 6.4. Cumulative distribution pattern expected if the trait was controlled by a single gene.

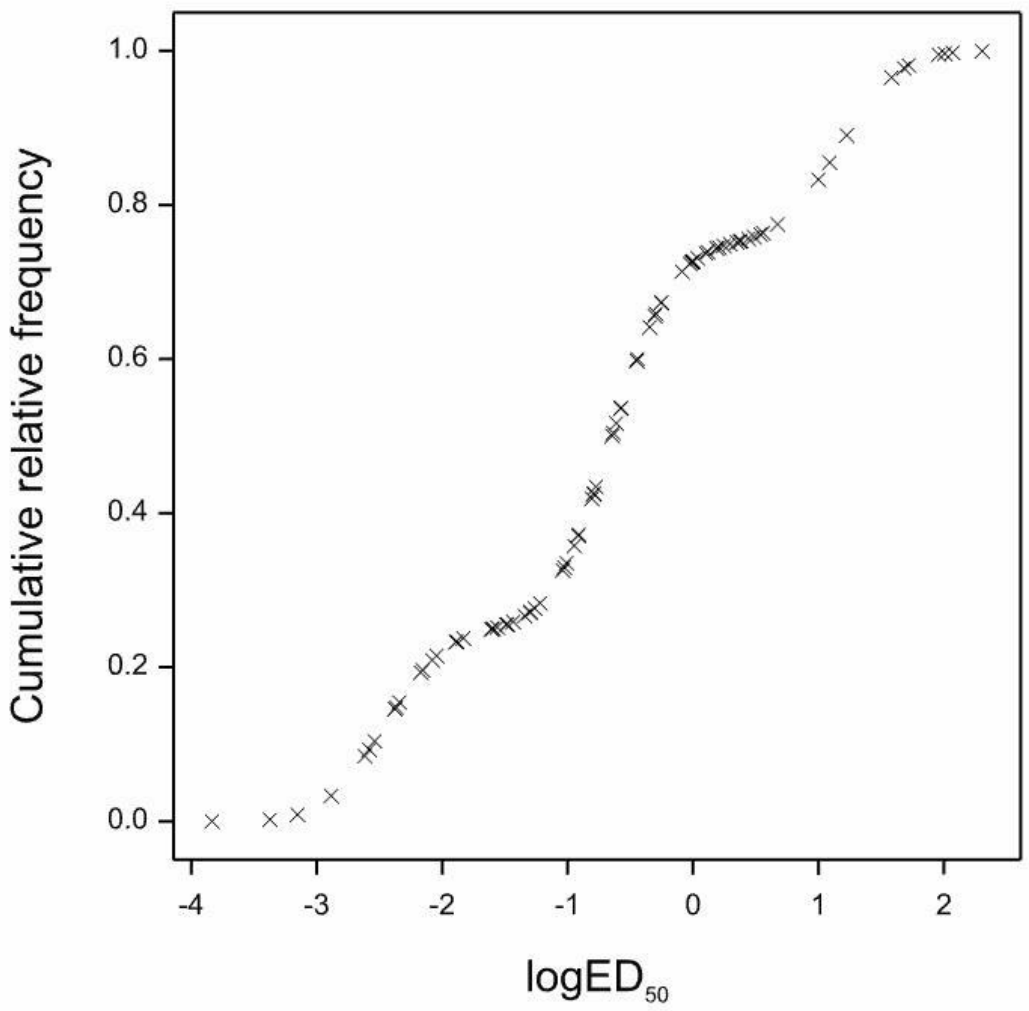


Figure 6.5. Cumulative distribution pattern expected if the trait was controlled by two additive genes.

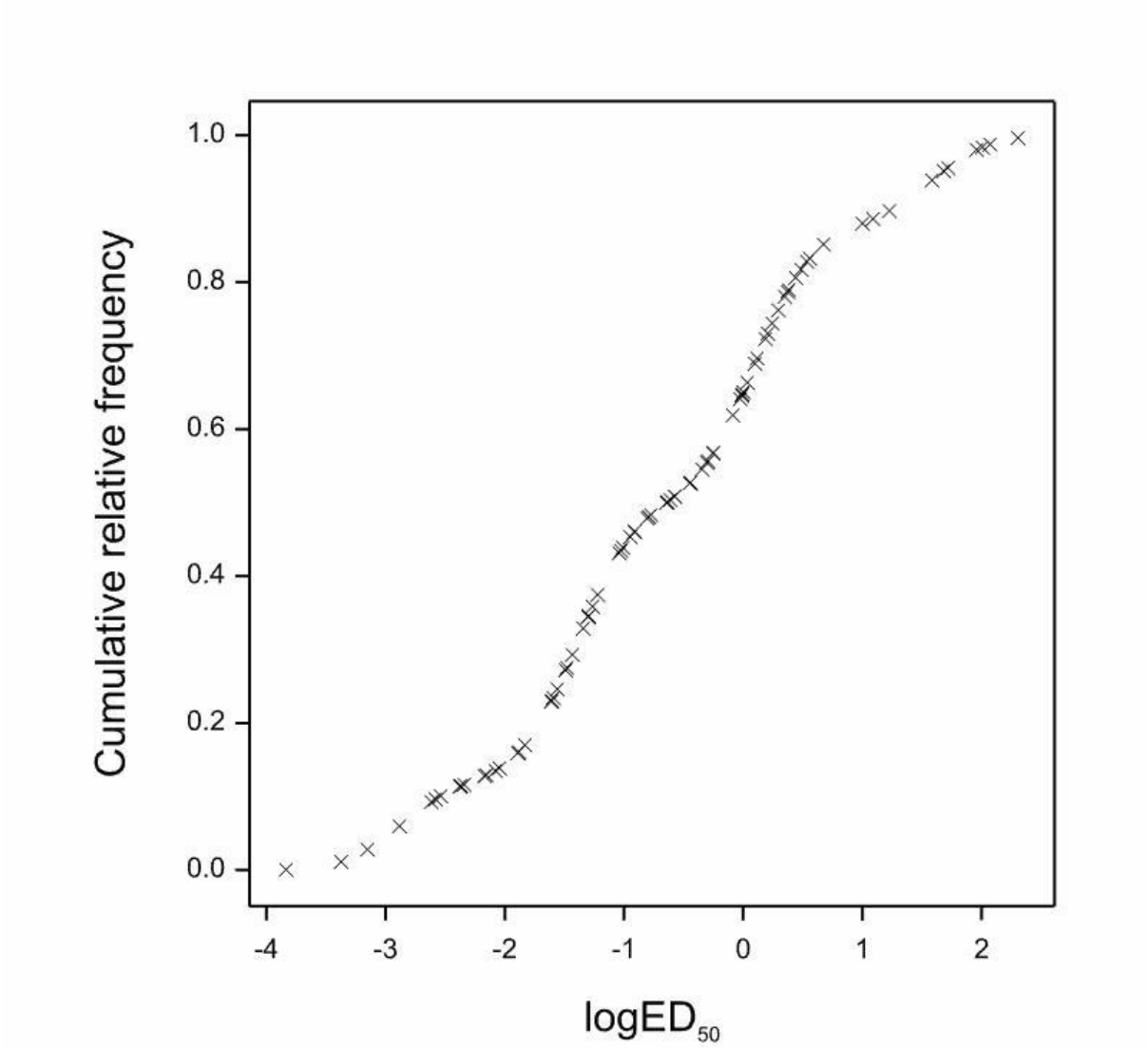


Figure 6.6. Cumulative distribution pattern expected if the trait was controlled by three additive genes.

6.5. Discussion

Resistance to DMI fungicides is probably under polygenic control (Figure 6.1) in at least some isolates of apple scab. From the other fungicide families used to control scab, complete resistance to benzimidazoles and strobilurins is known to be qualitative (Table 6.2),

although cases of partial resistance to strobilurin fungicides have also been reported to be quantitative (Koller, Parker et al. 2004). However, reports of both types of resistance (qualitative and quantitative) in a pathogen are not common, as qualitative resistance appears and spreads more quickly than quantitative resistance, and therefore is the one that is usually detected and reported. By comparison to these cases of mostly qualitative resistance, *V. inaequalis* seems to have quantitative resistance against other types of fungicide such as dodine (Table 6.2). Resistance to AP fungicides (Beresford, Follas et al. 2014) and SDHI fungicides (FRAC 2015) have been reported, but the mechanisms responsible for resistance to these classes of fungicide are still being studied, in the case of the SDHI fungicides it seems mutations in the *sdh* genes are involved (FRAC 2015).

An asymmetrical distribution in the progeny with levels of sensitivity beyond those of the parent isolates, with this effect being more pronounced on one of the side of the distribution, could indicate the existence of epistatic effects. It is worth noting that in the progeny distribution of the current study (Figure 6.1) there are more offspring with greater sensitivity than the sensitive parent than offspring more resistant than the resistant parent. This could be due to a gene combination which increased sensitivity that was not found in either of the two parents forming in some of the progeny (Table 6.3 and Figure 6.7). In additive scenarios, progeny with $\log ED_{50}$ values beyond those of the parents would also appear, but would be symmetrical with similar proportions on both sides.

Table 6.2. Resistance to different chemical families in apple scab.

Fungicide family [1]	Known resistance type	Mechanism
Dodine	Quantitative [2]	Not known
Benzimidazoles	Qualitative	Mutations in beta tubulin [3] gene
DMIs	Quantitative	Not known Overexpression of *CYP51A1* [4] very likely to play a role Transporters are another possibility [5]
Strobilurins	Qualitative and quantitative [6]	Mutations in *cytb* [6]

[1] The AP and SDHI fungicide families are not included in this table as there is still not much information about resistance control in these families; it seems that in the case of the SDHI family the *sdh* gene is involved (FRAC 2015).

[2] (Cox 2015).

[3] (Koenraadt, Somerville et al. 1992).

[4] (Schnabel and Jones 2001; Villani, Hulvey et al. 2016).

[5] (Vijaya Palani and Lalithakumari 1999).

[6] (Koller, Parker et al. 2004).

Table 6.3. Example of an asymmetrical cumulative distribution of the sensitivity phenotypes for a resistance trait controlled by four biallelic genes (2^4= 16 genotypes). Resistance alleles are marked as + or $+_A$ depending on the gene position (+ alleles are found in loci 2 and 4, and $+_A$ alleles in loci 1 and 3). Sensitive alleles are marked as – and contribute 0 points to the resistance. All + alleles contribute the same amount to the resistance (+1); a $+_A$ allele does not contribute if it is on its own, but together with another $+_A$ allele each contributes +1 so a $+_A$ $+_A$ combination would contribute a total of 2 points to the resistance . Taking these rules into consideration, the example cross has more progeny with a phenotype more sensitive than the sensitive parent than progeny with a phenotype more resistant than the resistant parent.

Parent isolates	Progeny	Progeny phenotype values
+A-+A+	----	0
Resistant	+A---	0 (allele +A on its own does not contribute)
(Phenotype	--+A-	0 (allele +A on its own does not contribute)
value = 3)	+A+--	1
	--+A+	1
-+--	---+	1
Sensitive	-+--	1
(Phenotype	-++A-	1
value = 1)	+A--+	1
	-++A+	2
	+A-+A-	2
	-+-+	2
	+A+-+	2
	+A - +A +	3
	+A++A-	3
	+A++A+	4

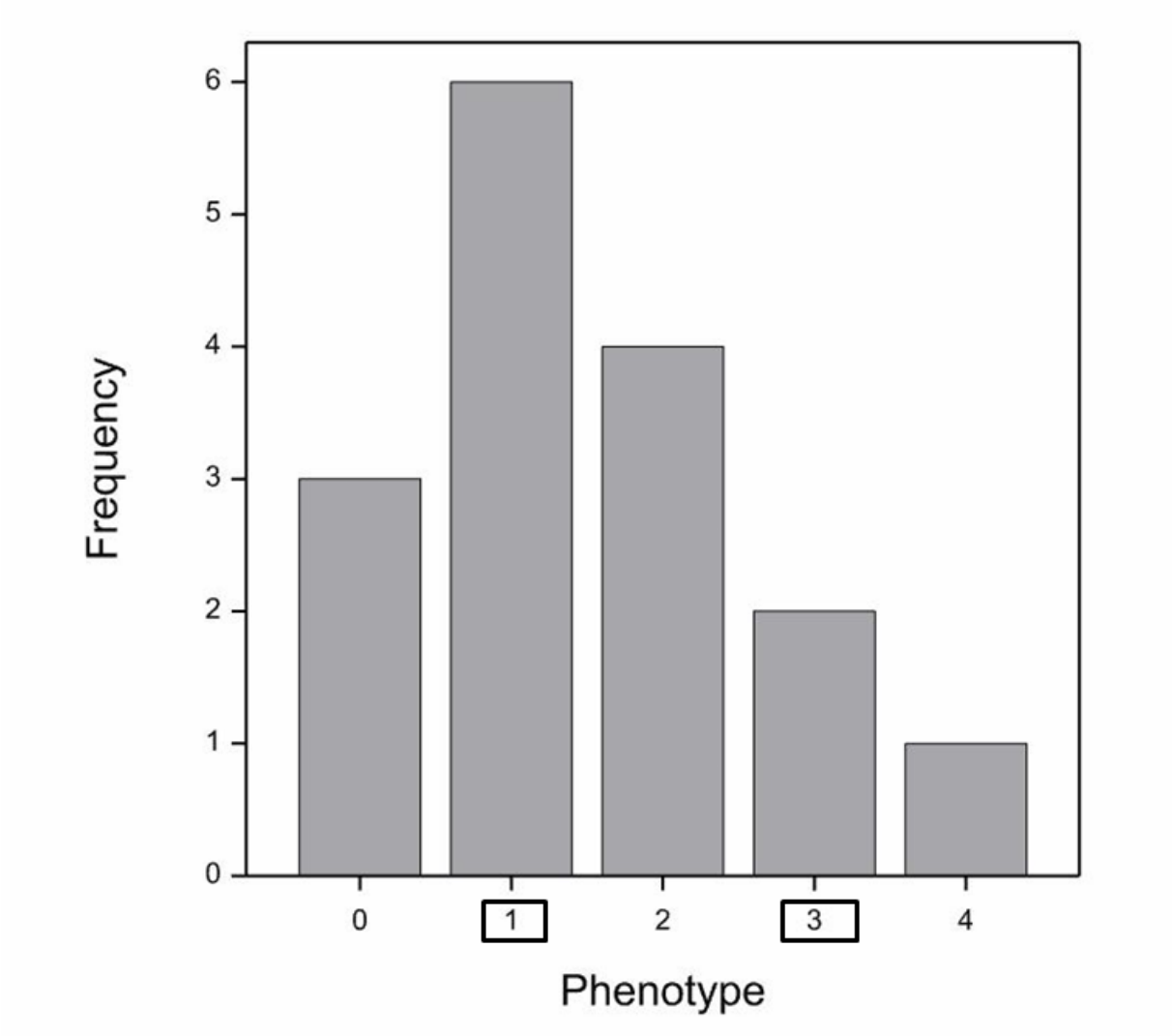


Figure 6.7. Distribution of the different phenotypes from Table 6.3. The phenotypes of the sensitive (1) and resistant (3) parent isolates are in rectangles.

In this chapter, the number of genes involved could not be reliably estimated. At this stage, it can only be said that the number is higher than one gene. In the QTL study in *Zymoseptoria tritici* (Lendenmann, Croll et al. 2015), genome areas containing QTLs linked to resistance contained from sixteen genes to 370 candidate genes. Genetic variability seemed to account for most of the variance between the progeny of the crosses in their study, and out of nine possibly connected QTL, three held about 34% of the additive variability.

From the current knowledge available about resistance mechanisms, it is possible to speculate what the most likely candidate genes are. Apart from the more evident upstream changes in the *CYP51A1* sequence (Schnabel and Jones 2001; Villani, Hulvey et al. 2016), other proteins in the biosynthesis of ergosterol could be involved. A study of QTLs in *Zymoseptoria* found the gene encoding for ERG6, a methyltransferase in the ergosterol biosynthesis route, was a good option (Lendenmann, Croll et al. 2015). In *Ustilago maydis*, a desaturase protein within the ergosterol synthesis route was capable of changing this pathway in a way beneficial to resistance (Joseph-Horne, Manning et al. 1995).

In the same analysis of QTLs in *Zymoseptoria* where ERG6 was designated as a potential candidate, the gene *PSK1* also came out as a possibility (Lendenmann, Croll et al. 2015). This protein is a polyketide synthase and has a role in melanisation. In *Cercospora beticola*, a group of genes of this family were expressed by a resistant isolate when the DMI tetraconazole was present, but not in a sensitive isolate (Bolton, Ebert et al. 2016). Melanin has been believed to be able to adhere to fungicide chemicals, which could possibly prevent them from reaching their target (Lendenmann, Croll et al. 2015). Another protein found in the QTLs of *Zymoseptoria* was an ABC transporter (Lendenmann, Croll et al. 2015). A member of the biosynthesis of phospholipids was suggested to also contribute; perhaps counteracting the fungicide effect on membrane characteristics (Lendenmann, Croll et al. 2015).

More research should be done to investigate the role of transporters in the resistance, as some laboratory mutants of *V. inaequalis* have been found to present this mechanism (Vijaya Palani and Lalithakumari 1999). It would be interesting to see if field isolates also had this mechanism, since in some other pathogen such resistant strains have not been found

(de Waard, Andrade et al. 2006). Transporters could also be important in cross-resistance related processes as some of these proteins allow the detoxification of several types of molecules, making them a likely mechanism of cross-resistance across fungicide classes (de Waard, Andrade et al. 2006). Apart from these genes, there could be compensatory changes at different loci that are necessary for the mutations that produce resistance to be viable.

In terms of the development of resistance, the present findings could point to an scenario where the resistance evolves more slowly because more changes are necessary in order to get high levels of resistance (Georgopoulos and Skylakakis 1986). In this type of multi-locus evolution all intermediate steps need to be advantageous so if at one point a step involves a fitness penalty, a delay in the evolution occurs as a compensatory mutation is needed before the next change can take place. This could partly explain why resistance has taken so long to develop in comparison to other chemical families. This does not rule out the existence of non-polygenic forms of control as other chemical families, such as the strobilurins, have demonstrated that both types of control are possible to find in the same pathogen (Koller, Parker et al. 2004). However, when the two pathways exist in the same pathogen, the single-gene option is likely to be more common because it involves less danger of incurring in a fitness penalty since only one change is necessary to confer resistance in contrast to multiple changes. It does seem as though generally there are more cases of single-gene resistance in plant pathogens possibly due to this reason, but also due to the fact that single-gene resistance emerges more quickly, having more chances to become widespread by the time cases of quantitative resistance appear, and is easy to study and detect since it involves a single change.

7. Effect of different factors on resistance evolution

7.1. Introduction

Fungicide resistance in *V. inaequalis* could have significant consequences for farmers due to a limited range of effective fungicides in the market, which means that it is necessary to monitor the situation so that it never comes to a point where the fungicides in use lose their efficacy and no substitutes are available (Chapman 2011). To be able to use fungicides more sustainably, anti-resistance strategies need to be implemented. But in order to draw up effective anti-resistance plans, we must first understand what elements are important for the spread of the resistance.

The spread of fungicide resistance is governed by principles that can be divided into two categories: mutation-related and selection pressure-related (Georgopoulos and Skylakakis 1986). The mutation-related aspect depends on the effect of the mutation itself on the dynamics of the pathogen-fungicide relationship and if the change is associated with a fitness cost that alters the probability of a resistant isolate to do well in the environment relative to sensitive (wild type) isolates (Georgopoulos and Skylakakis 1986). The selection pressure includes a series of factors that drive the phenotype of a less sensitive strain to grow in numbers. Some of the forces behind the selection pressure in the case of fungicide resistance are: the fungicide efficacy, the rate of degradation of the fungicide, the coverage of the fungicide and the frequency of the sprays (Kable and Jeffrey 1980; Skylakakis 1982; Georgopoulos and Skylakakis 1986; Chin 1987; Brent and Hollomon 2007; Hollomon 2015).

With some knowledge about these two elements (mutation-related and selection pressure-related), we can attempt to predict the evolution of the resistance. To achieve this, we must

develop mathematical models supported by the biology of the fungicide-pathogen interaction and available experimental data (Van den Bosch, Fraaije et al. 2015). Much of the mathematical modelling work published in the fungicide resistance field centres on the impact on resistance of fungicide mixtures and fungicide alternation or studies the selection phase of the resistance (Kable and Jeffrey 1980; Hobbelen, Paveley et al. 2011; van den Bosch, Paveley et al. 2011). Recently some research has attempted to delve into the emergence stage of the fungicide resistance process (Hobbelen, Paveley et al. 2014), and there have been papers with predictions tested against experimental data (Hobbelen, Paveley et al. 2011).

When designing a model that predicts the behaviour of fungicide resistance, both the mutation-related aspects and the selection pressure forces should be reflected in the model. For the mutation-related aspect, experimental data gathered on apple scab during the course of this PhD suggest polygenic resistance against tebuconazole. Polygenic resistance has been modelled before by other authors, although usually using broad pathogen definitions instead of being designed for an specific pathogen (Via 1986; Shaw 1989; Shaw 2000).

Other pathogen traits can also affect the evolution of resistance. It is known that shorter latency, greater aggressiveness and greater spore production all increase the rate of evolution (Skylakakis 1982; Brent and Hollomon 2007). The mode of spore dispersal also has an effect with dispersal over long distances speeding up the evolution (Brent and Hollomon 2007).

Regarding the effect of environmental conditions on the resistance, information of variability of spray depositions in apple trees is available (Xu, Wu et al. 2006). Leaf data from sprays

with a metal tracer indicated a great variability of spray deposition, with a coefficient of variation going from 46.1 to 66.6% (Xu, Wu et al. 2006).

Degradation studies have been carried out for a number of crops and chemicals. In apples, there has been research for myclobutanil, tebuconazole and difenoconazole: half-life values for tebuconazole on fruits ranged from 19.4 to 26 days for single dosage, or 19.8 to 28.9 days for double dosage (Patyal, Sharma et al. 2013); 6.3 and 10.2 days for difenoconazole (Guo, Li et al. 2010); and 13.3 to 24.8 days for myclobutanil (Hwang and Kim 2013). On leaves, the non-DMI fungicide thiophanate methyl has a half-life of 15 days (Soeda, Kosaka et al. 1972).

Combining all the above information, it would be possible to build a mathematical model and implement it as a computer simulation programme to understand how the resistance develops over time. As mentioned before, there are gaps in the knowledge of how certain factors affect the evolution of resistance, even more so in specific pathosystems. Polygenic control has been broadly studied, usually by considering a normal distribution of phenotypes (Via 1986; Shaw 1989), but it would be interesting to test how the involvement of different number of genes would affect the evolution rate. Also, polygenic control opens the possibility of different types of gene interaction. The results in previous chapters point to the possibility of epistatic interactions; it would be useful to gain some insight on how such interactions would affect the evolution of the resistance. It has been recently found that conidia may account for 20 to 50% of the primary inoculum (Passey, Robinson et al. 2017), and the mode of spore dispersal has been found before to have an effect on the evolution rate (Brent and Hollomon 2007); it would be important to determine the effects of a mixed composition of the primary inoculum on the evolution. DMIs are known for their strong

post-infection activity (Cross, Berrie et al. 2017), which includes pre-symptomatic activity, which prevent lesions for developing (if applied within three days of infection) or results in lesions that do not sporulate (if applied within five days of infection) (Schwabe, Jones et al. 1984; O'Leary and Sutton 1986), but may also have some post-symptomatic activity (Cross, Berrie et al. 2017), which usually affects spore production or germination (O'Leary and Sutton 1986; Sharma and Gupta 1995; Poblete and Latorre 2001). There is plenty of evidence for pre-symptomatic effects (Schwabe, Jones et al. 1984; O'Leary and Sutton 1986), while results for post-symptomatic effects are more mixed (O'Leary and Sutton 1986; Sharma and Gupta 1995; Poblete and Latorre 2001). However, it would be interesting to test how the presence of just one type of effect or both would alter the evolution.

7.2. Objectives

To predict how the resistance would spread under different conditions by using a model that includes isolates with the varying degree of resistance expected under single gene or polygenic control, and which are subject to variable spray deposition. In this model, the effects of different variables on the evolution of the resistance were investigated, including:

-Number of genes involved.

-Effect of epistasis: the influence of an activator/suppressor and genes interacting multiplicatively.

- Conidia as part of the primary inoculum: the effect of conidia making up a percentage of the primary inoculum as opposed to ascospores making up the whole inoculum.

-Post-symptomatic activity: the effect of post-symptomatic (antisporulant) activity,

represented in the model as a loss of reproduction ability (inability to produce conidia and ascospores) that occurs in isolates sprayed after the first five days following an infection, in addition to pre-symptomatic activity.

7.3. Materials and methods

7.3.1. Programming tools

NetLogo (Wilensky 1999) was selected as platform due to its agent-based modelling advantage of being able to follow the individual components of a system through time and space. NetLogo is a programmable modelling environment used to simulate the behaviour of a system (Wilensky 1999). Agent-based modelling, such the modelling done in NetLogo, allows users to divide the system into components and assign each separate entity its own behaviour, so that the process modelled evolves due to each component´s behaviour and also the interaction between components (Railsback and Grimm 2011). This modelling system allows the inclusion of spatial effects, and studies what happens to each individual element with time, rather than the outcome of the sum of the interactions. To achieve this, NetLogo simulates a process through a series of agents, which represent different components of the system.

The agents in NetLogo are the observer, links, turtles and patches (Wilensky 1999). The model described here includes three of these possible agents: the observer, the turtles and the patches. The observer can give instructions from outside the system taking the position of the user. The patches constitute the background of the system forming a grid of squares (each square would be a patch), and the turtles move over the patches. Depending on the system to be modelled, the agents will represent different components; for example, the

grid of patches can be used to represent a field (with each patch representing a tree) or a tree (with each patch representing a leaf). The same is true of the turtles; for example, each individual turtle can represent an animal, a cell or a fungal isolate. It is also possible for an individual turtle to represent a group of the aforementioned with the same or similar characteristics (for example, a group of genetically identical isolates).

The characteristics of the agents are represented by variables. Each agent is associated with a type of variable. There are global variables which contain a single value which is common to the whole system. Any agent can access a global variable. On the contrary, patch variables and turtle variables can only be accessed by the corresponding agents, and each individual agent has a different value for these types of variable.

NetLogo includes an interface tab, a programming tab and an info tab (Wilensky 1999). NetLogo uses a programming language known as a Logo dialect (Wilensky 1999). The system is coded in the code tab through the use of instructions known as commands. When a command is part of the NetLogo syntax it is referred to as a primitive. The user can also design their own commands by combining different primitives. These new commands are known as procedures. Commands are used to control the behaviour of the different agents. There are also reporters which are instructions used to calculate values. Again reporters can be a primitive or can be procedures designed by the user.

The simulation of the model is followed through an interface which can be modified with a number of buttons including the set up button that sets the starting point of a run by calculating the initial conditions of the system, including the initial value of all variables; a start button that gets the system moving; and there is also the possibility to add monitors and graphs to follow the evolution of a given variable or estimate; boxes that allow an input

or text label to be introduced or that report an output, and switches, choosers and slides to manually change the value of a variable. In the current model, apart from the set up and start buttons, a number of monitors and plots were included to ensure the correct functioning of the model.

In the info tab, the modeller can add an explanation and description of their model.

7.3.2. The outline of the model

The model describes the evolution of fungicide resistance in *V. inaequalis* over time. This model considers each year as a time window from 15th April to 30th August, following the use of DMIs from the green tip stage to the end of August, assuming late use of DMI sprays to control late infection of fruit, which results in post-harvest pin-point lesions. In the UK, sprays employed before the green tip stage are often protectants such as captan or fungicides with both protectant and curative effects such as dodine (Berrie and Xu 2003; Cross, Berrie et al. 2017).

The population of scab strains in the model go each year through a disease cycle characterised by four events, three of them happen repeatedly throughout the disease cycle: spore release, infection and conidial production. The disease cycle closes with a single event of sexual reproduction which generates the ascospore progeny that initiate a new disease cycle the next year in the spring. At the beginning of each spring, there are no isolates in the environment until ascospores (and overwintered conidia in some scenarios) start being released. Fungicide is applied, usually in ten days interval (unless specified otherwise), and has an effect on an isolate ability to form a lesion or produce spores.

7.3.3. Elements of the model

There are two elements interacting in the model. One of them is a population of isolates of *V. inaequalis* (represented by the collection of turtle agents in the model), and the other is the plant tissue (represented by the collection of patch agents in the model). Among the isolates, each isolate (one turtle agent) has its own value for each specific isolate variable and behaves independently. Likewise, the plant tissue is divided into patches, considered to be of around 0.093 m^2, and each patch (one patch agent) of tissue also has its own values for each plant tissue variable and behaves independently from other patches.

7.3.4. Variables included in the model:

The complete list of variables is given in Tables 7.1, 7.2 and 7.3.

Table 7.1. List of global variables present in the model.

Variable name	Description [1]
daycount	The number of days that have gone by since the start of a given year
years	The number of years that have passed since the start of a model run
Next-season	Day that marks the end of a year
spraying	Indicator of whether there has been any spray for the purpose of starting degradation
dayspray	Number of days that have passed since the last spray was applied, used for the degradation process
coverage	The chance a given patch receives some spray
	Unless specified otherwise, it was set to 40%
Spray-frequency	The number of days between sprays
	Unless specified otherwise, it was set to 10 days
dose-early	Dose-early: dose in the early part of the season before day 56 (10^{th} of June)
	Set to 330 mg/L (Systhane 20 EW label)
dose-fullleaf	Dose-fullleaf: dose after day 56
	Set to 132 mg/L (Systhane 20 EW label)
Change-spray	Day at which the dose sprayed changes from dose-early to dose-fullleaf
	Its value is 56

Variable name	Description [1]
coefficient-variation	The coefficient of variation of the dose (0.56, i.e. 56%, was the default value used, but other values, 2 i.e. 200% and 56 i.e. 5600%, were tested)
k	The degradation rate constant Unless specified otherwise, the constant was calculated for a fungicide half-life of 7 days
effect-limit	Time window after an infection (3 days) on which an isolate dies (curative effect) if sprayed with a dose above its sensitivity level
effect-limit2	Time window after an infection (5 days) on which an isolate would have its ability to reproduce (produce conidia and ascospores) removed if sprayed with a dose above its sensitivity level In scenarios where post-symptomatic activity has been included, this antisporulant effect has no time limit
n	The number of genes involved in the resistance
p	The frequency of each resistant allele
freq	The frequency of the most resistant phenotype at the start of a model run (0.001), which is used to calculate p

Variable name	Description [1]
sv	The contribution of an allele to the sensitivity
sc	
m	All used in the calculation of a theoretical mean sensitivity (me)
me	
i	
epistasis?	Indicator of the presence of epistatic effects in the model
epistasis-switch?	Indicating an activator/suppressor effect where the presence of one allele at one of the loci involved in the resistance dictates whether the rest have an effect or not
epistasis-multiplicative?	Indicating the epistasis type where each alleles multiplies instead of adding to the effect
rescale-epistasis	The sensitivity under the multiplicative epistasis scenarios is rescaled through this variable set at 10 in the present study
correction	The sensitivity under the multiplicative epistasis scenarios is corrected after rescaling by subtracting this variable that holds the number 0.1

Variable name	Description [1]
total	Total number of ascospores at the start of a model run and after sexual reproduction It is calculated under the assumption that there is rain during all the ascospore release period
ascosporeprod ascospores	The default number of ascospores produced by a mating (4) is given as an input at the start of a run through the variable ascosporeprod This number is then given to each isolate through the variable ascospores; the number then gets adjusted if there are not enough matings to rebuild the population for the next season in order to ensure a fixed number of isolates at the start of each season
successful-matings	Variable used to count the number of matings, and then recalculate the number of ascospores produced by a given mating if there are not enough matings to produce enough ascospores for the next season
rain-chance	The probability of a rain event on a given day
j	Variable used as an indicator of probability to determine whether a rain event takes place
release-day	Day intervals in which ascospores are released In each interval a different proportion of ascospores are released
percentage	Proportion of ascospores released in each day interval
numconidia	Number of conidia produced by an isolate during asexual reproduction It is set to 5 This variable is an input given at the start of a run and it is later recorded in the isolate variable numspo.

Variable name	Description [1]
distance-conidia distance-ascospores	Radius within which conidia can infect (distance-conidia, i.e. conidium dispersal distance) and radius within which ascospores can infect (distance-ascospores)
colour-cap	Establishing the sensitivity threshold (sensitivity=0.8 in the model sensitivity scale, where maximum resistance is 1) at which isolates appear in the model interface coloured as resistant isolates (black). Sensitive isolates appear brown
isolate-cap	Limiting the number of isolates a given patch of tissue can hold (the maximum is 10 isolates)
d	Recording the disease incidence (%) The percentage of patches of plant tissue with lesions
seve	Recording the disease severity (average number of lesions per patch of tissue)
rs	The seed fed to the random-number generator system in NetLogo. A given run uses the same value of rs to generate random numbers which ensures reproducibility of the results The number is also used as a run identifier.
limit	Stops a run if it reaches 50 years without maximum mean resistance being reached so a new run can start For some model validation scenarios, no time limit was established
rec	This variable ends a run and signals that results are to be recorded
replicate	When set to 1 starts a new run

[1] Certain parameters are explained in more detail in the rest of the chapter.

Table 7.2. List of isolate variables present in the model.

Variable name	Description [1]
Genes	Holding the combination of alleles for each gene position
sensitivity	Representing the ED_{50} of an isolate
numspo	Number of conidospores released by an isolate during an infection period It is set to 5
inc	Incubation time (latent period) after infection (13 days) before a new lesion starts sporulating
ascospores	Holding the number of ascospores produced during sexual reproduction (not all of these will be released during the next season; as release depends on rain events, some years will have a greater spore release than others) It is usually four ascospores per mating, but this value is changed if there are not enough matings to produce enough ascospores for the next season (each season starts with a number of ascospores set by the variable total)
released	Indicating spores that have been released, all conidia produced are released in this model (except the overwintered conidia in the scenario with mixed primary inoculum), but not all ascospores. Ascospore release depends on the number of rain events and the period within the season
dayint	Counting the days passed since the isolate infected
incubation-over	Signalling the end of the latent period

Variable name	Description [1]
non-reprod	Controlling which isolates stop reproducing after a spray with a dose above their sensitivity level has removed their ability to reproduce
repps	Controlling which spores reproduce sexually, and stopping newly produced ascospores from contributing to sexual reproduction in the same year they are produced
parent	Holding one of the parent isolates during sexual reproduction
mate	Holding the other parent during sexual reproduction
x	Variable used as an indicator of probability for isolate-related processes
l	Variable used to iterate over lists

[1] Certain parameters are explained in more detail in the rest of the chapter.

Table 7.3. List of plant tissue variables present in the model.

Variable name	Description [1]
lesions	Counts the number of lesions in a patch of tissue
newdose	The dose received after a spray
Mp, Sp, simap and mup	Variables used to calculate the lognormal distribution of the dose sprayed (newdose)
priord	Dose left after degradation
dose	Holding the dose after a spray (the sum of the newly sprayed dose and the dose left from previous sprays after degradation) Dose=priord+newdose
compare	Holding the prior dose (priord) or dose, but transformed into the scale used for the sensitivity variable in order to make decisions about the fungicide effect on a given isolate
z	Variable used as an indicator of probability for tissue-related processes

[1] Certain parameters are explained in more detail in the rest of the chapter.

7.3.4.1. Resistance parameters:

The evolution rate is monitored by following the change in a series of resistance parameters.

7.3.4.1.1. Sensitivity-related:

-Mean sensitivity of the population (the evolution of resistance in the population will be evaluated as a shift in the mean ED_{50} of the population)

In a given model run, the mean sensitivity (ED_{50}) starts always at 0.1 mg/L (low resistance) and ends at the maximum resistance value of 25 mg/L. These values were chosen from the tebuconazole ED_{50} data in Chapter 4 where the maximum ED_{50} was around 25 mg/L. However, it is difficult to make certain transformations on this scale, so it was mapped out onto a 0-1 scale with the following transformations:

Each value in the 0.1-25 scale needs to be multiplied by 10 to bring the scale to 1-250, then a natural logarithm is taken and finally the result is divided by 5.521461.

In the model, the frequency of the most resistant phenotype is kept at 0.001 at the beginning of a model run. As all resistant allele frequencies are the same for all loci to simplify calculations, this would translate in higher allele frequencies as the number of genes involved increased. This created a problem if the minimum level of the sensitivity scale (most sensitive phenotype) was also 0 (in the 0-1 scale), as the higher allele frequencies meant more isolates with a higher number of resistant alleles and that shifted the mean of the population from 0. To avoid this problem and keep the mean sensitivity of the population at 0 at the start independently of the number of genes, it is necessary to carry out a change in the sensitivity scale by first widening the range, and then shifting it back so the maximum resistance is kept at 1. To do this, the theoretical mean phenotype (me) of the population is

calculated as follows:

First, the frequency of each phenotype is calculated through the following equation:

$$(1-p)^{(n-i)}p^{i}\binom{n}{i}$$

Where p is the frequency of the resistant allele in each locus, 1-p would be the frequency of the non-resistant allele, i the number of loci with a resistant allele for that phenotype and n the total number of loci that can contribute to the resistance.

Then the frequency of each phenotype is multiplied by the sensitivity of that phenotype. To calculate the sensitivity of each phenotype, the steps specified below in the sensitivity section were followed which vary according to the type of gene interactions.

After that, the results from each phenotype are added and that gives the theoretical mean phenotype.

The mean phenotype is then used to recalculate each phenotype sensitivity value applying the following transformations:

1) Widening the scale

$$Phenotype\_rescaled = Phenotype * \frac{1}{(1-me)}$$

2) Shifting the scale back

$$Phenotype_{newscale} = Phenotype\_rescaled - \left(\frac{1}{(1-me)} - 1\right)$$

The change in scale with these transformations ensured all model runs started with a mean

sensitivity of 0 and ended when the mean sensitivity of the population reached maximum resistance (1).

7.3.4.1.2. Control-related parameters:

-Incidence of infection (the proportion of patches of tissue with lesions).

-Severity of the disease (average number of lesions per patch of tissue).

7.3.4.2. Isolate variables:

- Number of genes (n). The genes with allele variants that confer resistance. The chance that for each locus an allele is resistant is given by the following formula:

$$(0.001)^{\frac{1}{n}}$$

Where n is the total number of genes which can affect the sensitivity. The use of this formula guarantees that the frequency of the most resistant phenotype is kept the same at the start of a run independently of the number of genes.

-Sensitivity. The level of resistance of a given isolate is first calculated in a 0-1 sensitivity scale (where 0 is very sensitive and 1 is very resistant), as explained in the mean sensitivity section, and depends on the number of genes variable (n), as explained below:

- Number of genes (n): the interaction between the number of genes and the phenotype, sensitivity, depends on the type of gene interactions considered:
  a) Additive

  Each resistant allele adds an amount to the resistance equal to:

$$\frac{1}{n}$$

b) Switch-type epistasis:

The contribution of each gene is the same as in the additive case, but there is an extra gene that acts as an activator/ suppressor of the whole system, and which does not add to the phenotype. This gene will also have a functional and non-functional allele, and only the right allele (the functional allele for an activator and the non-functional for a suppressor) allows the other genes to contribute to the resistance. Note that this activator/suppressor gene affects all other resistance genes in the present model, in the environment activators/suppressors may only affect some of the genes required for resistance and not all.

c) Epistasis multiplicative:

Instead of each gene adding an amount to the sensitivity, each gene multiplies by:

$$11^{\left(\frac{1}{n}\right)}$$

A change of scale (from 0-1 to 1-11) is needed to avoid multiplying by 0, the minimum resistance level in the 0-1 scale. For that reason, multiplicative resistance was calculated on a scale from 1 to 11, and then transformed to the 0-1 scale by dividing all sensitivity values by 10 and then subtracting 0.1.

After calculating the phenotypes in the 0-1 sensitivity scale, the transformations described in the mean sensitivity section in this chapter are applied to ensure that the mean sensitivity of the population is always 0 at the start of each run and 1 at the end of a run. That means that after the transformations, the minimum value in the sensitivity scale (indicating the most

sensitive phenotype) is always below 0 (except for the single-gene scenario) and changes depending on the number of genes, while the maximum value is always 1 (maximum resistance) independently of the number of genes.

-Mating type. As the isolates from the model randomly choose their mates from a short distance radius, this variable was added to stop isolates picking their own asexual progeny as mates, as these would have the same mating-type. Mating type ratio at the start of a run is considered to be 1:1 in the population.

7.3.4.3. Plant tissue variables:

-Dose - the actual concentration of fungicide in mg/L on a portion of susceptible tissue (patch), rather than the concentration sprayed. This dose is computed as the sum of the dose left from the previous spray after degradation and the new dose sprayed. The dose received in each patch of plant tissue follows a log normal distribution, computed through the equations recommended in Railsback and Grimm (2011) for a log normal distribution, with a mean equal to the dose sprayed and the standard deviation being 0.56 times the mean – this last figure comes from the coefficient of variation in Xu, Wu et al. (2006). The sprayed dose changes on the 10th of June when it is assumed the trees are in full leaf, following the recommendations in the label for Systhane 20EW (Dow Agrosciences).

Degradation of the fungicide is computed through the following equation:

$$Dose_{day\ after\ spray} = Dose_{sprayed} * e^{(k*day\ after\ spray)}$$

where Dose $_{day\ after\ spray}$ is the dose after a number of days have passed from a spray (day after spray), Dose $_{sprayed}$ is the dose that was sprayed, day after spray is the number of days counted from the day of spraying (where the day of spraying is 0), k is a degradation

constant which is calculated as:

$$k = \frac{(ln\ 0.5)}{7\ days}$$

Where 7 days is the half-life being used for the fungicide.

Doses (and degraded doses) are transformed from mg/L to the sensitivity scale to decide the effect of a given dose on an isolate.

7.3.5. How the model works

The whole model dynamic for a given year is summarised in Figure 7.1.The liberation of ascospores takes place through rain events; released ascospores establish new infections which, after a latency period, form a new sporulating lesion. Rain events are generated randomly with a chance of 33% that is derived from the average nine days of rainfall expected during the months of April and May in the Kent area as stated in the East Malling weather averages table in the Met Office website (1981-2010). The release of ascospores is not uniform but varies with time (Table 7.4). Tests were carried out in the model validation section of this chapter to make sure the average yearly release was around 2500 spores (actual average=2520 spores).

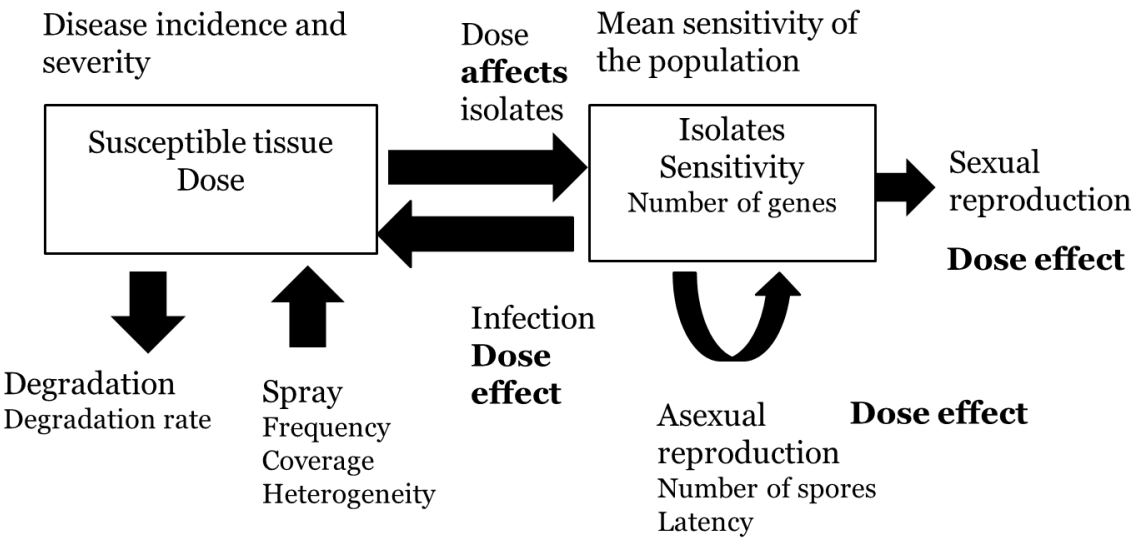


Figure 7.1. Summary of the dynamics of the model in a given year.

Table 7.4. Ascospore percentage of the total inoculum available released in different periods of the year.

Days after 15th April [1]	Percentage of ascospore released [2,3,4]
5.0	2.0%
10.0	10.0%
15.0	15.0%
20.0	20.0%
30.0	8.0%

[1] The days listed refer to until which day number after 15th April ascospores are released at the corresponding percentage. For example, from 15th April until 20th April (5 days after 15th April) when there is rain 2% of ascospores are released.

[2] The percentage is relative to 2500 ascospores (approximately the total number released, on average).

[3] Based on the relative number of ascospores released given in Figure 4 in Aylor (1998).

[4] Ascospores that are not released do not react to the fungicide, reproduce or contribute to the mean sensitivity, and are eliminated at the end of the year.

In the model, an isolate reproduces asexually (produces conidia) by producing five new isolates– a representation of 25000 spores/cm^2 which is what isolates of *V. inaequalis* are said to produce on average in a day (Albert and Lewis 1962). When it rains, the new conidia infect new sites in a short distance radius. Infection by both ascospores and conidia needs a latent period of 13 days before sporulation (producing conidia) can take place for the first time. This latency period is an intermediate value within the recorded intervals ranging from 9 to 17 days (Vaillancourt and Hartman 2000). Once the latent period is over, new conidia are produced and released during rain events.

After infection takes place, the fungicide is employed and, upon meeting a concentration of fungicide greater than ED_{50}, isolates suffer from the effect of the fungicide. Two types of response to the fungicide are included: if it has been three days or less since the particular isolate infected, the isolate dies (curative effect)– this means that the isolate stops contributing to the mean sensitivity computed – if it has been five days or less since infection, the isolate loses its ability to produce conidia and ascospores; however, these isolates still contribute to the mean sensitivity, as they go on to develop into a lesion after their latency period is over that could be sampled during field experiments . Both of these effects were observed by Schwabe, Jones et al. (1984), and are part of the pre-symptomatic activity of DMI fungicides. After there has been a spray, isolates will continue being subjected to the fungicide action while it degrades. Thus, in the model, fungicide dose does not remain static; it is constantly changing through degradation and new spray events. The degradation depends on a kinetic constant which sets the rate at which the degradation process eliminates the fungicide residue. On the other hand, the fungicide dose gets renewed by new sprays. The spraying program in the model is characterised through a

frequency, a degree of coverage and a degree of variability. The frequency refers to the spray interval (number of days between sprays), the coverage to the chance a given patch – and thus a given isolate – gets some spray, and the heterogeneity to each patch receiving a different random dose following a log-normal distribution.

At the end of each season new ascospores are produced via sexual reproduction; they will get released the next year. Isolates choose mates from within a short distance. For a mating to be successful, isolates need to have opposing mating-types – this ensures that an isolate does not choose its own asexual progeny as mates. As the average number of isolates released has to be the same each year (around 2500), the number of ascospores produced in total is always the same – calculated on the basis of the case in which ascospores are released every day during the ascospore release period. When there are not enough matings to produce the required number of spores, the system compensates by having each mating produce more spores. If, on the contrary, more spores are produced than needed to re-establish the initial inoculum the next season, some spores are eliminated at random until the number of spores matches that of the total produced each year. Each spore produced in a given mating inherits a specific allele combination, with a 50% chance to inherit the allele of a given parent for each loci. The sensitivity of that spore is then calculated, as explained in the description of the sensitivity variable, based on the allele combination inherited. Each spore has a 50% chance of inheriting each mating-type.

7.3.6. Model assumptions and limitations

The influence of weather factors in this model is virtually not considered; an average latency value is used throughout the duration of the model runs and rain chance is kept at 33%, as mentioned before. Both conidia and released ascospores always infect in the model;

something that would not occur in nature, as weather conditions play a big role in determining infection success. Another weather factor which is not included is the effect of rain on the loss rate of the fungicide residue (Xu, Murray et al. 2008) which could result in a greater loss rate than that generated by the degradation rate.

Alleles that increase resistance all have the same effect on the phenotype, and are present at an identical frequency in the initial population. In the field, alleles probably have varying contributions to sensitivity and appear at different frequencies.

Effects of distance on conidiospore and ascospore infection are not part of this model. An isolate infects within a certain radius with constant probability. This radius is larger for ascospores (900 units) than for conidia (3 units). In the real environment, spores would be much more likely to colonise an area that is close to them than a remote one.

A log normal variation of the dose has been used, although this type of distribution fits fruits better than it does leaves (Xu, Wu et al. 2006).

Fungicide half-life is taken as 7 days which is not far off from those calculated for other chemicals. This number comes from considering doses in fruits have to be below a certain threshold to comply with regulations. For myclobutanil the threshold is 0.6 mg/kg (HSE 2017). Taking an average of 30 tonnes of apples in a hectare and considering our program sprays at a rate of 66 g of chemical per hectare (2.2 mg/kg under the assumption of 30 tonnes of apples per hectare), seven days were taken as a good value to take the quantity per kg under the threshold in the recommended 14 days preharvest interval (Systhane 20EW label, Dow Agrosciences).

Regarding the changes in the availability of plant tissue with time, the model only considers

a scarcity of infection sites later in the season by placing a limit on the number of isolates that can infect a given patch, this also serves to keep the number of isolates from growing too large and slowing down the speed at which the model runs. In nature, new tissue would become available during the growing season as new leaves are produced – more slowly in the late part of the season (Carisse, Jobin et al. 2008). This part of the tissue cycle is not part of this model, which may have an effect on some of the results derived from it.

Fitness levels for the resistant strains have not been considered. Very different behaviours have been described for other pathogens and fungicide pairs in regards to fitness. However, as far as is reported in the literature reviewed for this thesis, no fitness penalty has been found so far in field isolates of *V. inaequalis* resistant to DMI fungicides (Chapman 2011).

In the current model, there is a mechanism to ensure enough spores are produced for the next year which consists in having each mating produce more spores if there are not enough matings; however, in nature, a shortage of matings would result in an allee effect (Taylor and Hastings 2005), which may change the dynamics of the pathogen population in the following year.

7.3.7. Comparison with other models

The model described here differs from previous models in the fungicide resistance literature in a number of ways. Previous fungicide resistance models usually relied on mathematical equations, difference or differential equations, to model the behaviour of the system. The current model uses difference equations, but not differential equations, to calculate values for a number of parameters.

Many fungicide resistance models are deterministic, although stochastic models have been

developed, specially to describe the emergence and invasion phase which includes an element of mutation (van den Bosch and Gilligan 2008). The current model does include some stochasticity as a number of events, i.e. rain, are controlled by probabilities.

Past models tended to monitor the frequencies of the different phenotypes or their absolute numbers with time (Kable and Jeffrey 1980; Skylakakis 1981; Chin 1987), rate of increase of the phenotypes (Shaw 1989; Shaw 2000) or diseased area containing each phenotype (Hobbelen, Paveley et al. 2011; Hobbelen, Paveley et al. 2014). The current model follows the change of the mean sensitivity of the population with time.

The time step in the current model is one day. A few of the other models used one spray (Kable and Jeffrey 1980) or one pathogen generation (Chin 1987) as their time step.

Regarding the influence of crop growth dynamics on the resistance, past models were split between those that did not consider this aspect and those that did (van den Bosch and Gilligan 2008). The model in this chapter only very vaguely includes a density effect by placing a cap on the number of isolates that can infect a given patch, but does not include the initial phase of tissue growth when the behaviour of the system is closer to density independence.

The model in this chapter includes heterogeneity of the dose and polygenically controlled resistance. The first aspect was modelled in (Shaw 2000) using a logistic probability distribution for the log-dose, and the second in (Shaw 1989) with a normal probability distribution for the sensitivity trait. The model described here uses a log normal distribution of the dose (log-dose would be distributed normally) as there is some evidence for log normal distribution of the dose in the experimental study by Xu, Wu et al. (2006). The

distribution of the sensitivity phenotype in this model is the result of assigning each isolate an allele for each locus associated to the sensitivity (the chance of getting a given allele comes from the frequency of the allele in the whole population), and then calculating the phenotype of the isolate from its allele combination. The model from (Shaw 1989) also includes sexual reproduction, recalculating the mean sensitivity after sexual reproduction on the basis of heritability. Sexual reproduction in this chapter is based on the pathogen's epidemiology, with isolates reproducing with nearby isolates.

The model presented here shares common elements with other models in the fungicide resistance literature like including fungicide coverage, fungicide degradation and dose (Kable and Jeffrey 1980; Chin 1987; Shaw 2000; Hobbelen, Paveley et al. 2014).

Some aspects not included in this model which feature in some of the other fungicide resistance models in the literature are an infectious period for a lesion (Hobbelen, Paveley et al. 2011), which is the period in which a lesion is capable of producing spores and generating new lesions; an assumption that not all spores land or infect susceptible tissue (Hobbelen, Paveley et al. 2011), and fitness penalties (Chin 1987; Shaw 1989; Hobbelen, Paveley et al. 2014).

The major difference with past models is the scale at which the crop and pathogen dynamic is studied. In past models, the crop is usually studied from a field (or lager) scale, while the pathogen is studied in terms of population and subpopulations with different phenotypes. In the model in this chapter, the use of an agent modelling system means that the crop is studied in terms of subunits known as patches that together constitute a field, while the pathogen is studied in terms of individual isolates that form a population, so the scale of the interactions in the system is much smaller, and also has an added spatial component as the

field has two-dimensions.

There does not seem to be other models in the fungicide resistance literature that use this approach, but a number of models in the wider plant pathology literature do include a spatial element. Zadoks and Kampmeijer (1977) developed a mathematical model that included a feature very similar to the patches in an agent based model. They divided the background subjected to pathogen infection, which they called a block, into smaller pieces which they called compartments. These compartments could be grouped into subsets called units. The approach was a way to model different scales within a system, the block could, for example, represent a field, the units a tree or plant, and the compartments in a unit leaves within the plant. The same concept of compartments was used by Mundt, Leonard et al. (1986) to study epidemics in fields with mixtures of cultivars with different susceptibility to the pathogen by assigning different compatibility values (which represented different host genotypes) to the compartments so that only compartments with the same compatibility value than the compartment from which the epidemic initiated were considered to be susceptible. For their spore dispersal, they used a modified version of the Gregory model but, as this model only allows studying the dispersal in one direction, they added some parameters that allowed studying the dispersal in all directions. A more recent example that incorporates a spatial component is the model by Mikaberidze, Mundt et al. (2015) where the field is described in terms of two-dimensions (width and length), and integrates equations over time and the two dimensions of space. This model also includes a dispersal function in which dispersal depends on the distance to the point of origin of the spore. This dependence of the dispersal on distance, as mentioned in the assumptions and limitations section in this chapter, is not part of the model described in this chapter where the

probability of a spore to infect a patch within a given radius is independent of the distance to the source. Although the models by Zadoks and Kampmeijer (1977) and Mundt, Leonard et al. (1986), which used the compartments approach, and the model by Mikaberidze, Mundt et al. (2015) subdivide the crop system into smaller units, the scales they use for the pathogen are larger than one isolate (the scale used in this chapter) as they talk about propagules and fractions of propagule or density of infected hosts. Also, although the model by Mundt, Leonard et al. (1986) considered individual characteristics for different crop tissue subsets (different compatibility values), it did not consider subsets with independent trait values for the pathogen population; in contrast, the current model incorporates independent behaviour for subsets of both the pathogen and the host.

7.3.8. Model validation

To make sure the model was working as intended, different scenarios where the outcome is predictable or known were tested (Table 7.5). Most scenarios were tested assuming four-gene control, but a few scenarios were also tested with one gene.

Table 7.5. Scenarios and values tested during the model validation for different parameters.

Factor studied	Values/scenario tested
Coverage (%)	80, 60, 40, 20
Spray frequency	10 days, 7 days
Fungicide half-life	15 days, 7 days, 3 days
Degradation	Fungicide does not degrade
Spraying	No sprays
Population size	Static (isolates do not reproduce sexually or asexually)

Besides these tests, it was also checked that doses sprayed followed a log-normal distribution and the average ascospore release was around 2500 spores.

7.3.9. Test scenarios

After the model was validated, the evolution of fungicide resistance was studied in the following scenarios:

7.3.9.1. Number of genes and epistasis

A summary of the different scenarios studied and the number of genes tested in each case appears in Table 7.6. The way the different types of gene interactions in the table (additive, switch-type epistasis or multiplicative epistasis) affect the sensitivity is detailed in the description of the isolate variables, under the variable number of genes (n).

Table 7.6. Different types of gene interactions tested in the model and the number of genes tested in each case.

Scenario considered	Number of genes tested
Additive control	1,2,4,6,8,10
Activator/suppressor (switch-type epistasis)	2,4,8
Multiplicative epistasis	2,4,8

7.3.9.2. Coefficient of variation

The effect of a change of the coefficient of variation from 0.56 to 2 was studied in the same scenarios presented in Table 7.6. For the two epistasis scenarios, the same number of genes in Table 7.6 were used and for the additive scenario one, two, four and eight genes were used.

7.3.9.3. Conidia as primary inoculum

A scenario with four-gene control in which the primary inoculum was made up of conidia and ascospores was tested, as recent research has found that conidia could make up from 20% to 50% of the primary inoculum (Passey, Robinson et al. 2017). For this scenario, a modification of the model was used with some added variables (Table 7.7). Instead of eliminating all conidia before the next season starts, a number of conidia are allowed to survive onto the next year (overwintering conidia). These overwintering conidia from the previous season are then released the next year during rain events over the course of two weeks. From the total number of overwintering conidia, 18% are released per rain event. The chance of release per rain event was adjusted so that the average release of conidia through the years was about 20% of the average ascospore release (around 500 conidia released on average). As with ascospores, non-released overwintering conidia do not respond to the fungicide, reproduce or contribute to the mean sensitivity of the population, and are eliminated at the end of the season.

Table 7.7. List of additional variables included in the modification of the model used in the conidia as primary inoculum scenario.

Variable type	Variable name	Description
Global	conidia-num	Number of conidia released on average (500)
	conidia-release	Percentage of conidia released per rain event from the average release held in conidia-num (18%)
	con-total	Total number of overwintering conidia surviving each year It is calculated assuming there is rain in every day of the release period
	con-inoculum	Holding all conidia
	asc	Holding all ascospores
Isolate	conidia	This variable helps differentiate isolates that are overwintering conidia (or progeny of overwintering conidia) from those that are not It is set to 1 in overwintering conidia (and their progeny) and 0 in the ascopores (and their progeny)

7.3.9.4. Post-symptomatic activity

The effect on the evolution of including post-symptomatic activity, having isolates respond to the fungicide after the five-day time window, was studied in the scenarios detailed in

Table 7.8. After the five-day time window post-symptomatic activity was included as a removal of an isolate's ability to reproduce (produce conidia and ascospores).

Table 7.8. Scenarios tested with the inclusion of post-symptomatic activity, in addition to pre-symptomatic activity, and number of genes tested in each scenario.

Scenario considered	Number of genes tested
Additive control	4,8,10
Activator/suppressor (switch-type epistasis)	4,8
Multiplicative epistasis	4,8
Conidia as primary inoculum	4

7.3.10. Model runs

For each scenario (both during the model validation and test scenarios), different values were tried for each parameter, and a record was kept of how they affected the model in terms of the mean sensitivity of the population, disease incidence, severity of the disease and speed at which the changes take place –measured in number of years. The sensitivity distribution of the isolates at the end of each year was also recorded.

A model run ends when the mean sensitivity reaches the maximum resistance value (1) or, if the mean sensitivity never progresses to that maximum value, at 50 years from the start (although some of the model validation scenarios were run for a longer period), in which case the mean sensitivity at that point is recorded.

For each set of parameters, the model was run around ten to 20 times (model validation) and 20 to 30 times for the test scenarios (around ten to 34 times for post-symptomatic activity scenarios) to ensure conclusions were as precise as possible due to stochasticity.

These numbers of runs were chosen after comparing results via a two sample t-test under the same scenario with ten and 100 runs.

7.3.11. Data handling

Graphs and statistical tests were carried out with Genstat 16 (Payne, Murray et al. 2013).

Error bars were calculated as 2SEM (standard error of the mean) on either side of the mean for each individual scenario (SEMs were calculated for each of the values of the variables studied in each scenario tested).

7.4. Results

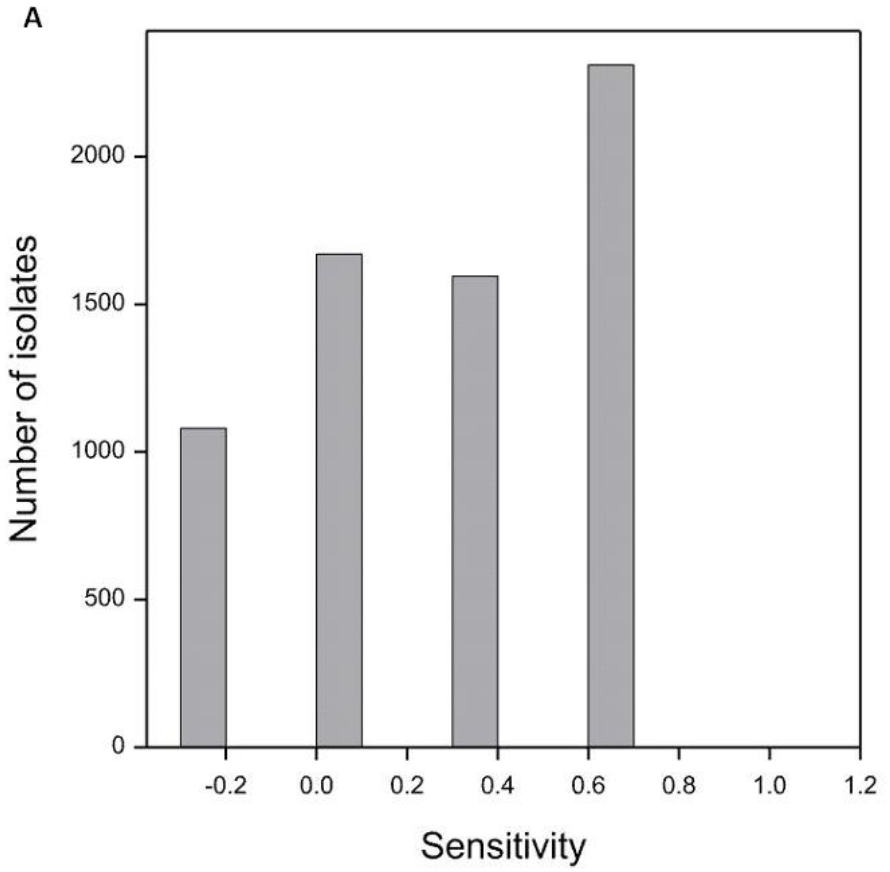
7.4.1. Model validation

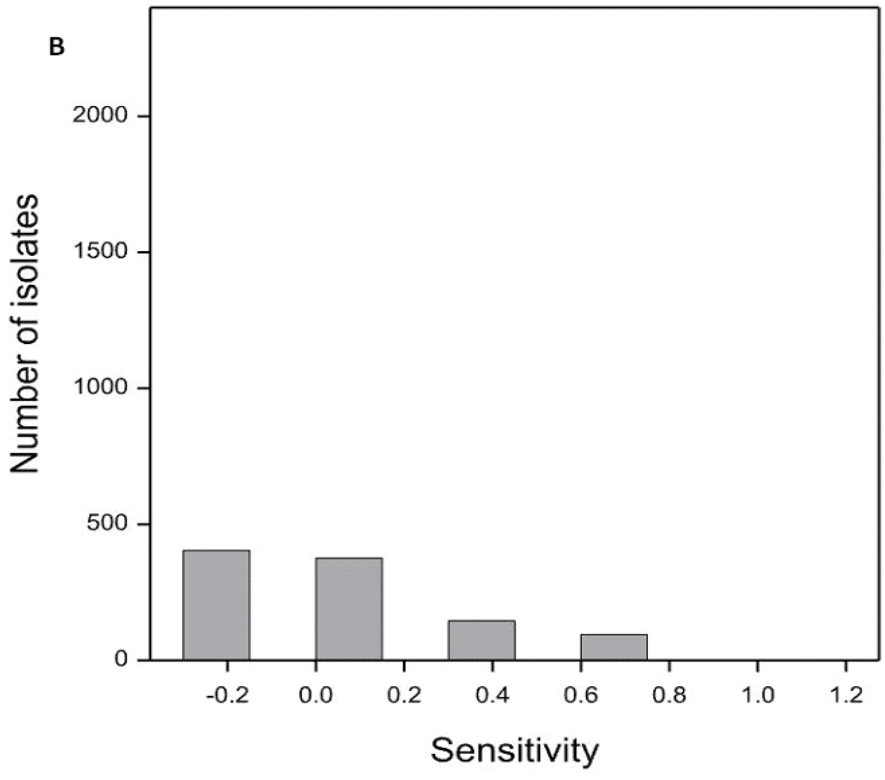
7.4.1.1. Observations

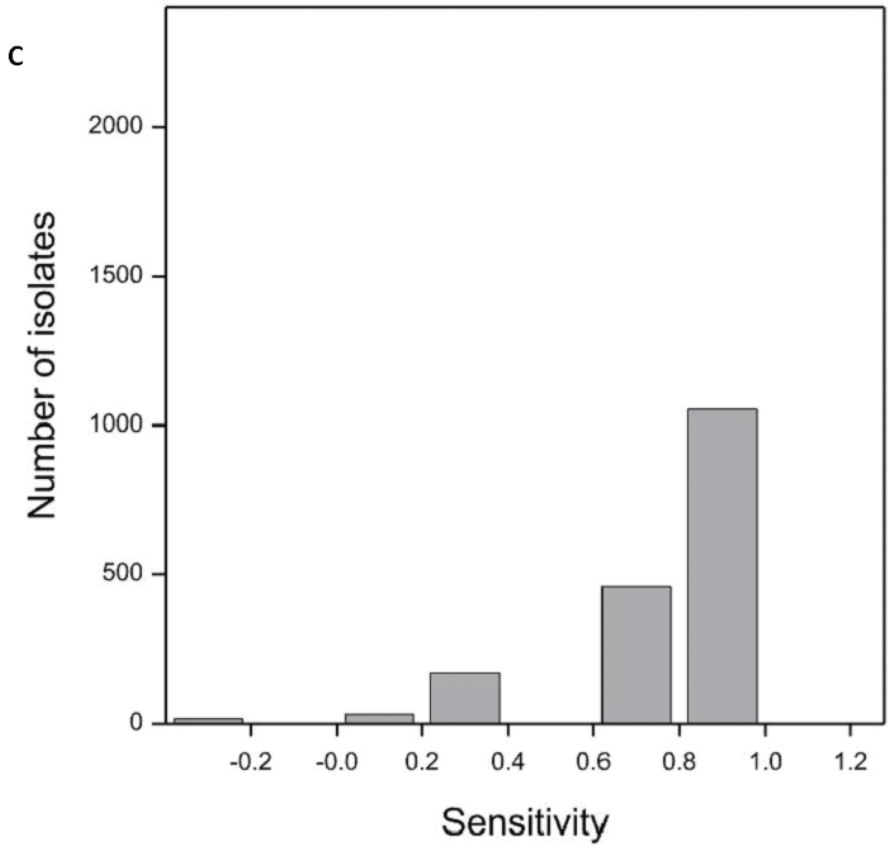
Scenarios with low selection pressure (quick degradation of the fungicide, lower coverage) evolved as expected, slowing down the evolution rate. On the other hand, a slow-down of the evolution, instead of the expected acceleration, was observed in scenarios with high selection pressure such as: high coverage (80% and 60%), no degradation resulting in fungicide accumulation and degradation half-life of the fungicide of 10 days or above. These superficially counter-intuitive results seem to be a correct consequence of the assumptions of the model. Only pre-symptomatic effects of the fungicides have been included in this model, as evidence for post-symptomatic effects is more vague and inconclusive(O'Leary and Sutton 1986; Poblete and Latorre 2001). Pre-symptomatic effects only affect those infections that have not yet developed into a lesion, and therefore, infections that have turned into lesions are no longer affected by subsequent sprays; this means that in scenarios where

doses are higher at the end of a year, either because of fungicide accumulation due to slower degradation or due to greater fungicide input through higher coverage or more frequent sprays, isolates that infect at the end of the season are more likely to be affected because the trees are holding higher doses of fungicides. Even the most resistant isolates are often affected by the fungicide at this point, slowing down resistance progression when compared with scenarios where there are lower doses of fungicide at the end of the season (Figures 7.2A, 7.2B, 7.2C).

It is important to mention that in nature the fungicide may not accumulate to the rates seen in this model, as part of it would be washed off by rain (Xu, Murray et al. 2008), and also new tissue that is fungicide free would keep growing during the season (at least until July) (Carisse, Jobin et al. 2008).


A
Number of isolates
2000
1500
1000
500
0
-0.2
0.0
0.2
0.4
0.6
0.8
1.0
1.2
Sensitivity


B
Number of isolates
2000
1500
1000
500
0
-0.2
0.0
0.2
0.4
0.6
0.8
1.0
1.2
Sensitivity



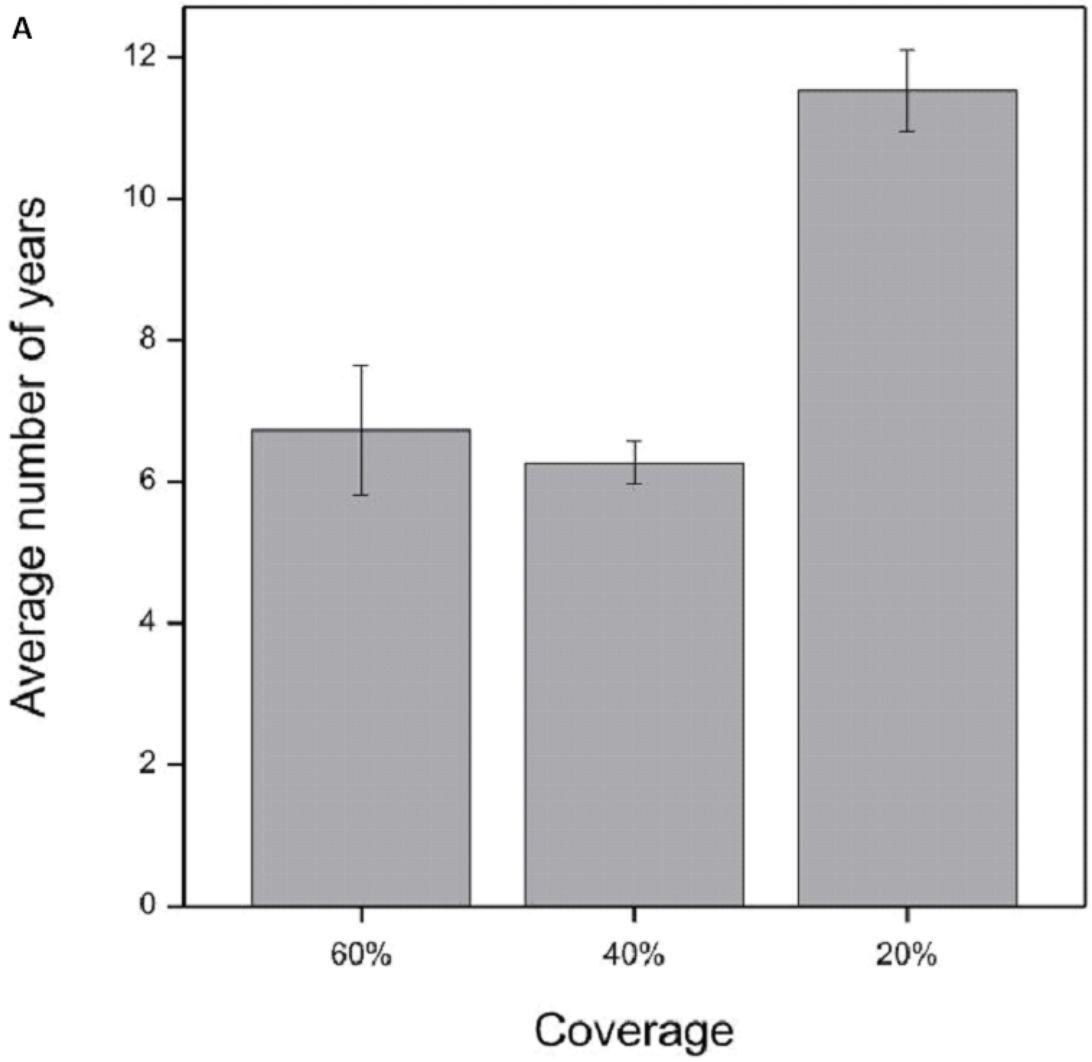
Figures 7.2A, 7.2B and 7.2C. Number of isolates for each sensitivity phenotype (an explanation of how the sensitivity is computed can be found under the description of the variables mean sensitivity and sensitivity in section 7.3.4; in the sensitivity scale used, higher numbers indicate higher resistance) at the end of the first year of sprays for a case of four-gene control and 40% coverage (A), 60% coverage (B) and 40% coverage and post-symptomatic activity, where isolates are affected at any point of the disease cycle and isolates affected by the fungicide are not included in the figure (C). The greater selection pressure (60% coverage) means that sensitivity is shifted toward less resistance because the greater fungicide doses at the end of the year result in more isolates being killed at the end of the year (when isolates are more resistant) than at the start of the year (when they are

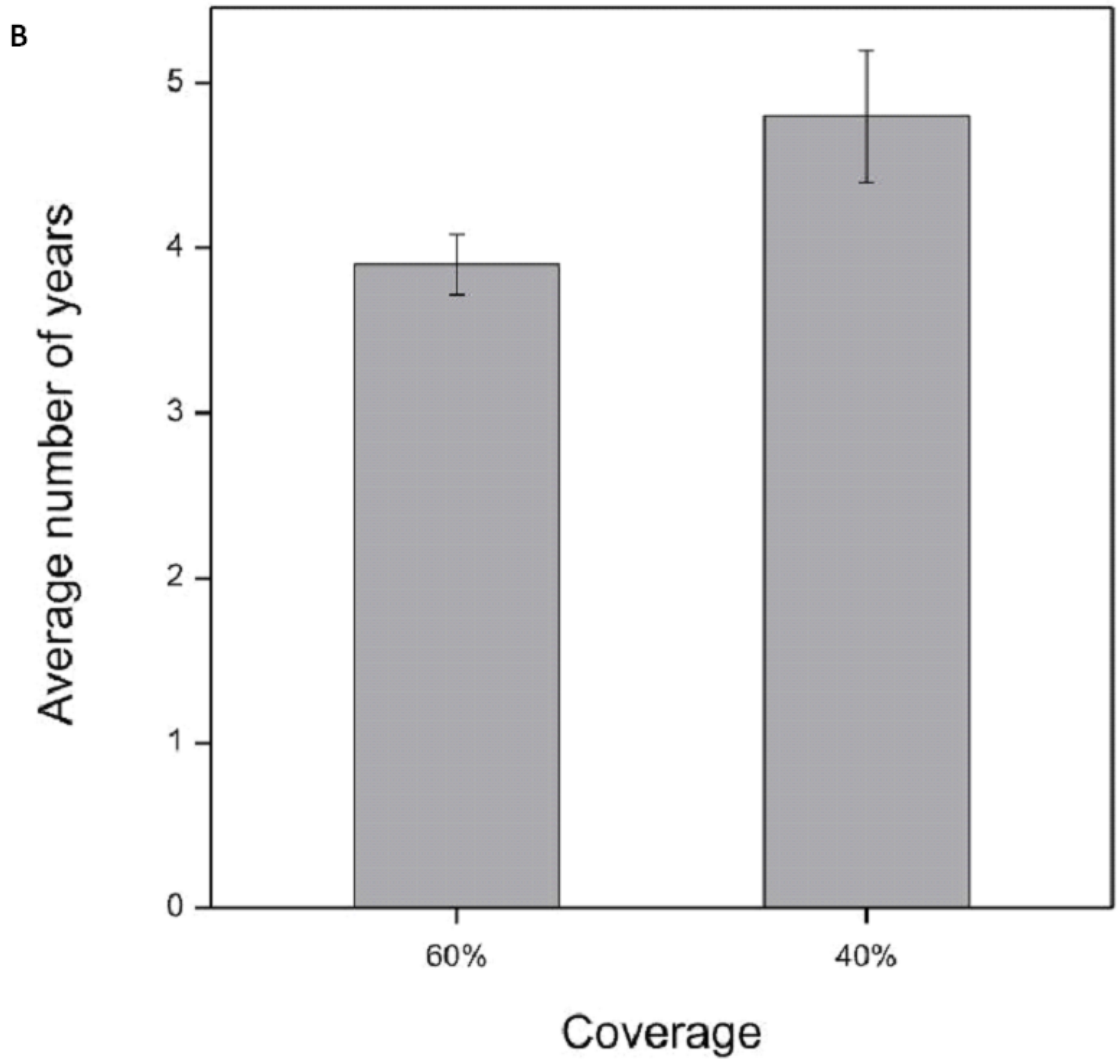
more sensitive). Since only pre-symptomatic activity is considered (A and B), isolates are only affected in a short time window after infection, which means the sensitive isolates that survived at the start of the season can no longer be affected by the sprays at the end of the season. When post-symptomatic activity is included (C), and if isolates affected by the fungicide are not counted, the number of isolates at each sensitivity level follows a trend where there is an increased number of isolates with higher resistance levels.

7.4.1.2. Coverage

With four-gene control, a coverage of 80% proved too high to sustain a population and 60% seemed to result in a slow-down of the evolution rate compared to 40% coverage; however, a test with one gene control and 60% coverage resulted in a speed up in the evolution rate when compared with lower coverage (40%). Lower coverage percentages of 40% and 20% gave expected results (Figures 7.3A, 7.3B) with four-gene control, with the higher coverage (40%) resulting in a faster evolution.

A





Figures 7.3A and 7.3B. Average (mean) number of years to reach maximum resistance under different degrees of coverage (measured as the chance of an individual patch to receive some spray during a fungicide application) with four-gene (A) and one-gene (B) control. Error bars are calculated as 2SEM on either side.

7.4.1.3. Spray frequency

In the four genes scenario, a shorter spray interval gave expected results (Figure 7.4): an increase of the evolution rate.

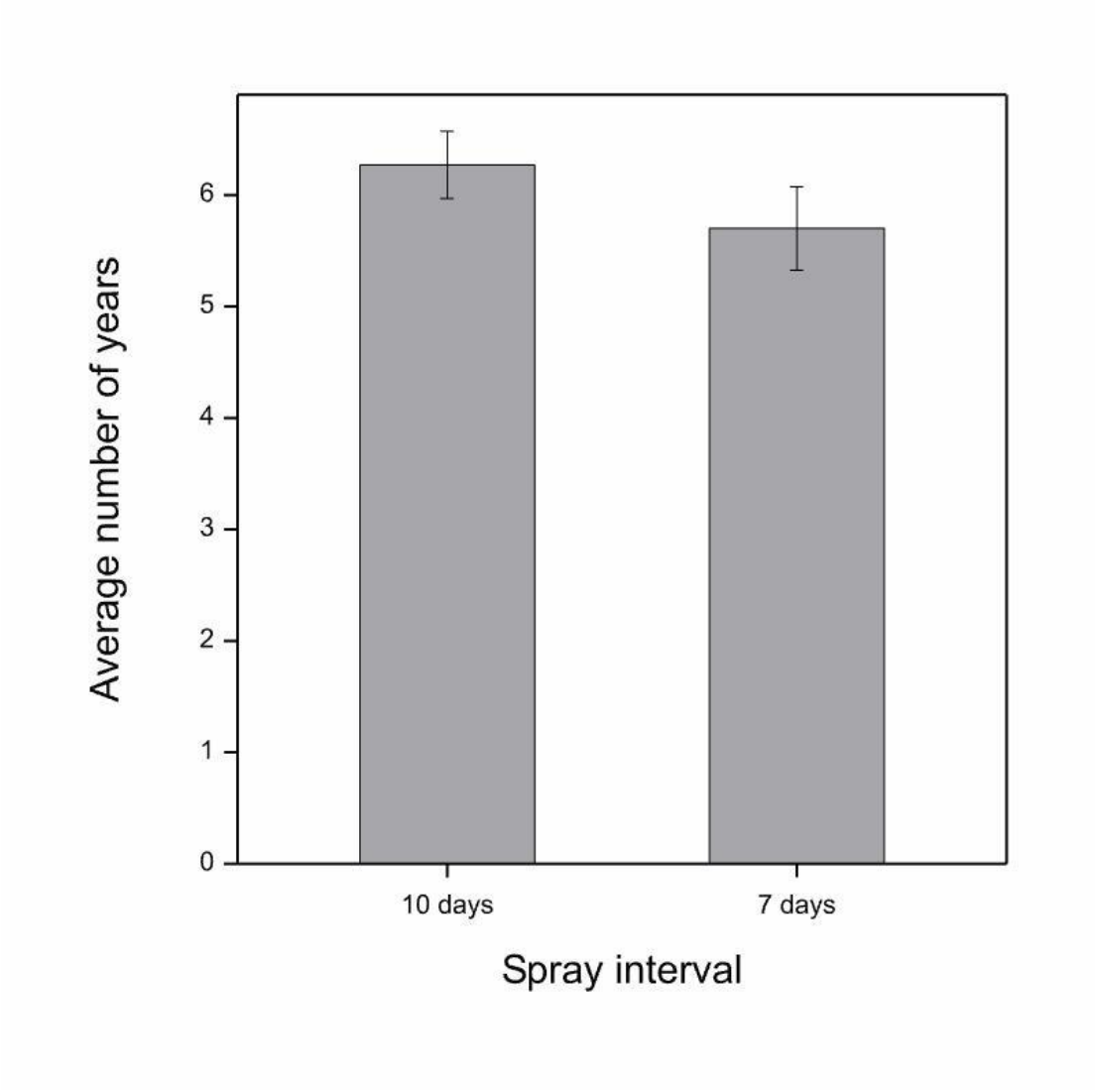


Figure 7.4. Average (mean) number of years to reach maximum resistance under two different spray intervals with 40% coverage. Error bars are calculated as 2SEM on either side.

7.4.1.4. Fungicide half-life

All values tested above seven days slowed down the evolution rate, even in the single gene scenario. There are a number of studies on fungicide residue that point to a slow decay of some chemicals, with the possibility of fungicide accumulation after successive sprays (Potter, Wauchope et al. 2001; Cabizza, Dedola et al. 2012). However, as mentioned before losses of fungicide residue due to rain (Xu, Murray et al. 2008) could prevent this accumulation from happening. A quicker degradation rate (under 7 days) also slowed down the evolution due to reduced selection pressure (Figure 7.5).

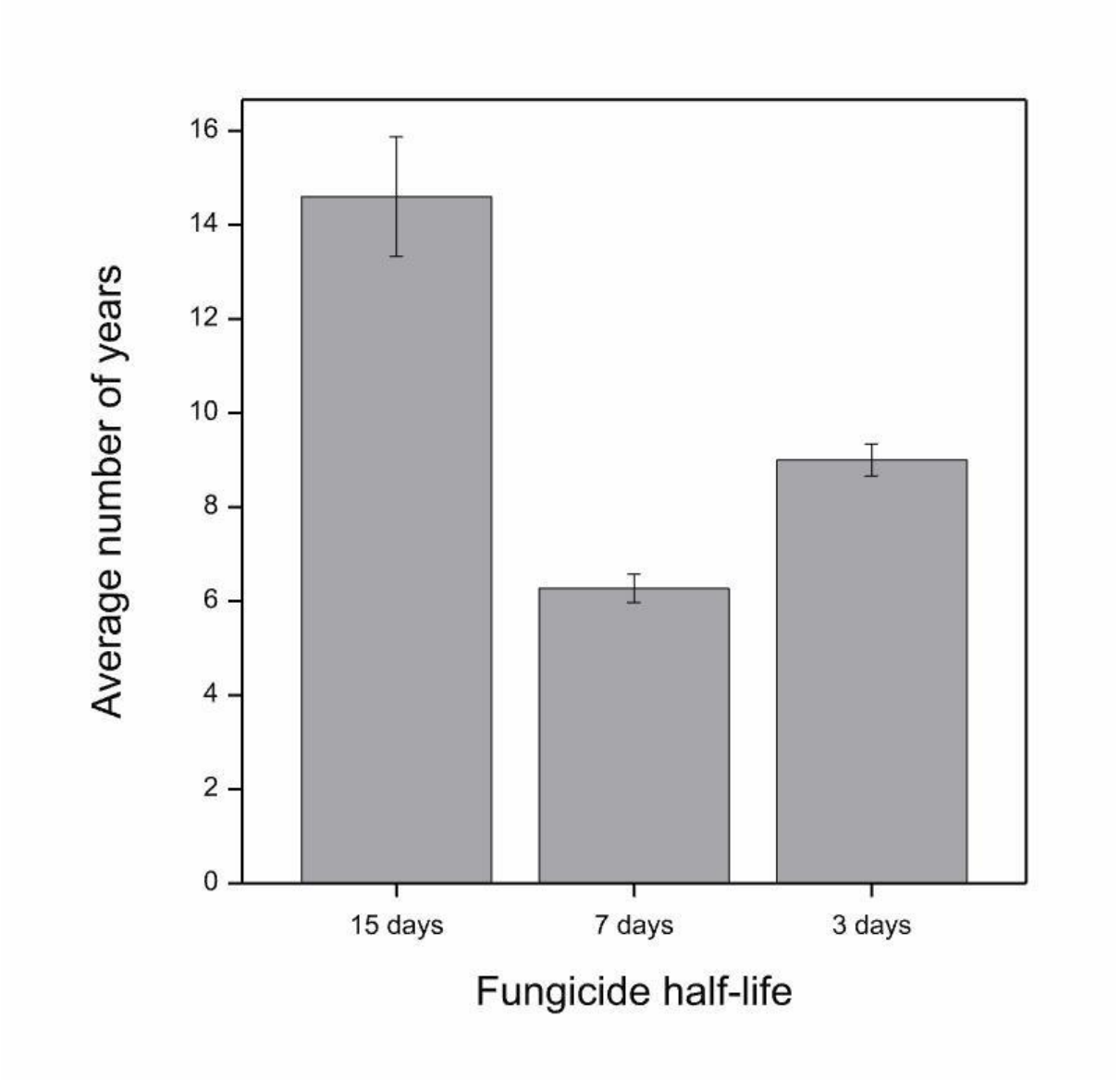
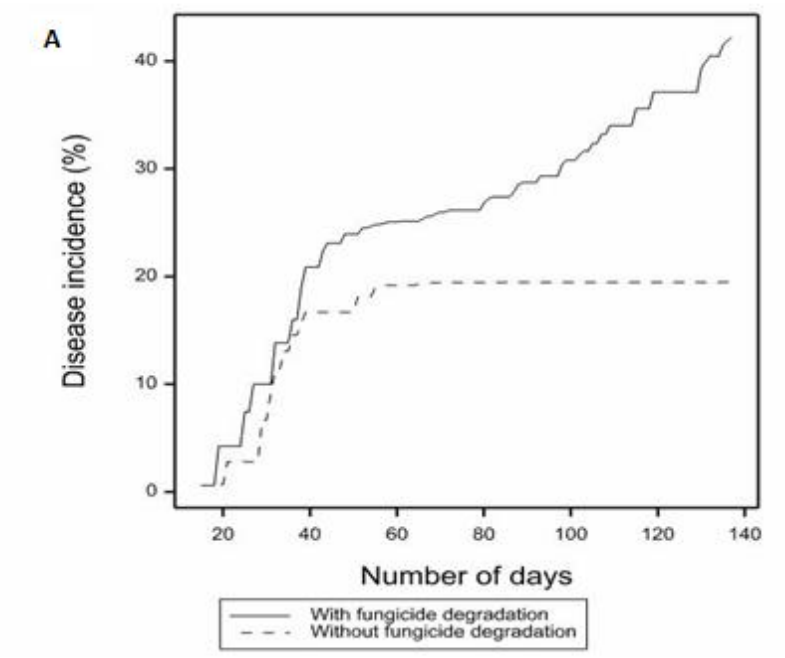
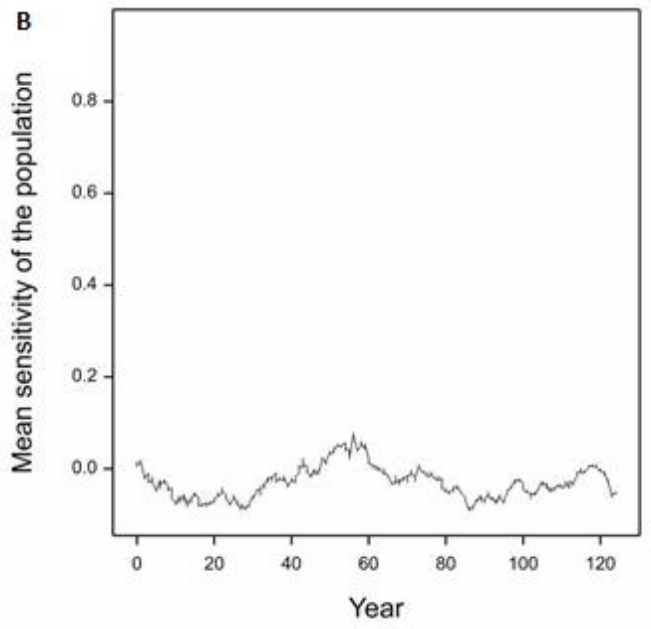


Figure 7.5. Average (mean) number of years to reach maximum resistance under fungicide half-life values of 15, 7 and 3 days with four-gene control. Error bars are calculated as 2SEM on either side.

7.4.1.5. Degradation

If the fungicide did not degrade, and therefore built up in the model environment, it resulted in greater control (Figure 7.6A), and the mean sensitivity of the population was kept low (Figure 7.6B).





Figures 7.6A and 7.6B. Disease incidence evolution over a year in a model scenario where fungicide degradation is included and in a scenario with no degradation and fungicide build up (A) and mean sensitivity of the isolate population (in the model scale, where maximum resistance is 1) over a number of years when there is no fungicide degradation (B).

7.4.1.6. No sprays

When fungicide sprays were taken out of the model, the mean sensitivity remained low for the duration of the test (Figure 7.7).

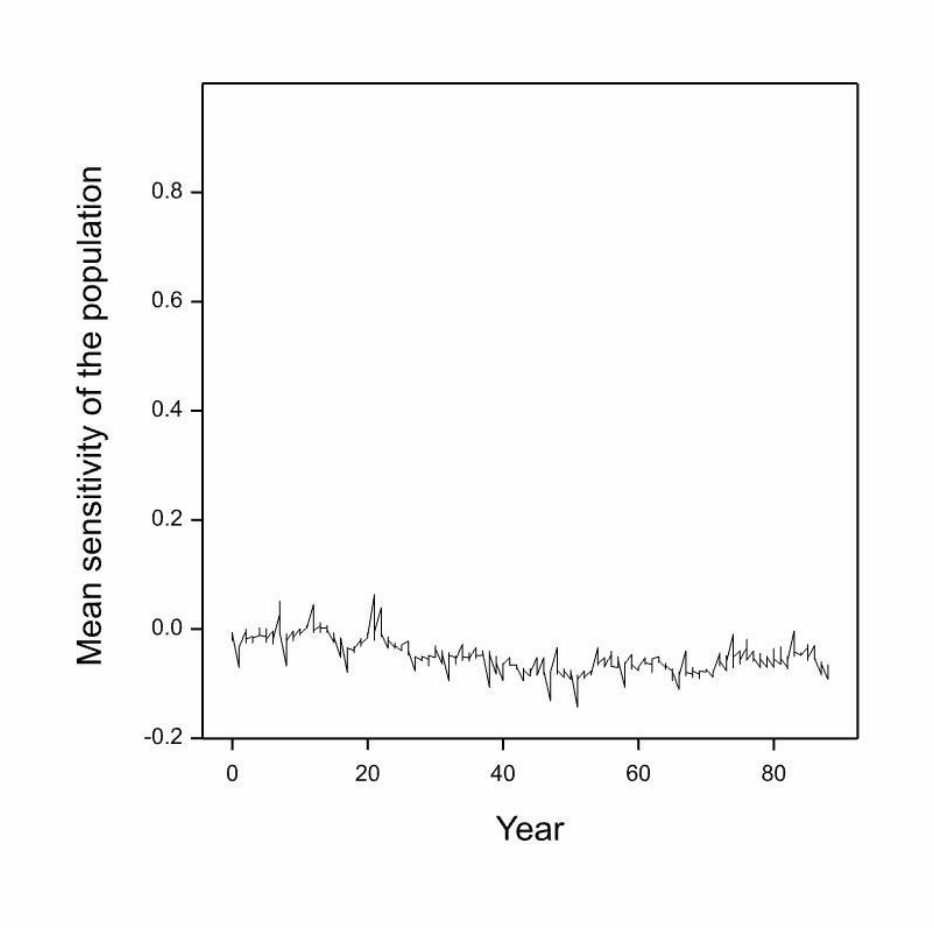


Figure 7.7. Evolution of the mean sensitivity of the population (in the model scale, where maximum resistance is 1) over the years.

7.4.1.7. Non-reproducing (static) population

If after the initial spore release isolates do not produce either conidia or ascopores, the disease incidence after the first round of infections remains the same as no new lesions are formed (Figure 7.8).

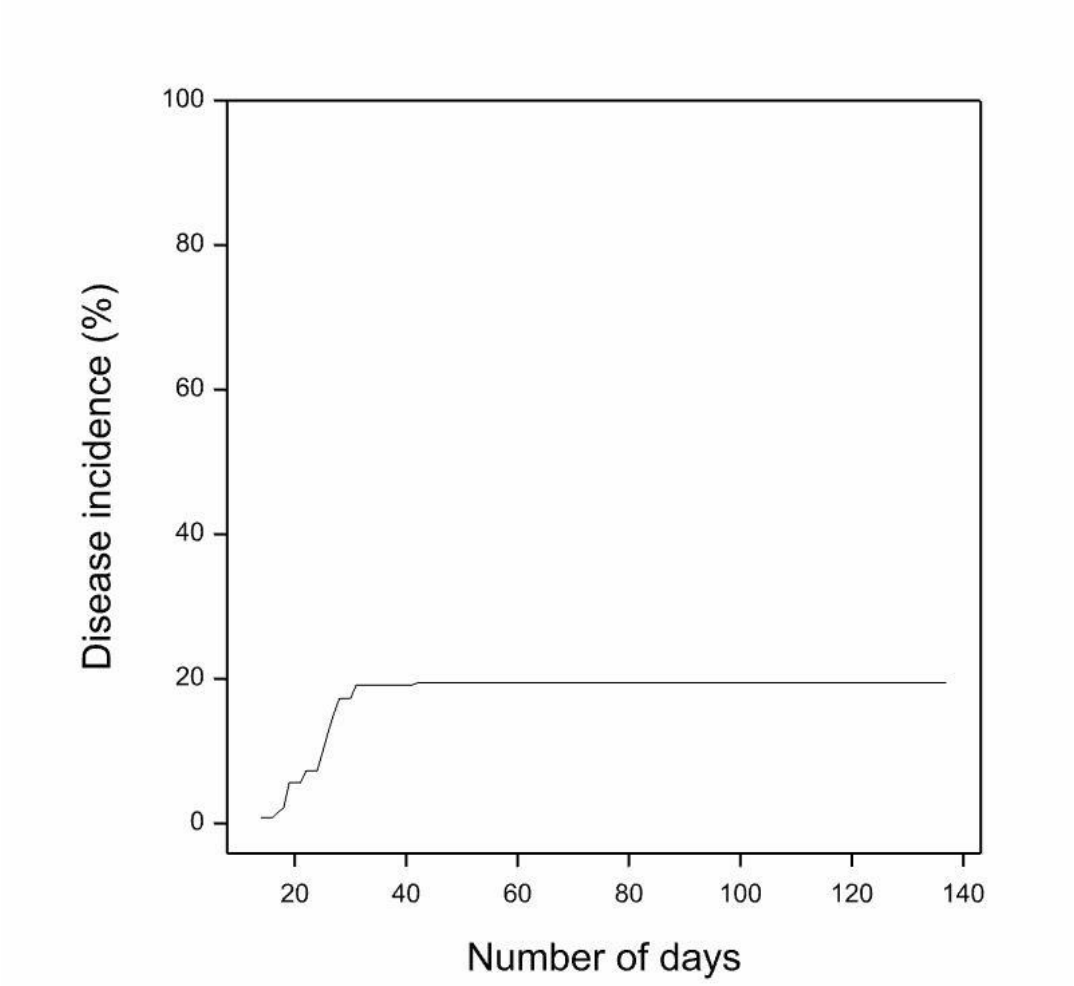


Figure 7.8. Disease incidence over time if isolates do not reproduce.

7.4.1.8. Dose distribution

The dose in the model followed a log-normal distribution, and doses computed both during early season and after full leaf fitted a log-normal distribution well (Figures 7.9, 7.10).

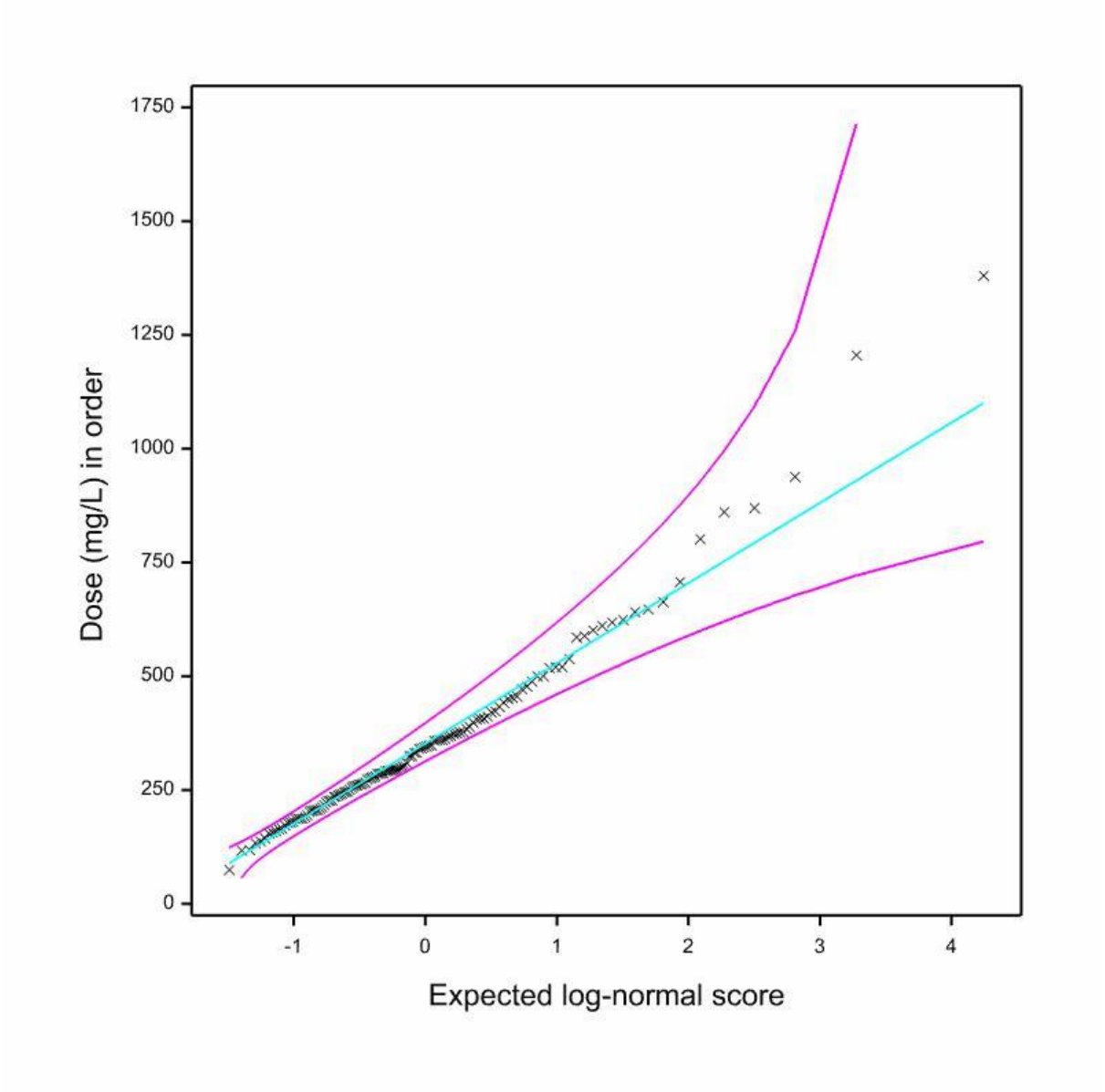


Figure 7.9. Dose received by a single patch of tissue over the years during the early part of the season.

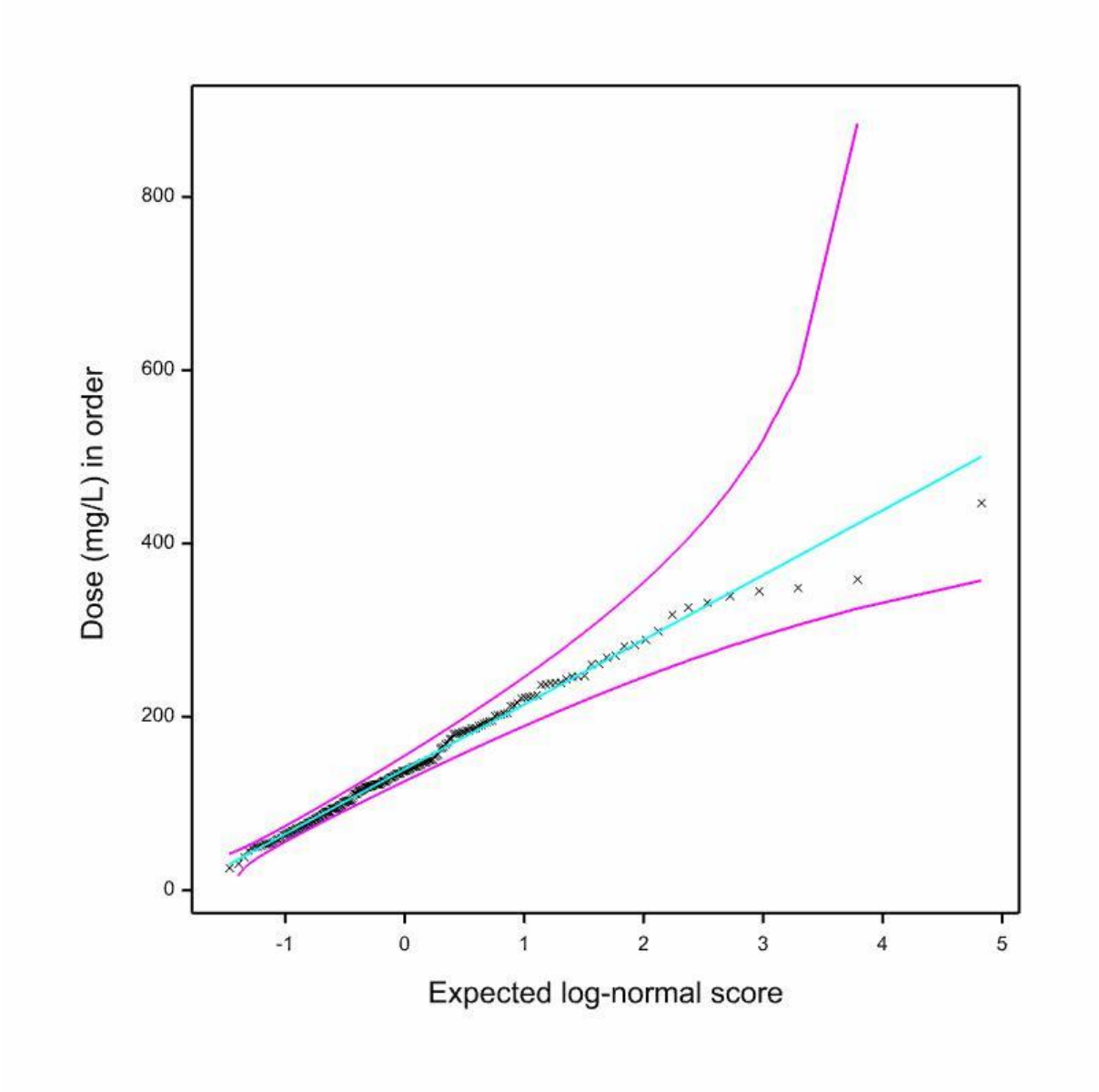


Figure 7.10. Dose received by a single patch of tissue over the years after full leaf stage.

7.4.1.9. Ascospore release

The number of spores released is around 2500 (average=2520 spores).Each year the number of spores released fluctuates as it depends on the rain chance and the timing of the rain (Figure 7.11).

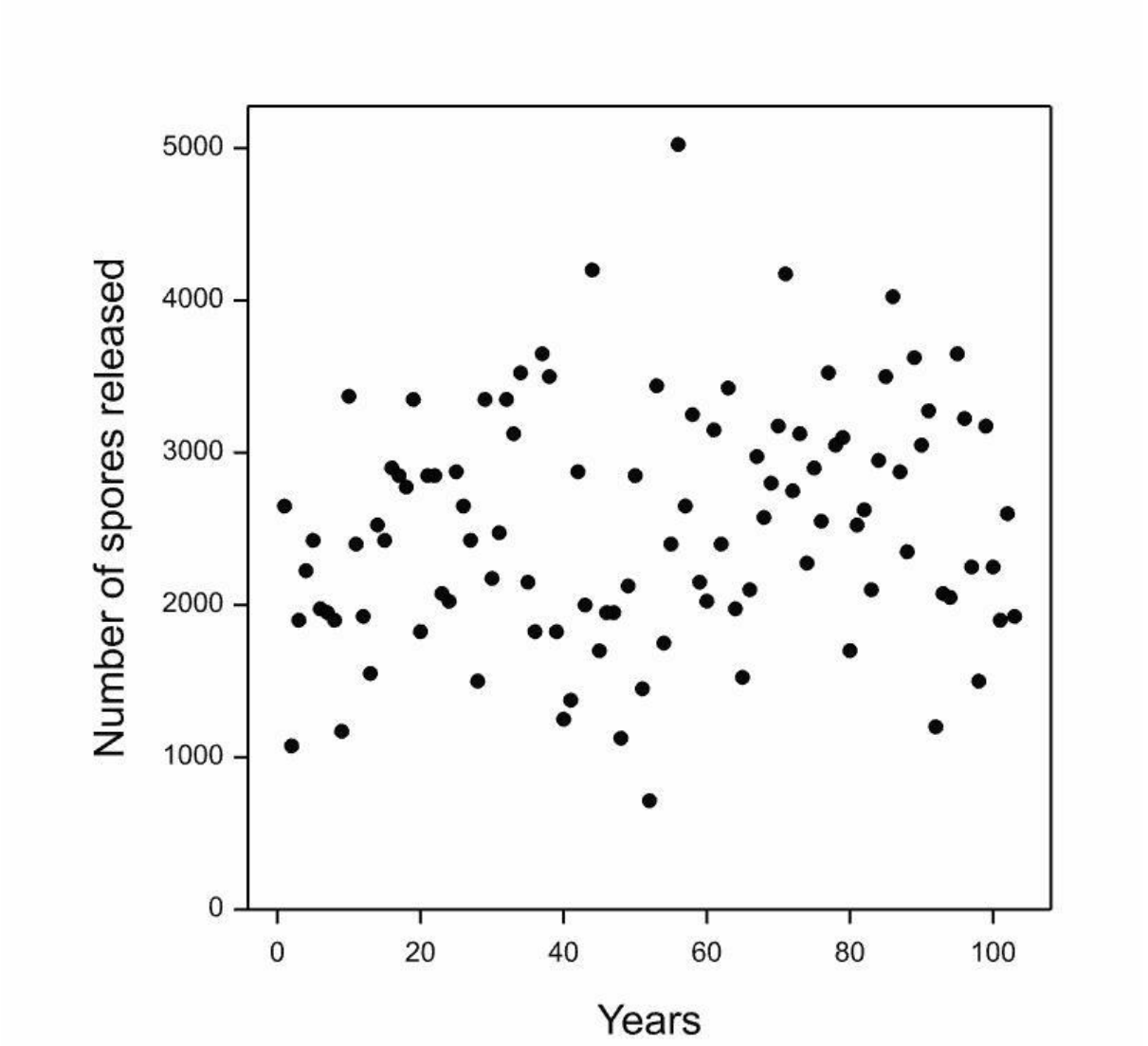


Figure 7.11. Number of ascospores released each year over more than 100 years.

7.4.2. Test scenarios

7.4.2.1. Gene interactions

The type of gene interactions and the number of genes both affected the time it took the mean sensitivity to reach the maximum level of resistance (Figure 7.12). The time increased with the number of genes in the additive scenario. The requirement of an

activator/suppressor that modifies the activity or expression of the other genes slows down the evolution of resistance relative to the additive scenario; in contrast, when each gene multiplies rather than adds to the trait, the evolution is quicker for a larger number of genes (eight genes), and similar or slower for four genes and two genes. In the epistasis scenarios, evolution was substantially slower for the two-gene scenarios. As there was one case where full resistance never evolved in one of the runs (Table 7.9), it seems the slow-down is due to alleles disappearing from the population or being found in low proportions. Cases where resistance did not evolve were observed in the single-gene scenario (Table 7.9), which were also due to the extinction of the resistance allele in the population.

Table 7.9. Account of cases in the test runs where the evolution did not progress to maximum resistance (mean sensitivity of 1 in the 0-1 mean sensitivity scale).

Scenario	Gene control	Evolution pattern	Proportion of runs with the pattern [1]
Pre-symptomatic activity	Single-gene	Resistance not evolving [2]	16/53
	Switch-type epistasis and two-gene control	Full resistance is not reached [3]	1/22
Post-symptomatic activity	Four-gene control and additive interactions	Resistance not evolving	8/29
		Full resistance is not reached	4/29
	Eight-gene control and additive interactions	Resistance not evolving	3/33
		Full resistance is not reached	6/33
	Ten-gene control and additive interactions	Resistance not evolving	6/42
		Full resistance is not reached	2/42

Scenario	Gene control	Evolution pattern	Proportion of runs with the pattern [1]
Post-symptomatic activity	Four-gene control with switch-type epistasis	Resistance not evolving	72/90
		Full resistance is not reached	5/90
	Eight-gene control with switch-type epistasis	Resistance not evolving	9/21
		Full resistance is not reached	1/21
	Four-gene control with multiplicative epistasis	Resistance not evolving	76/95
		Full resistance is not reached	9/95
	Eight-gene control with multiplicative epistasis	Resistance not evolving	14/28
		Full resistance is not reached	4/28
Post-symptomatic activity with primary inoculum including conidia as well as ascospores	Four-gene control with additive interactions	Resistance not evolving	14/40

[1] This proportion is relative to the total number of runs for that scenario.

[2] Resistance not evolving: the population of isolates is quickly eliminated, so there is no evolution of resistance.

[3] Full resistance is not reached: mean sensitivity rises above 0, but does not reach the maximum value of 1.

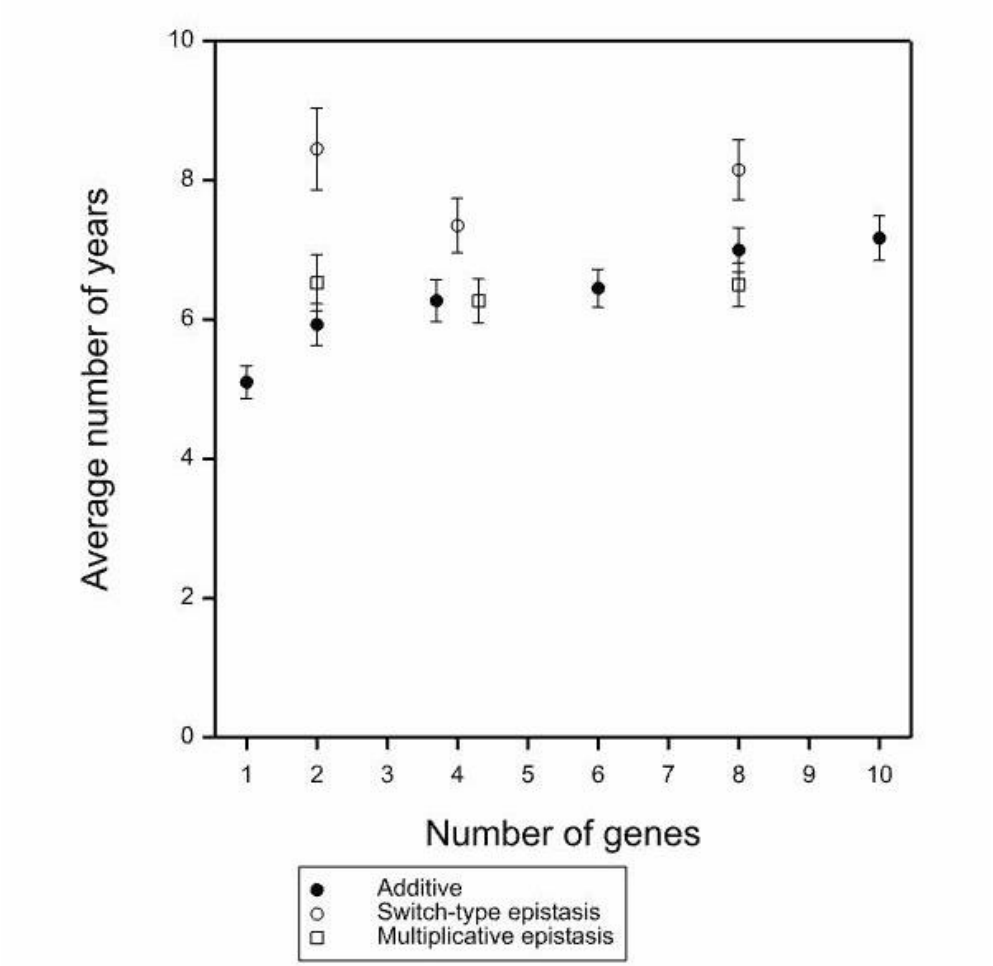
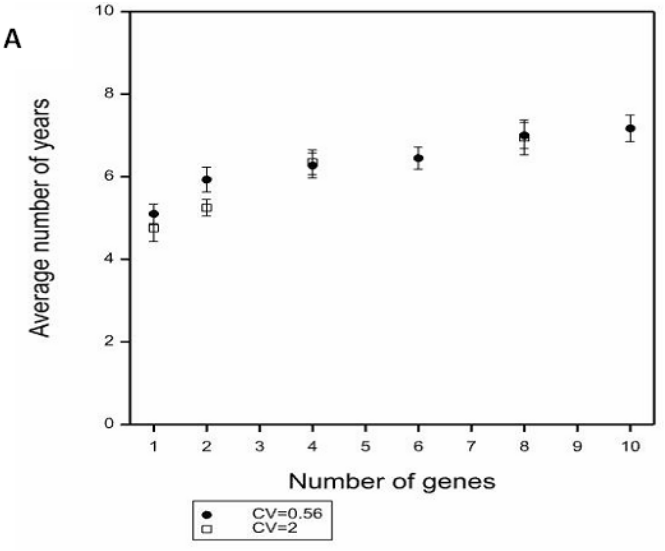


Figure 7.12. Average (mean) number of years for the mean sensitivity of the population to reach maximum resistance when resistance is controlled by different number of genes under three types of gene interactions (additive, epistasis where control depends of the presence of an activator or suppressor, or a type of epistasis where genes contribute to the resistance multiplicatively). Superimposed points have been separated along the x axis. Error bars are calculated as 2SEM on either side. Only scenarios where the maximum resistance was reached are included in this graph (Table 7.9).

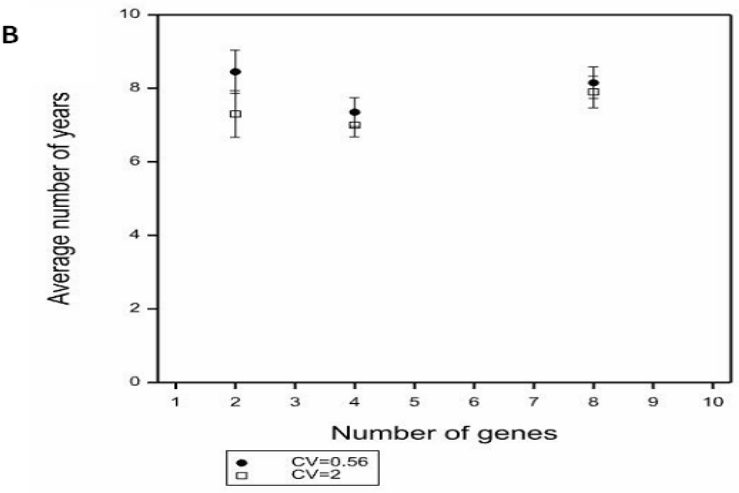
7.4.2.2. Coefficient of variation

Increasing the coefficient of variation in spray distribution seemed to speed up evolution (Figures 7.13A, 7.13B, 7.13C), clearly for the cases with epistasis background and slightly for the additive case. It seems likely that the low coefficient of variation (0.56) increases selection pressure, resulting in a higher mortality of isolates at the end of each year due to a higher fungicide input, as explained in the observations section of this chapter.

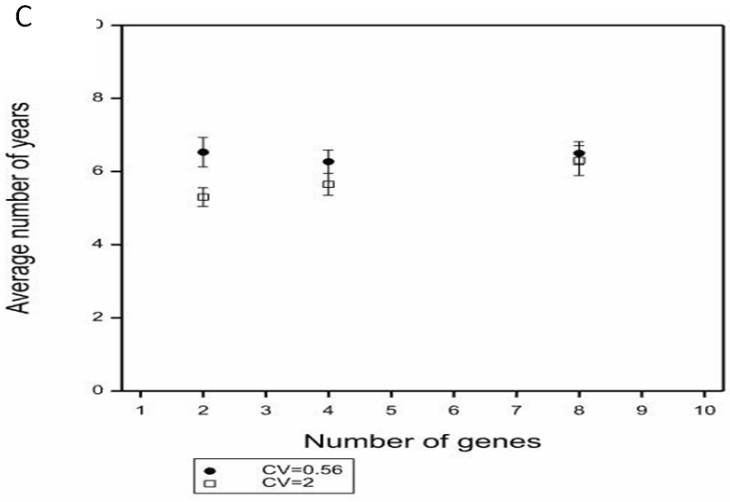
A


Average number of years
Number of genes
CV=0.56
CV=2

B


Average number of years
Number of genes
CV=0.56
CV=2

C


Average number of years
Number of genes
CV=0.56
CV=2

Figures 7.13A, 7.13B and 7.13C. Average (mean) number of years for the mean sensitivity of the population to reach maximum resistance under sprays with doses with different coefficient of variation (CV) when resistance is controlled by different number of genes with three types of interactions: additive (A), epistasis where control depends of the presence of an activator or suppressor (B) or epistasis where genes contribute to the resistance multiplicatively (C). Error bars are calculated as 2SEM on either side.

7.4.2.3. Conidia as primary inoculum

The presence of conidia as primary inoculum does not seem to greatly affect the time it takes to reach maximum resistance (Figure 7.14).

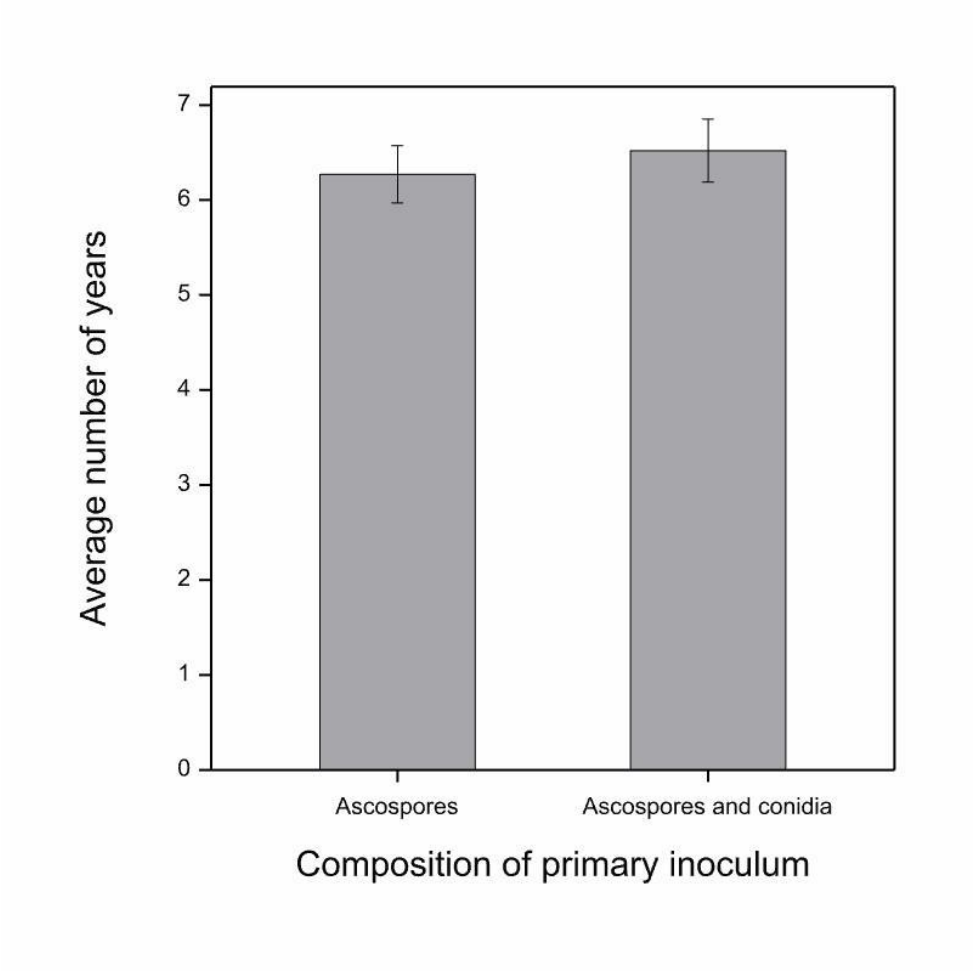


Figure 7.14. Average (mean) number of years for the mean sensitivity of the population to reach maximum resistance with primary inoculum composed of only ascospores or of overwintered conidia and ascospores. Both scenarios were tested considering four additive gene control, and other parameters were set to the defaults specified in Tables 7.1, 7.2 and 7.3. Error bars are calculated as 2SEM on either side.

7.4.2.4. Post-symptomatic activity

If the effect on reproduction continues through the lifetime of an isolate, evolution sped up (Figure 7.15). Trends for the additive and switch epistasis scenarios were maintained. On the other hand, multiplicative epistasis generated an increase in the evolution rate (Figure 7.16),

and the addition of overwintering conidia to the primary inoculum slows down evolution (Figure 7.17). All post-symptomatic scenarios presented cases that did not reach full resistance or where the disease was quickly eliminated in the first year (Table 7.9). Some scenarios presented a rather high number of such cases, especially scenarios with either type of epistasis. As an example, for the post-symptomatic scenarios considering only additive interactions, there were the following cases where maximum resistance was not reached:

For four-gene control, in eight out of 29 cases the population was quickly eliminated, and from the cases that survived past that first year, four out of 21 cases did not evolve to maximum resistance, but instead only reached a lower resistance level. For eight-gene control, in three out of 33 runs the population was eliminated early, and from the remaining cases, six out of 30 did not reach maximum resistance. For the ten-gene scenario, the proportions were six out of 42 (cases where the disease was quickly eradicated) and two out of 36 (the cases, out of those remaining, that did not reach maximum resistance). The proportion of runs where isolates were quickly eliminated and the proportion of runs where isolates did not reach full resistance were not found to be different across the range of gene numbers by a Chi-square test ($p=0.130$ and $p=0.173$, respectively).

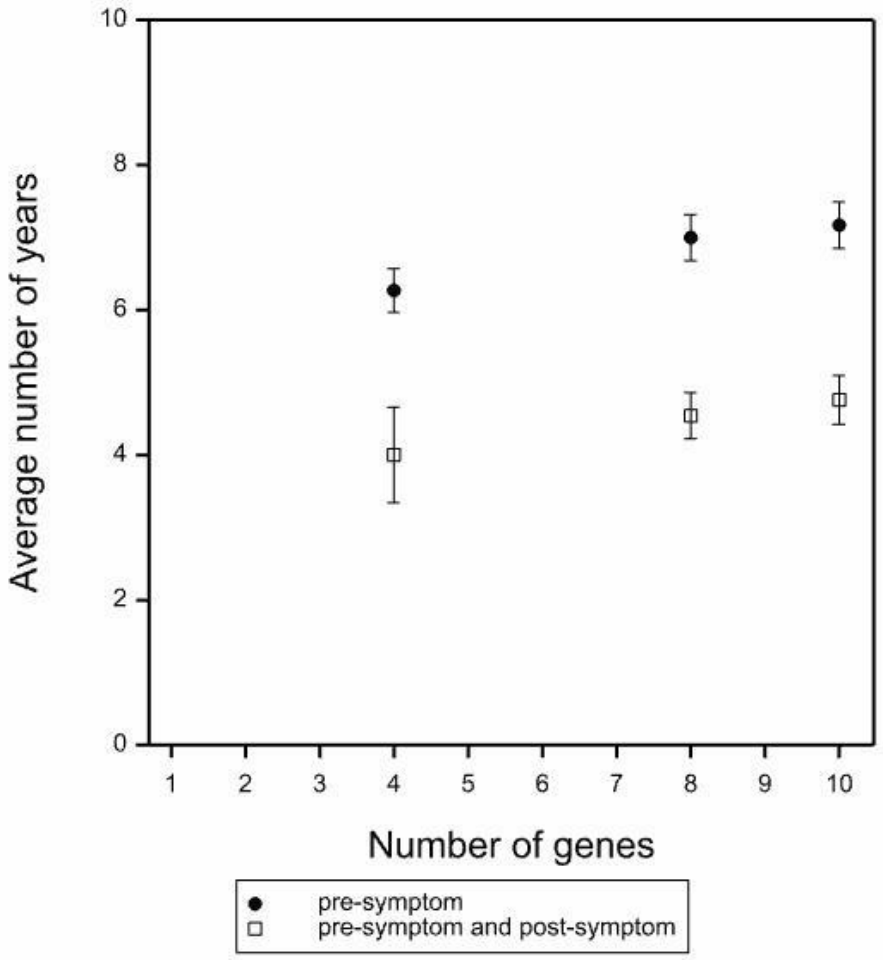


Figure 7.15. Average (mean) number of years for the mean sensitivity of the population to reach maximum resistance if the fungicide only has pre-symptomatic activity or both pre-symptomatic and post-symptomatic activities with resistance controlled by different number of genes in an additive scenario. Error bars are calculated as 2SEM on either side. In the post-symptomatic scenario, only cases evolving to maximum resistance are included in the graph (Table 7.9).

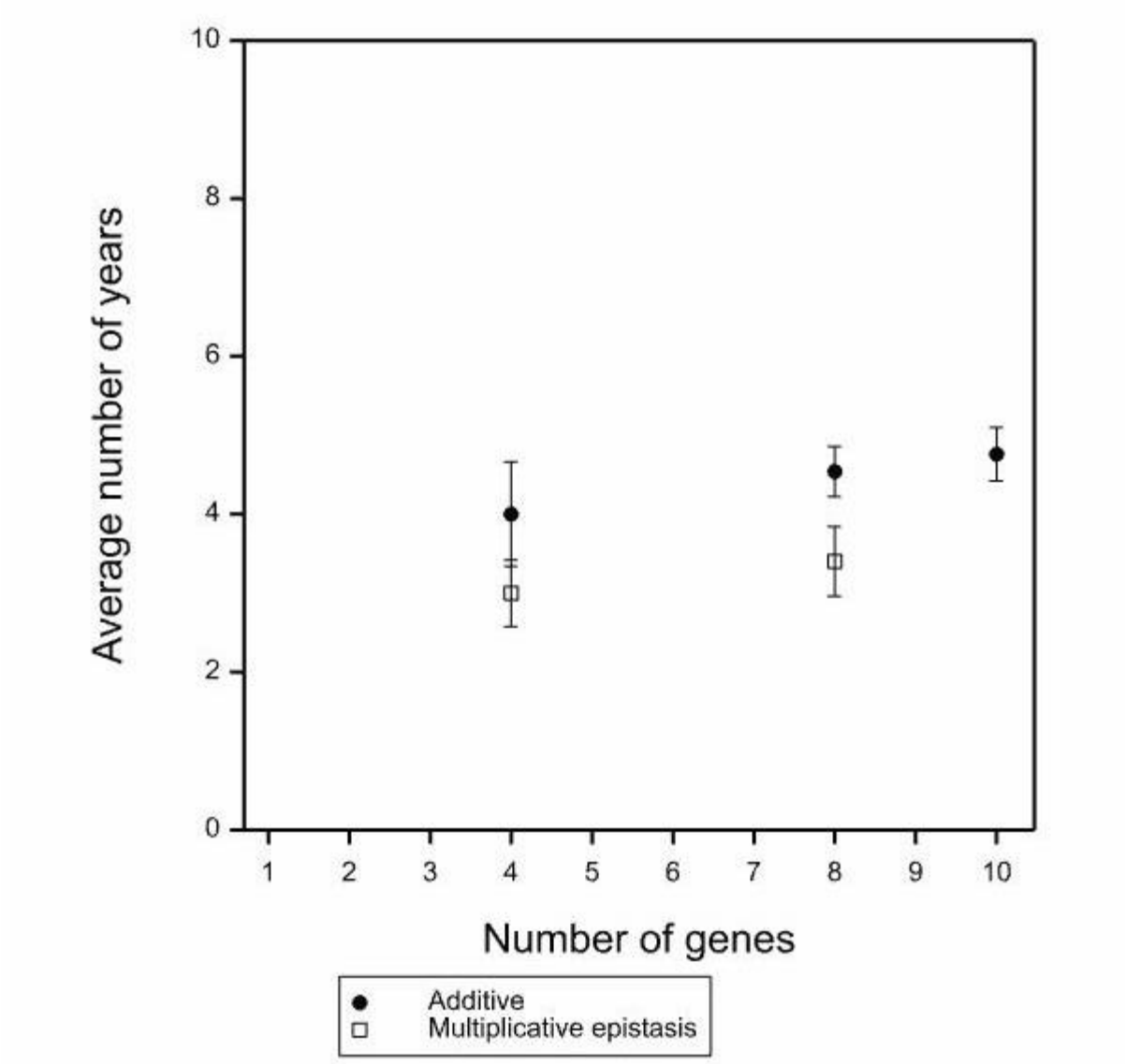


Figure 7.16. Average (mean) number of years for the mean sensitivity of the population to reach maximum resistance if the fungicide presents both pre-symptomatic and post-symptomatic activities and the resistance is controlled by different number of genes under additive or multiplicative epistasis scenarios. Error bars are calculated as 2SEM on either side. Only cases were maximum resistance was reached have been included in this graph (Table 7.9).

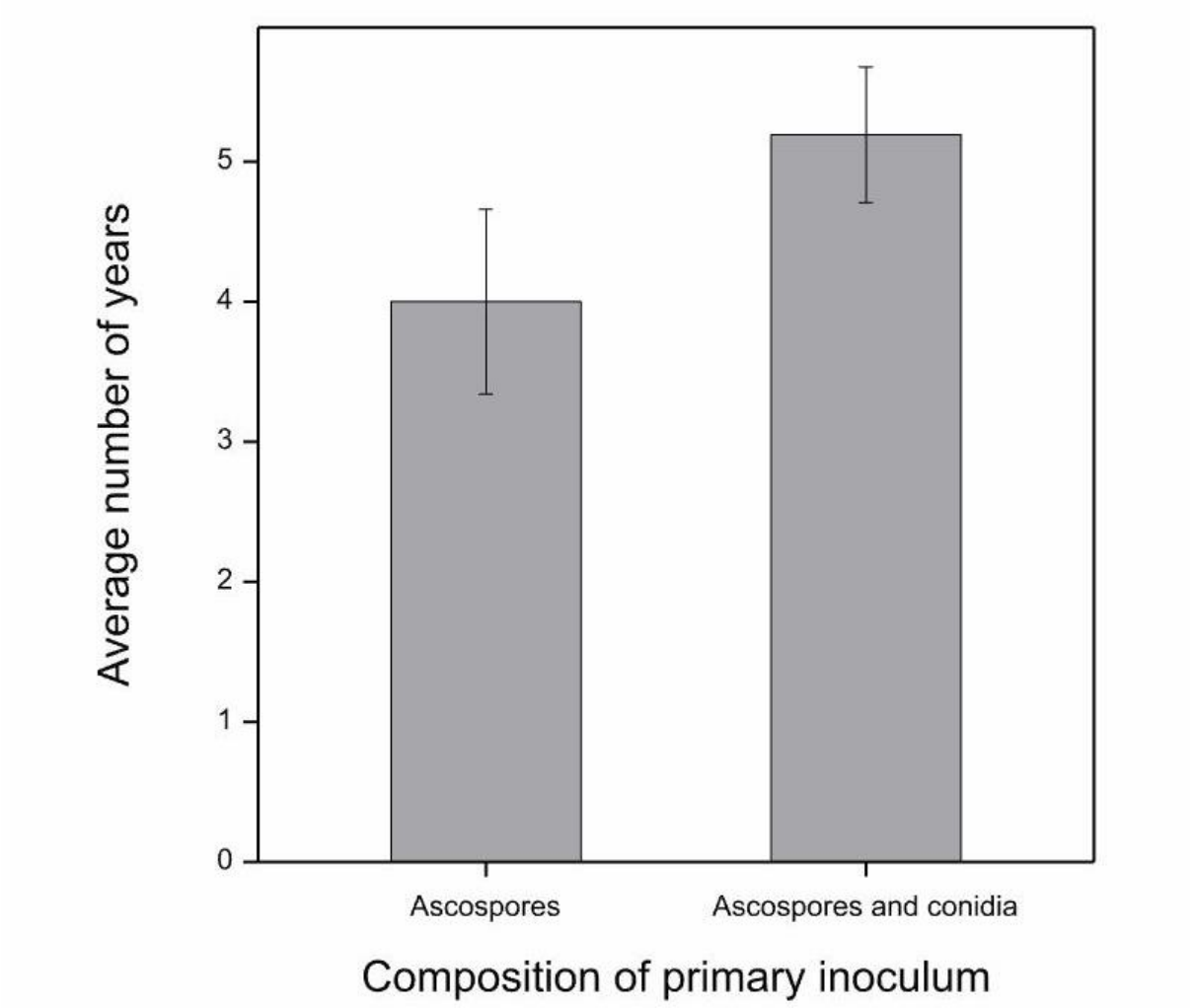


Figure 7.17. Average (mean) number of years for the mean sensitivity of the population to reach maximum resistance with primary inoculum composed of only ascospores or of overwintered conidia and ascospores, if the fungicide has strong post-symptomatic activity in addition to pre-symptomatic activity. Error bars are calculated as 2SEM on either side. Only cases that evolved to maximum resistance are included in this graph (Table 7.9).

7.5. Discussion

The model presented here has the advantage over pre-existing models of looking into a polygenic resistance behaviour and the effect of other factors such as epistasis in resistance evolution.

The results in this study contradict findings from other studies that suggested higher coverage (Kable and Jeffrey 1980; Skylakakis 1982), persistence of the fungicide (Chin 1987) or variability of the dose (Shaw 2000) would result in slower evolution. However, the difference in the findings is likely to be a product of differences in the initial assumptions and design of the model. The previous studies mentioned usually represented the fungicide effect as an elimination of isolates regardless of the stage of infection the isolate was at (pre-symptomatic or post-symptomatic), which implies all isolates affected by the fungicide ceased to be included in the mean sensitivity of the population and isolates with higher resistance at the end of the year would not get disproportionately affected by the higher fungicide concentrations that may build up at the end of the year. In the current study, only pre-symptomatic activity has been included in most scenarios. Evidence for post-symptomatic activity is vaguer, with some articles finding some activity and others finding no or weak activity (O'Leary and Sutton 1986; Poblete and Latorre 2001). This pre-symptomatic activity can only occur in a short time window after infection, and includes both effects on lesion development and effects on conidospore and ascospore production (Schwabe, Jones et al. 1984; O'Leary and Sutton 1986). As fungicide concentrations increase over the course of a year in the model in high selection scenarios, isolates that are actively infecting towards the end of the year encounter high fungicide concentrations, which often even the most resistant isolates cannot survive. This leads to a shift toward lower sensitivity of the population and slower evolution rate, as sensitive isolates at the beginning of the year may survive the lowest concentrations present at the time and contribute genes to the following year, while toward the end of the year resistant isolates may not survive the high concentrations accumulated. However, this pattern of evolution could potentially not be present in the field because, as mentioned throughout this chapter, the fungicide may not

accumulate to the rates observed in this model. Fungicide residues could be washed off by rain (Xu, Murray et al. 2008) or have a faster degradation rate, preventing accumulation, and at the same time new tissue without any residue would keep appearing during part of the season (Carisse, Jobin et al. 2008), minimising the effect of fungicide accumulation on the evolution rate. Isolates that have impaired reproduction due to the fungicide effect, but have been able to produce a lesion have been included in the calculation of the mean sensitivity, which also would shift the mean sensitivity toward less resistance. The decision to include these isolates in the mean sensitivity comes from the way isolates are sampled for sensitivity tests. Isolates are sampled from leaf lesions and tested for sensitivity, then used to calculate the mean sensitivity of a population (Xu, Gao et al. 2010; Chapman 2011; Pfeufer and Ngugi 2012). This process does not usually require isolates to be pre-tested for spore production unless the research team is also interested in this aspect.

The current study points to the evolution of resistance slowing down with more genes controlling it. It is known in practice that qualitative single-gene resistance evolves quickly, while quantitative polygenic resistance tends to be slower (Brent and Hollomon 2007). Routes to resistance that depend on a mutation on a regulatory component (activator/suppressor) to contribute to the resistance would lead to a slower evolution than cases where mutations on these regulatory components are not required; in this study, only a scenario in which the whole route depended on the regulatory component was tested, but it is likely that if only one component needs the action of the regulator it would still lead to a slow-down as those two alleles will still need to be inherited together for the whole system to work. The effect of epistasis in which alleles multiply rather than add to the resistance is less clear from the results, although it seems that resistance is sped up to a certain point

which seems more clear with a higher coefficient of variation and the inclusion of post-symptomatic activity. Other factors have been said to affect the evolution when epistatic interactions are required. For example, the rate of evolution is less if there is negative selection for another associated trait (Wade 2000).

Including conidia as a component of the primary inoculum did not significantly affect the evolution rate if only pre-symptomatic activity is considered; only a very slight delay occurred. However, if post-symptomatic activity was also included, overwintering conidia produced a bigger delay on the evolution. Previous studies have argued for conidia to partially account for the variability of sensitivity within an orchard, with different parts of an orchard containing isolates with different levels of sensitivity (Gao, Berrie et al. 2009).Our findings may give some idea of the effect of overwintering conidia on the evolution of resistance in the whole orchard population. Previous studies have supported faster evolution with long range dispersal (Brent and Hollomon 2007), so it could be that having more spores with short range dispersal in the primary inoculum (in the model spores disperse uniformly within a radius, which is much larger for ascospores than conidia) is causing a slow-down of the evolution in the model.

As mentioned before, studies about post-symptomatic effects have reported mixed results; of those that have found an effect, reduced conidiospore production and spore germination (O'Leary and Sutton 1986; Sharma and Gupta 1995; Poblete and Latorre 2001) or burn-out of lesions (Cross, Berrie et al. 2017) have been cited as effects. In the current study, a few tests with post-symptomatic activity in addition to the pre-symptomatic activity were carried out to determine the effect this kind of activity would have on the evolution and to make sure the general trends observed when only pre-symptomatic activity was included were not

altered. On the whole, the inclusion of post-symptomatic activity seems to speed up evolution as, in the current study, eliminating reproduction of isolates (both for conidia and ascospores) beyond the pre-symptom window resulted in faster evolution. Effects on ascospore production have been described when treating fallen leaves and as a result of within season spraying (O'Leary and Sutton 1986); these effects would probably count as a post-symptomatic effect, although one that cannot be appreciated until the end of the season. By extrapolation from the current results, this also probably speeds up evolution.

For the additive and switch-type epistasis cases, inclusion of post-symptomatic effects did not alter the trends observed in the simulations with pre-symptomatic effects only. The scenario including conidia as part of the primary inoculum showed slower evolution, whereas multiplicative epistasis accelerated evolution of resistance. However, these last two trends do seem to be present, although much more vaguely, in the pre-symptomatic results where the conidia as a component of the primary inoculum scenario shows a very small delay and multiplicative epistasis seems to be speeding up the evolution at times, more clearly with a greater coefficient of variation. Therefore, the results presented here are probably in most cases valid for fungicides with strong post-symptomatic activity as well as pre-symptomatic.

In general, the factors studied only had small effects on the evolution rate (40% increase in the time to reach maximum resistance for the ten-gene additive scenario relative to the single-gene scenario, the four-gene and eight-gene switch-type epistasis scenarios only increased the time by 17% or less relative to the equivalent additive scenarios, the inclusion of conidia in the primary inoculum only achieved a 4% increase, and there was a maximum 36% decrease with post-symptomatic activity scenarios with additive interactions and 46%

decrease when increasing spray coverage from 20% to 40%). This leaves open the question of why resistance to myclobutanil has evolved so slowly and not become widespread. One answer could be all the apple trees grown in homes and abandoned orchards which may act as a reservoir for sensitive strains.

8. General discussion

This thesis was mainly designed to understand the genetics behind the variability in sensitivity to DMI fungicides Systhane (myclobutanil) and Folicur (tebuconazole) in *V. inaequalis*. Previous work (Schnabel and Jones 2001; Jobin and Carisse 2007; Xu, Gao et al. 2010) had suggested it was likely that the resistance was under polygenic control, as field isolates showed a spectrum of sensitivity levels rather than just a sensitive and an insensitive phenotype. Experiments were designed to confirm this hypothesis and to elucidate some of the mechanisms involved, including the importance and role of the fungicide target *CYP51A1*. The latter has been shown to participate in resistance development in a number of pathogens either through changes in expression patterns or mutations in its gene sequence (Mullins, Parker et al. 2011; Cools, Hawkins et al. 2013). In addition, the prospective use of tebuconazole as a substitute for myclobutanil, which has been shown to be losing efficacy in a number of countries (Jobin and Carisse 2007; Gao, Berrie et al. 2009; Chapman 2011; Beresford, Wright et al. 2012; Pfeufer and Ngugi 2012; Villani, Biggs et al. 2015), was also studied.

To achieve the aims set before the start of the project, the work needed to start with a group of isolates with different backgrounds of myclobutanil use. For determining the sensitivity levels of these isolates there was a range of techniques available; those most widely used like measuring the diameter of single spore isolates or the germ tube of spores washed off a leaf disc were time-consuming or had the danger of screening more than one genotype (Raposo, Colgan et al. 1995; Xu, Gao et al. 2010) and would take time which could be used for other experiments. In an attempt to save time, an automated method based on a microplate and absorbance readings was considered as a faster alternative that would

allow for the testing of a large number of isolates in a smaller amount of time. Such a method does not seem to have been developed for use in apple scab, and thus it needed to be adapted for this pathogen. Unfortunately, the adaptation of the microplate format developed during this thesis failed, leading back to the use of the more time-consuming traditional methods.

During the sensitivity tests, increased resistance was observed in *V. inaequalis* isolates from orchards with disease control problems relative to isolates from an unsprayed orchard. In the tests, tebuconazole was not found to be a good substitute for myclobutanil, as the two chemicals showed a strong cross-resistance.

A selection of isolates from those screened with varying degrees of sensitivity was chosen to sequence the *CYP51A1* gene to determine what role, if any, it may play in the development of resistance. Only two isolates, sensitive to the fungicide, had mutations and both of them were silent. Other research studies (Schnabel and Jones 2001; Villani, Hulvey et al. 2016) that have tried to find mutations in the *CYP51A1* gene sequence have failed to discover any mutant alleles connected with resistance in *V. inaequalis*. Previous studies have found a link between overexpression of the gene and resistance (Schnabel and Jones 2001; Villani, Hulvey et al. 2016); one of the most recent found repetitive elements upstream the gene which correlated with resistance to difenoconazole (Villani, Hulvey et al. 2016). The current study found three isolates that were likely to have insertions upstream the gene; however, neither of them had a high level of resistance.

The main finding of this thesis is the confirmation of polygenic control in the resistance to a DMI fungicide (tebuconazole) , which was one of the initial hypotheses that had led to this thesis and which other authors (Jobin and Carisse 2007) had also proposed as an explanation

to the patterns of the sensitivity phenotypes observed in isolate populations. The distribution of the progeny of a sensitive- resistance cross followed the pattern that would be expected under polygenic control. In addition, the progeny showed some epistasis as the mean sensitivity of the ascospore offspring was significantly below the mean of the parent isolates.

These results led to a modelling study to understand how a polygenic resistance trait would evolve. In most cases, resistance evolves slower the more genes involved. With epistasis under a regulating element (activator/suppressor), resistance evolves more slowly than in the additive scenario. Conidia as primary inoculum (Gao, Berrie et al. 2009; Passey, Robinson et al. 2017) would either not have any great effect on evolution or slow evolution down if the fungicide has a significant post-symptomatic activity, defined in the theoretical model in this thesis as all antisporulant activity happening after the first five days following an infection. If post-symptomatic activity (antisporulant activity) on conidia and ascospore production is strong, the evolution is faster. If there is only pre-symptomatic activity, which occurs in isolates sprayed during the first five days after an infection and results in either no lesions developing or only lesions that do not produce spores, higher selection pressure scenarios driven by greater coverage or slower degradation of the fungicide, contrary to what could be expected, slow down evolution. The same effect happens with less variability in the dose distribution. This effect comes from pre-symptomatic activity only occurring in a short time window (three days from infection for preventing lesion development and five days to stop spore production) (Schwabe, Jones et al. 1984; O'Leary and Sutton 1986) and accumulation of the fungicide, due to a greater fungicide input or slow fungicide elimination depending on the scenario, towards the end of the season in all these scenarios. These two

factors combined result in more isolates, including highly resistant isolates, being affected in higher numbers at the end of each year, while more sensitive isolates at the start of the year are more likely to develop into lesions without being affected by the fungicide. It is important to point out that this may not occur in the field as other factors such as rain that rinses off the chemical (Xu, Murray et al. 2008) or differences in degradation rate would prevent fungicide accumulation. The model in this thesis did not include many aspects of tissue dynamics such as the continuous emergence of new plant tissue during part of the season (Carisse, Jobin et al. 2008) which would still not have received any sprays and would probably greatly diminish the chances of fungicide accumulation on the plant tissue in nature. Also, there are still a lot of pathogen and fungicide characteristics for which their impact on fungicide evolution is not well understood or may be even unknown. So the model predictions in this thesis are theoretical and within a strict set of conditions.

Reports across the world indicate that fungicide resistance to DMIs in *V. inaequalis* is on the rise (Chapter 1, Table 2), and the research carried out in this thesis (Chapter 4) adds to this trend. However, the rate of evolution has not followed the trend expected, a quick evolution of resistance which becomes widespread. It was thought this could be due to a percentage of primary inoculum comprising conidia (Passey, Robinson et al. 2017) slowing down evolution and placing a limit on how the resistance spreads, but the model predictions in this thesis do not seem to support this (Chapter 7). Other factors that could explain why resistance has not yet become widespread could be the presence of apple trees in gardens which are not sprayed and could act as refugia for sensitive strains. In any case, the increase of resistance makes it important to continue investing in developing new products to replace those chemicals in danger of falling out of use, and to understand the basis of resistance

development so as to design strategies to increase the life of current and future products. It does not seem as if mutations in the sequence of *CYP51A1* are an important resistance mechanism in *V. inaequalis* (Schnabel and Jones 2001; Villani, Hulvey et al. 2016), as there have not been any studies that have found isolates displaying this mechanism. Apart from overexpression of the demethylase gene (Schnabel and Jones 2001; Villani, Hulvey et al. 2016), other possible candidate causes of resistance identified in other studies have been other genes in the ergosterol biosynthesis pathway, proteins related to melanisation, proteins involved in phospholipid biosynthesis and transport proteins (de Waard, Andrade et al. 2006; Lendenmann, Croll et al. 2015) like the ABC family of transporters. More than one mechanism of resistance can be present, resulting in polygenic resistance like that observed in the sensitive- resistance cross of the current study (Chapter 6). The presence of more than one gene would slow down resistance evolution (Chapter 7); the extent at which the evolution gets slowed would depend on the number of genes, being slower with higher number of genes (Chapter 7). As epistasis can also be involved in cases of polygenic control (Chapters 6 and 7), the rate of evolution may be slower (if the epistasis requires a regulatory element), or be unchanged, or possibly increased if the genes interact multiplicatively. However, even though all these factors (epistasis and number of genes) can affect the evolution of the resistance, the magnitude of their effects is quite small (Chapter 7).

To increase our knowledge of fungicide resistance to DMI chemicals in *V. inaequalis* a number of steps need to be taken. The optimisation of the microplate method in this thesis failed, but adjustments in type of inoculum, like trying to use spores instead of fragmented mycelia, might yield better results. As some isolates show poor sporulation *in vitro* (Kirkham 1957) it may be important to find ways to increase sporulation in order to make a potential

automation method based on spores to be viable. Further research on genetic elements upstream of the *CYP51A1* gene associated to both overexpression and resistance may help discover new pieces of important information. Since the thesis here confirmed the existence of polygenic control in the sensitivity to DMI fungicides (Chapter 6), it is important to find what other mechanisms that do not involve the *CYP51A1* gene can result in fungicide resistance. Research could start with some of the candidates that have been listed above. As the presence of a resistance mechanism does not guarantee that a resistant isolate can establish a resistant population, more research into fitness costs would also help us estimate the likelihood of field resistance. Chapman (2011) did some *in vitro* studies of fitness by comparing the growth and conidia production of isolates (sensitive and resistant) in PDA plates with no fungicide. However, as in nature isolates would be growing in plant tissue, fitness tests involving plant material would be better to determine if resistant isolates suffer any fitness cost.

9. Bibliography

Acquaah, G. (2007). Principles of Plant Genetics and Breeding, Blackwell Publishing.

Adedeji, F. O. (1986). "Effect of fertilizer on microbial decomposition of leaf litter of a regenerating bush-fallow in sub-humid tropical Nigeria." Agriculture, Ecosystems and Environment **18**: 155-166.

Albert, J. J. and F. H. Lewis (1962). "Effect of repeat applications of dodine and of captan on apple scab foliage lesions." Plant Disease Reporter **46**(3): 163-167.

Aylor, D. E. (1998). "The Aerobiology of Apple Scab." Plant Disease **82**(8): 838-849.

Barbara, D. J., A. L. Roberts, et al. (2008). "Virulence characteristics of apple scab (*Venturia inaequalis*) isolates from monoculture and mixed orchards." Plant Pathology **57**: 552-561.

Beresford, R. M., G. B. Follas, et al. (2014). "Fungicide resistance management strategy for apple black spot (*Venturia inaequalis*) in New Zealand." Retrieved 2018, from https://resistance.nzpps.org/index.php?p=crops/apples_blackspot.

Beresford, R. M., P. J. Wright, et al. (2012). "Sensitivity of Venturia inaequalis to myclobutanil, penconazole and dodine in relation to fungicide use in Hawke's Bay apple orchards." New Zealand Plant Protection **65**: 106-113.

Beresford, R. M., P. J. Wright, et al. (2013). "Resistance of Venturia inaequalis to demethylation inhibitor and dodine fungicides in four New Zealand apple-growing regions." New Zealand Plant Protection **66**: 274-283.

Berrie, A. and J. Cross (2006). "Development of an integrated pest and disease management system for apples to produce fruit free from pesticides residues - Aspects of disease control." IOBC, WPRS, Bulletin **29**(1): 129-138.

Berrie, A. and X. M. Xu (2003). "Managing apple scab (*Venturia inaequalis*) and powdery mildew (*Podosphaera leucotricha*) using Adem^{TM}." International Journal of Pest Management **49**(3): 243-249.

Bolton, M. D., M. K. Ebert, et al. (2016). "RNA-sequencing of *Cercospora beticola* DMI-sensitiveand -resistant isolates after treatement with tetraconazole identifies common and contrasting pathway induction." Fungal Genetics and Biology **92**: 1-13.

Bowen, K. J., C. H. Mesarich, et al. (2011). "*Venturia inaequalis* the causal agent of apple scab." Molecular Plant Pathology **12**(2): 105-122.

Brent, K. J. and D. W. Hollomon (2007). Fungicide Resistance in Crop Pathogens : How can it be managed? FRAC.

Brown, S. K. and K. E. Maloney (2005). Biotechnology of Fruit and Nut Crops. R. E. Litz, CABI Publishing.

Burchill, R. T. (1968). "Field and laboratory studies of the effect of urea on ascospore production of *Venturia inaequalis* (Cke.) Wint." Annals of Applied Biology **62**: 297-307.

Burchill, R. T., K. E. Hutton, et al. (1965). "Inhibition of the Perfect Stage of *Venturia inaequalis* (Cooke) Wint., by Urea." Nature **205**: 520-521.

Bus, V. G., E. H. Rikkerink, et al. (2011). "Revision of the nomenclature of the differential host-pathogen interactions of *Venturia inaequalis* and *Malus*." Annual Reviews Phytopathology **49**: 391-413.

Cabizza, M., F. Dedola, et al. (2012). "Residues behaviour of some fungicides applied on two greenhouse tomato varieties different in shape and weight." Journal of Environmental Science and Health, Part B **47**(5): 379-384.

Carisse, O. and M. Dewdney (2002). "A review of non-fungicidal approaches for the control of apple scab." Phytoprotection **83**(1): 1-29.

Carisse, O., T. Jobin, et al. (2008). "Predicting apple leaf emergence from degree-day accummulation during primary scab period." Canadian Journal of Plant Science **88**: 229-238.

Carisse, O. and J. R. Pelletier (1994). "Sensitivity distribution of *Venturia inaequalis* to fenarimol in Quebec apple orchards." Phytoprotection **75**(1): 35-43.

Carter, H. E., B. A. Fraaije, et al. (2014). "Alterations in the predicted regulatory and coding regions of the sterol 14α-demethylase gene (*CYP51*) confer decreased azole sensittivity in the oilseed rape pathogen *Pyrenopeziza brassicae*." Molecular Plant Pathology **15**(5): 513-522.

Chapman, K. S. (2011). "Identification of Resistance to Multiple Fungicides in Field Populations of *Venturia inaequalis*." Plant Disease **95**(8): 921-926.

Chin, K. M. (1987). "A Simple Model of Selection for Fungicide Resistance in Plant Pathogen Populations." The American Phytopathological Society **77**(5).

Collier, R. and M. A. Else (2014). UK Fruits and Vegetable Production - Impacts of Climate Change and Opportunities for Adaptation. Climate Change Impact and Adaptation in Agricultural Systems. J. Fuhrer and P. J. Gregory, CAB International.

Cools, H. J., N. J. Hawkins, et al. (2013). "Constraints on the evolution of azole resistance in plant pathogenic fungi." Plant Pathology **62**: 36-42.

Cox, K. D. (2015). Fungicide Resistance in *Venturia inaequalis*, the Causal Agent of Apple Scab, in the United States. Fungicide Resistance in Plant Pathogens: Principles and a Guide to Practical Management. H. Ishii and D. W. Hollomon, Springer**:** 433-438.

Cox, K. D., N. L. Russo, et al. (2008). "QoI qualitative resistance and *CYP51A1* upstream anomalies in NY populations of the apple scab pathogen *Venturia inaequalis*." Phytopathology **98**

Cross, J., A. Berrie, et al. (2017). Apple Canker (*Neonectria ditissima*). Apple Best Practice Guide, AHDB Horticulture. Retrieved July, 2017, from

http://apples.ahdb.org.uk/apple-canker.asp.

Cross, J., A. Berrie, et al. (2017). Apple powdery mildew – additional information. Apple Best Practice Guide, AHDB Horticulture. Retrieved July, 2017, from

http://apples.ahdb.org.uk/apple-powdery-mildew-additional-information.asp#link4.

Cross, J., A. Berrie, et al. (2017). Apple scab - additional information. Apple Best Practice Guide, AHDB Horticulture. Retrieved July, 2017, from

http://apples.ahdb.org.uk/apple-scab-additional-information.asp#link1.

Cross, J., A. Berrie, et al. (2017). Crown Rot and Collar Rot (*Phytophthora cactorum, Phytophthora syringae*). Apple Best Practice Guide, AHDB Horticulture. Retrieved July, 2017, from

http://apples.ahdb.org.uk/Crown-Rot-and-Collar-Rot.asp.

Cusin, R., L. F. Revers, et al. (2017). "New biotechnological tools to accelerate scab-resistance trait transfer to apple." Genetics and Molecular Biology **40**: 305-311.

de Waard, M. A., A. C. Andrade, et al. (2006). "Impact of fungal drug transporters on fungicide sensitivity, multidrug resistance and virulence." Pest Management Science **62**: 195-207.

Deising, H. B., S. Reimann, et al. (2008). "Mechanisms and significance of fungicide resistance." Brazilian Journal of Microbiology **39**(2): 286-295.

Delye, C., F. Laigret, et al. (1997). "A mutation in the 14α-Demethylase Gene of *Uncinula necator* That Correlates with Resistance to a Sterol Biosynthesis Inhibitor

" Applied and Environmental Microbiology **63**: 2966-2970.

Dyer, P. S., J. Hansen, et al. (2000). "Genetic Control of Resistance to the Sterol 14α-Demethylase Inhibitor Fungicide Prochloraz in the Cereal Eyespot Pathogen Tapesia yallundae." Applied and Environmental Microbiology **66**(11): 4599-4604.

Errampalli, D. (2004). "Distribution of Myclobutanil Fungicide Sensitivities among Populations of *Venturia inaequalis*, the Causal Agent of Apple Scab, in Ontario." ISHS Acta Horticulturae **638**: 157-162.

FAOSTAT (2014). Search : production quantity of apples in all countries.

Ferree, D. C. and I. J. Warrington (2003). Apples: Botany, Production, and Uses, CABI Publishing.

Flores, F. J. and C. D. Garzon (2013). "Detection and assessment of chemical hormesis on the radial growth *in vitro* of oomycetes and fungal plant pathogens." Dose-Response **11**: 361-373.

FRAC (2015). "List of species Resistant to SDHIs April 2015." Retrieved 2018, from http://www.frac.info/docs/default-source/sdhi-wg/sdhi-references/list-of-species-resistant-to-sdhis-april-2015.pdf?sfvrsn=2d144a9a_2.

FRAC (2017). "FRAC Code List 2017." Retrieved July, 2017, from http://www.frac.info/docs/default-source/publications/frac-code-list/frac-code-list-2017-final.pdf?sfvrsn=fab94a9a_2.

FRAC (2017). "General recommendations for use (SBI Fungicides)." Retrieved July, 2017, from

http://www.frac.info/working-group/sbi-fungicides/general-use-recommendations.

FRAC (2017). "Monitoring Methods." Retrieved September, 2017, from http://www.frac.info/monitoring-methods.

FRAC (2017). "Resistance Overview- Mechanisms of Fungicide Resistance." Retrieved July, 2017, from http://www.frac.info/resistance-overview/mechanisms-of-fungicide-resistance.

FRAC (2017). "Resistance Overview- What is Resistance?". Retrieved July, 2017, from http://www.frac.info/resistance-overview.

Frac, M., A. Gryta, et al. (2016). "Fast and Accurate Microplate Method (Biolog MT2) for Detection of *Fusarium* Fungicides Resistance/Sensitivity." Frontiers in Microbiology **7**: 1-16.

Gao, L. Q., A. Berrie, et al. (2009). "Within- and between-orchard variability in the sensitivity of *Venturia inaequalis* to myclobutanil, a DMI fungicide, in the UK." Pest Management Science **65**: 1241-1249.

Georgopoulos, S. G. and G. Skylakakis (1986). "Genetic variability in the fungi and the problem of fungicide resistance." Crop Protection **5**(5): 299-305.

Gessler, C. and I. Pertot (2012). "Vf scab resistance of Malus." Trees **26**(1): 95-108.

Gladieux, P., X. G. Zhang, et al. (2008). "On the Origin and Spread of the Scab Disease of Apple: Out of Central Asia." PLoS ONE **3**(1): 1-14.

Granade, T. C., M. F. Hehmann, et al. (1985). "Monitoring of Filamentous Fungal Growth by *In Situ* Microspectrophotometry. Fragmented Mycelium Absorbance Density, and ^{14}C Incorporation: Alternatives to Mycelial Dry Weight." Applied and Environmental Microbiology **49**(1): 101-108.

Grierson, W. (2014). Fruit Development, Maturation, and Ripening. Handbook of Plant and Crop Physiology. M. Pessarakli, CRC Press.

Guo, C., J. Z. Li, et al. (2010). "Determination and Safety Evaluation of Difenoconazole Residues in Apples and Soils." Bulletin of Environmental Contamination and Toxicology **85**: 427-431.

Henriquez, J. L., O. Sarmiento, et al. (2011). "Sensitivity of *Venturia inaequalis* chilean isolates to difenoconazole, fenarimol, mancozeb, and pyrimethanil." Chilean Journal of Agricultural Research **71**(1): 39-44.

Hignett, R. C., J. H. Carder, et al. (1977). "Origin and Characteristics of *Venturia inaequalis* Melanoproteins Active in the Apple Scab Disease." Journal of General Microbiology **102**: 207-210.

Hobbelen, P. H. F., N. D. Paveley, et al. (2011). "Derivation and testing of a model to predict selection for fungicide resistance." Plant Pathology **60**: 304-313.

Hobbelen, P. H. F., N. D. Paveley, et al. (2014). "The Emergence of Resistance to Fungicides." PLoS ONE **9**(3): 1-14.

Hollomon, D. W. (2015). Fungicide Resistance: 40 Years on and Still a Major Problem. Fungicide Resistance in Plant Pathogens: Principles and a Guide to Practical Management. H. Ishii and D. W. Hollomon, Springer.

HSE (2017). Maximum Residue Level Database.

HSE (2017). Pesticide Register of UK Authorised Products.

Hwang, J. I. and J. E. Kim (2013). "Residue Patterns of Fungicides, Flusilazole and Myclobutanil in Apples." Current Research on Agriculture and Life Sciences **31**(4): 272-279.

Jackson, J. E. (2003). The Biology of Apples and Pears. Cambridge University Press.

Jobin, T. and O. Carisse (2007). "Incidence of Myclobutanil- and Kresoxim-Methyl-Insensitive Isolates of *Venturia inaequalis* in Quebec Orchards." Plant Disease **91**(10): 1351-1357.

Jones, E., S. Michael, et al. (2012). Basics of Assay Equipment and Instrumentation for High Throughput Screening. Assay Guidance Manual, NCBI.

Joseph-Horne, T. and D. W. Holomon (1997). "Molecular Mechanisms of Azole Resistance in Fungi." FEMS Microbiology Letters **149**: 141-149.

Joseph-Horne, T., N. J. Manning, et al. (1995). "Defective Δ5 (6) desaturase as a cause of azole resistance in *Ustilago maydis*." FEMS Microbiology Letters **127**(1-2): 29-34.

Kable, P. F. and H. Jeffrey (1980). "Selection for Tolerance in Organims Exposed to Sprays of Biocide Mixtures: A Theoretical Model." The American Phytopathological Society **70**(1).

Kearse, M., R. Moir, et al. (2012). "Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data." Bioinformatics **28**(12): 1647-1649.

Kirkham, D. S. (1957). "Relationship between Cultural Characters and Pathogenicity in *Venturia inaequalis* and *Venturia pirina*." Journal of General Microbiology **16**: 360-373.

Koenraadt, H., S. C. Somerville, et al. (1992). "Characterization of Mutations in the Beta-Tubulin Gene of Benomyl-Resistant Field Strains of *Venturia inaequalis* and Other Plant Pathogenic Fungi." Phytopathology **82**: 1348-1354.

Koller, W., D. Parker, et al. (2005). "Fungicide Resistance of Apple Scab: Status Quo and Management Options." New York Fruit Quarterly **13**(1): 9-17.

Koller, W., D. M. Parker, et al. (1991). "Baseline sensitivities of *Venturia inaequalis* to Sterol Demethylation Inhibitors." Plant Disease **75**(7): 726-728.

Koller, W., D. M. Parker, et al. (2004). "A Two-Phase Resistance Response of *Venturia inaequalis* Populations to the QoI Fungicides Kresoxim-Methyl and Trifloxystrobin." Plant Disease **88**: 537-544.

Koller, W. and W. F. Wilcox (1999). "Evaluation of Tatics for Managing Resistance of *Venturia inaequalis* to Sterol Demethylation Inhibitors." Plant Disease **83**(9): 857-863.

Koller, W. and W. F. Wilcox (2000). "Interactive Effects of Dodine and the DMI Fungicide Fenarimol in the Control of Apple scab." Plant Disease **84**(8): 863-870.

Koller, W., W. F. Wilcox, et al. (1997). "Detection and Quantification of Resistance of *Venturia inaequalis* Populations to Sterol Demethylation Inhibitors." Disease Control and Pest Management **87**(2): 184-190.

Kunz, S., H. Deising, et al. (1997). "Acquisition of Resistance to Sterol Demethylation Inhibitors by Populations of *Venturia inaequalis*." Phytopathology **87**(12): 1272-1278.

Leadbeater, A. (2014). Plant Health Management: Fungicides and Antibiotics. Encyclopaedia of Agriculture and Food Systems. N. K. van Alfen, Academic Press, Elsevier.

Leadbeater, A. and U. Gisi (2010). The Challenges of Chemical Control of Plant Diseases. Recent developments in Management of Plant Diseases. U. Gisi, I. Chet and M. L. Gullino, Springer.

Lendenmann, M. H., D. Croll, et al. (2015). "QTL Mapping of Fungicide Sensitivity Reveals Novel Genes and Pleiotropy with Melanization in the Pathogen *Zymoseptoria tritici*." Fungal Genetics and Biology **80**(53-67).

Locke, T., A. Berrie, et al. (2011). "Fungicide Resistance in Apple and Pear Pathogens." Retrieved July, 2017, from

http://adlib.everysite.co.uk/adlib/defra/content.aspx?id=13015.

Lucas, G. B., C. L. Campbell, et al. (1992). Introduction to Plant Diseases: Identification and Management, Springer.

Lundholt, B. K., K. M. Scudder, et al. (2003). "A Simple Technique for Reducing Edge Effect in Cell-Based Assays." Journal of Biomolecular Screening **8**(5): 566-570.

MacHardy, W. E. and D. M. Gadoury (1989). "Revision of Mills's Criteria for Predicting Apple Scab Infection Periods." Ecology and Epidemiology **79**(3): 304-310.

MacHardy, W. E., D. M. Gadoury, et al. (2001). "Parasitic and Biological Fitness of *Venturia inaequalis*: Relationship to Disease Management Strategies" Plant Disease **85**(10): 1036-1051.

Mavroeidi, V. I. (2004). Triazole Resistance in *Mycosphaerella graminicola*: Status, Genetic Basis and Effects of Low Doses and Mixtures. PhD thesis, University of Reading..

Mazzola, M. (2002). Biological Disease Control in the Production of Apple. Biological Control of Crop Diseases. S. Gnanamanickam, Marcel Dekker.

McDonald, J. M., D. P. Rice, et al. (2016). "Sex speeds adaptation by altering the dynamics of molecular evolution." Nature **000**: 1-15.

Meletiadis, J., J. W. Mouton, et al. (2001). "Comparison of Spectrophotometric and Visual Readings of NCCLS Method and Evaluation of a Colorimetric Method Based on Reduction of a Soluble Tetrazolium Salt, 2,3-Bis {2-Methoxy-4-Nitro-5-[(Sulfenylamino) Carbonyl]-2H-Tetrazolium-Hydroxide}, for Antifungal Susceptibility Testing of Aspergillus Species." Journal of Clinical Microbiology **39**(12): 4256-4263.

Met Office. (1981-2010). "Averages table for East Malling." Retrieved July, 2017, from http://www.metoffice.gov.uk/public/weather/climate/u107dpz9j.

Mikaberidze, A. and B. A. McDonald (2015). Fitness Cost of Resistance: Impact on Management. Fungicide Resistance in Plant Pathogens: Principles and a Guide to Practical Management. H. Ishii and D. W. Hollomon, Springer.

Mikaberidze, A., C. C. Mundt, et al. (2015). "Invasiveness of plant pathogens depends on the spatial scale of host distribution " Ecological Applications **26**: 1238-1248.

Mondino, P., L. Casanova, et al. (2015). "Sensitivity of *V. inaequalis* to trifloxystrobin and diefnoconazole in Uruguay." Journal of Phytopathology **163**: 1-10.

Mullins, J. G. L., J. E. Parker, et al. (2011). "Molecular Modelling of the Emergence of Azole Resistance in *Mycosphaerella graminicola*." PLoS ONE **6**(6): e20973.

Mundt, C. C., K. J. Leonard, et al. (1986). "Computerized Simulation of Crown Rust Epidemics in Mixtures of Immune and Susceptible Oat Plants with Different Genotype Unit Areas and Spatial Distributions of Initial Disease." Phytopathology **76**: 590-598.

Myers, J. A., B. S. Curtis, et al. (2013). "Improving accuracy of cell and chromophore concentration measurements using optical density." BMC Biophysics **6**(4).

Norrgard, K. (2008). "Genetic variation and diseases: GWAS." Nature Education **1**(1): 87.

O'Leary, A. L. and T. B. Sutton (1986). "Effects of Postinfection Applications of Ergosterol Biosynthesis-Inhibiting Fungicides on Lesion Formation and Pseudothecial Development of *Venturia inaequalis*." Disease Control and Pest Management **76**(1): 119-124.

Oliver, R. P. and H. G. Hewitt (2014). Fungicides in Crop Protection, CABI.

Passey, T. A. J., J. D. Robinson, et al. (2017). "The relative importance of conidia and ascospores as primary inoculum of *Venturia inaequalis* in a southeast England orchard." Plant Pathology: 1-7.

Passey, T. A. J., M. W. Shaw, et al. (2016). "Differentiation in populations of the apple scab fungus *Venturia inaequalis* on cultivars in a mixed orchard remain over time." Plant Pathology **65**: 1133-1141.

Patyal, S. K., I. D. Sharma, et al. (2013). "Dissipation kinetics of trifloxystrobin and tebuconazole on apple (*Malus domestica*) and soil - a multi location study from north western Himalayan region." Chemosphere **92**(8).

Payne, R., D. Murray, et al. (2013). An Introduction to the Genstat Command Language, 16th edn. Hemel Hempstead, VSN International.

Pelaez Montoya, J. E., L. E. Vasquez David, et al. (2006). "Use of a Micro titre Plate Dilution Assay to Measure Activity of Antifungal Compounds Against *Mycosphaerella Fijiensis*, Morelet." Revista Facultad Nacional de Agronomia **59**(2): 3425-3433.

Pfeufer, E. E. and H. K. Ngugi (2012). "Orchard Factors Associated with Resistance and Cross Resistance to Sterol Demethylation Inhibitor Fungicides in Populations of Venturia inaequalis from Pennsylvania." Phytopathology **102**(3): 272-282.

Poblete, J. A. and B. A. Latorre (2001). "Pre-infection and curative activity of sterol inhibitor fungicides against *Venturia inaequalis* on apples." Ciencia e Investigación Agraria **28**(3): 145-150.

Potter, L., R. D. Wauchope, et al. (2001). "Accumulation and decay of chlorothalonil and selected metabolites in surface soil following foliar application to peanuts." Environmental Science & Technology **35**(13): 2634-2639.

Railsback, S. and V. Grimm (2011). Agent-Based and Individual-Based Modelling: A Practical Introduction. Princeton, Princeton University Press.

Raposo, R., R. Colgan, et al. (1995). "Application of an Automated Quantitative Method to Determine Fungicide Resistance in *Botrytis cinerea*." Plant Disease **79**(3): 294-296.

Rivero Casique, R. (2015). Phenology and Effect of Climate on Apple Cultivars (*Malus Domestica* Borkh.). Master's thesis, Norway, Norwegian University of Life Sciences**:** 12-17.

Rosenberger, D., K. Cox, et al. (2012). "A New Fungicide for Controlling Summer Diseases on Apples?" New York Fruit Quarterly **20**(4): 9-13.

Sang, H., J. Hulvey, et al. (2015). "A pleiotropic drug resistance transporter is involved in reduced sensitivity to multiple fungicide classes in *Sclerotinia homoeocarpa* (F.T. Bennett)." Molecular Plant Pathology **16**(3): 251-261.

Sansavini, S., S. Tartarini, et al. (2000). "Scab (*Venturia inaequalis*) resistance resistance in apple: the *Vf* gene and polygenic resistance in the breeding strategy at DCA-Bologna." Acta Horticulturae **595**: 29-32.

Schnabel, G. and A. L. Jones (2001). "The 14α-Demethylase (*CYP51A1*) Gene is Overexpressed in *Venturia inaequalis* Strains Resistant to Myclobutanil" Phytopathology **91**(1): 102-110.

Schwabe, W. F. S., A. L. Jones, et al. (1984). "Greenhouse Evaluation of the Curative and Protective Action of Sterol-Inhibiting Fungicides against Apple Scab." Disease Control and Pest Management **74**(249-252).

Sharma, J. N. and V. K. Gupta (1995). " Reduction in fungicide usage by monitored control of apple scab (Venturia inaequalis (Cke.) Wint.)." International Journal of Tropical Plant Diseases **13**(2): 167-175.

Shaw, M. W. (1989). "A model of the evolution of polygenically controlled fungicide resistance." Plant Pathology **38**: 44 -55.

Shaw, M. W. (2000). "Models of the Effects of Dose Heterogeneity and Escape on Selection Pressure for Pesticide Resistance." Phytopathology **90**(4): 333-339.

Shaw, M. W. (2006). "Is there such a thing as a fungicide resistance strategy? A modeller's perpective." Aspects of Applied Biology **78**: 95-103.

Silveira Gomes, L. I., L. B. J. Bibiano, et al. (2014). "Baseline sensitivity of Brazilian *Mycosphaerella fijiensis* isolates to protectant and systemic fungicides." Tropical Plant Pathology **39**(2): 172-177.

Skylakakis, G. (1981). "Effects of Alternating and Mixing Pesticides on the Buildup of Fungal Resistance." The American Phytopathological Society **71**(11): 1119-1121.

Skylakakis, G. (1982). "The development and use of models describing outbreaks of resistance to fungicides." Crop Protection **1**(3): 249-262.

Soeda, Y., S. Kosaka, et al. (1972). "The Fate of Thiophanate-methyl Fungicide and Its Metabolites on Plant Leaves and Glass Plates." Agricultural and Biological Chemistry **36**(6): 931-936.

Stammler, G., S. Miessner, et al. (2013). Pathogens with low QoI resistance risk. 17th International Reinhardsbrunn Symposium, Reinhardsbrunn, Germany.

Stanis, V. F. and A. L. Jones (1985). "Reduced Sensitivity to Sterol-Inhibiting Fungicides in Field Isolates of *Venturia inaequalis*." Phytopathology **75**(10): 1098-1101.

Stevic, M., P. Vuksa, et al. (2010). "Resistance of *Venturia inaequalis* to demethylation inhibiting (DMI) fungicides." Zemdyrbyste=Agriculture **97**(4): 65-66.

Strand, L. L. (1999). Integrated Pest Management for Apples and Pears. University of California, Agriculture and Natural Resources.

Swezey, S. L., P. Vossen, et al. (2000). Organic Apple Production Manual. University of California, Agriculture and Natural Resources.

Taylor, M. T. and A. Hastings (2005). "Allee effects in biological invasions." Ecology Letters **8**: 895-908.

Turechek, W. W. (2004). Apple Diseases and their Management. Diseases of Fruits and Vegetables: Diagnosis and Management. S. A. M. H. Naqvi, Kluwer Academic Publishers.

University of Hertfordshire. (2017). PPDB: Pesticide Properties DataBase.

Vaillancourt, L. and J. R. Hartman (2000, 2005). "Apple scab. The Plant Health Instructor.". Retrieved May, 2015, from http://www.apsnet.org/edcenter/intropp/lessons/fungi/ascomycetes/Pages/AppleScab.aspx .

van den Bosch, F., B. Fraaije, et al. (2015). The Use of Mathematical Models to Guide Fungicide Resistance Management Decisions. Fungicide Resistance in Plant Pathogens: Principles and a Guide to Practical Management. H. Ishii and D. W. Hollomon, Springer.

van den Bosch, F. and C. A. Gilligan (2008). "Models of Fungicide Resistance Dynamics." Annual Review of Phytopathology **46**: 123-147.

van den Bosch, F., N. Paveley, et al. (2011). "The dose rate debate: does the risk of fungicide resistance increase or decrease with dose?" Plant Pathology **60**(4): 597-606.

van den Brink, H. J. M., H. J. G. M. van Nistelrooy, et al. (1996). "Increased resistance to 14α-demethylase inhibitors (DMIs) in *Aspergillus niger* by coexpression of the *Penicillium italicum* eburicol 14α-demethylase (*cyp51*) and the *A. niger* cytocrome P450 reductase (*cprA*) genes." Journal of Biotechnology **49**: 13-18.

Via, S. (1986). Pesticide Resistance: Strategies and Tatics fro Management. Washington D.C., National Academy Press.

Vijaya Palani, P. and D. Lalithakumari (1999). "Resistance of *Venturia inaequalis* to the ergosterol biosynthesis-inhibiting fungicide, penconazole [1-(2-(2,4-dichlorophenylpentyl)-1H-1,2,4-triazole]." Mycological Research **103**(9): 1157-1164.

Villani, S. and K. Cox (2013). "CYP51A1 upstream anomalies and overexpression in myclobutanil and difenoconazole resistant Venturia inaequalis isolates." Phytopathology **103**(6): 153-153.

Villani, S. M., A. R. Biggs, et al. (2015). "Prevalence of myclobutanil resistance and difenoconazole insensitivity in populations of *Venturia inaequalis*." Plant Disease **99**: 1526-1536.

Villani, S. M., J. Hulvey, et al. (2016). "Overexpression of the *CYP51A1* Gene and Repeated Elements are Associated with Differential Sensitivity to DMI Fungicides in *Venturia inaequalis*" Disease Control and Pest Management **106**(562-571).

Wade, M. J. (2000). Epistasis as a Genetic Constraint within Populations and an Accelerant of Adaptive Divergence among Them. Epistasis and the Evolutionary Process. J. B. Wolf, E. D. Brodie and M. J. Wade, Oxford University Press.

Wilcox, W. F. (1992). "Development and Evaluation of an Integrated, Reduced-Spray Program Using Sterol Demethylation Inhibitor Fungicides for Control of Primary Apple Scab." Plant Disease **76**(7): 669-677.

Wilensky, U. 1999. NetLogo. http://ccl.northwestern.edu/netlogo/. Center for Connected Learning and Computer-Based Modeling, Northwestern University. Evanston, IL.

Witte, J. S. (2010). "Genome-Wide Association Studies and Beyond." Annual Review of Public Health **31**: 9-20.

Xu, X. M., L. Q. Gao, et al. (2010). "Are insensitivities of Venturia inaequalis to myclobutanil and fenbuconazole correlated?" Crop Protection **29**: 183-189.

Xu, X. M., R. A. Murray, et al. (2008). "The effects of temperature, humidity and rainfall on captan decline on apple leaves and fruit in controlled environment conditions." Pest Management Science **64**(3): 296-307.

Xu, X. M., P. Wu, et al. (2006). "Variability in initial spray deposit in apple tress in space and time." Pest Management Science **10**: 947-956.

Zadoks, J. C. and P. Kampmeijer (1977). "The role of crop populations and their deployment, illustrated by means of a simulator, EPIMUL76." Annals of the New York Academy of Sciences **287**: 164-190.