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Understanding and minimising fungicide resistance

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Contents

Introduction

The emergence of fungicide resistance is a major challenge facing agriculture. With increasing regulation and costs limiting the development of new fungicides, farmers remain reliant on a relatively small group of actives, many of which are decreasingly effective as major crop disease pathogens develop resistance to them. This volume provides an authoritative review on the wealth of research on understanding the development of fungicide resistance in agricultural crops and the establishment of preventative measures which can be implemented to limit its spread and the consequent impact of disease on yields.

The chapters are split into two parts: Part 1 chapters focus on understanding and managing resistance, discussing topics such as the factors driving resistance emergence, the molecular basis of the resistance, the tools developed for tracking resistant pathogens as well as the fine balance between disease control and resistance selection. Chapters also review fungicide resistance risk assessment, minimising fungicide resistance in crop cultivation, the evolutionary biology of fungicide resistance, as well as the role of extension in fungicide resistance management and key challenges in developing new fungicides. Chapters in Part 2 examine resistance in key groups of fungicides as case studies, specifically drawing attention to sterol biosynthesis inhibitor (SBI), quinone outside inhibitor (Qol), succinate dehydrogenase inhibitor (SDHI), Anilinopyrimidine (AP) and oxysterol binding protein inhibitor (OSBPI) fungicides.

Part 1 Understanding and managing resistance

Part 1 opens with a chapter that provides an overview of how pathogens develop resistance to fungicides. Chapter 1 begins by first focusing on how fungicide resistance can be detected and measured. It then moves on to analyse the different mechanisms of resistance, drawing specific attention to target site mutation, target site overexpression and multidrug resistance. This is followed by a section on the evolution of resistance, highlighting how this evolution has been divided into two stages: emergence and selection. The chapter concludes by emphasising the importance of improving fungicide effectiveness to in turn, decrease their usage.

The next chapter reviews molecular evolution and mechanisms of fungicide resistance in plant pathogenic fungi. Chapter 2 reviews mechanisms of resistance to the most widely-used, broad-spectrum groups of fungicide over the last 50 years, including methyl benzimidazole carbamate (MBC)

fungicides, azoles, amines including morpholines and piperidines, succinate dehydrogenase inhibitors (SDHI) and quinone-outside inhibitors (QoI, often known as strobilurins). For each class of fungicide the chapter discusses the fungal target and explores how resistance operates at the gene and protein level. It also focuses on powdery mildews as an example because they are notorious for the speed at which they have evolved resistance to most of the major classes of fungicide.

The subject of Chapter 3 is tracking the development of fungicide resistance. It starts by first providing an overview of the key objectives and requirements in tracking fungicide resistance. The chapter then moves on to review phenotyping tracking of fungicide resistance, which is followed by a discussion on developments in genotyping techniques. A section on in-field detection and quantification techniques is also included. The chapter also highlights developments in sequencing techniques, such as the use of genome-wide association studies as well as nanopore and other long-read sequencing approaches.

Chapter 4 draws attention to crop disease control efficacy and selection for resistance. It first focuses on control versus selection, highlighting the theory behind fungicide application programmes and provides examples of predictions and a test using already published data. The chapter also reviews reducing selection while maintaining control, focusing specifically on fungicide mixtures. Building on this discussion as a foundation, the chapter moves on to focus on the possibility of using two at-risk fungicides and what needs to be considered if mixtures such as these were to be developed. The chapter also review integrated pest management measures and mutual protection, before concluding by emphasising the importance of developing long-term disease control methods.

The next chapter focuses on fungicide resistance risk assessment. Chapter 5 reviews the development and scientific rationale of the widely used risk matrix approach to fungicide resistance risk assessment. This type of approach relies on previous experience of resistance with a particular fungicide mode of action, pathogen and agronomic system to assess the level of risk. In contrast to this, the chapter also reviews a more recent trait-based approach that doesn't rely on prior knowledge. A section that considers future trends in fungicide resistance risk assessment is also provided.

The subject of Chapter 6 is good practice in minimising the development of fungicide resistance in crop pathogens. The chapter begins by first providing an overview of resistance management guidance and how this has evolved over time. It then moves on to consider the three key evolutionary phases of fungicide resistance: the emergence phase, selection phase and adjustment phase. The chapter also describes governing principles of resistance evolution as a foundation of guidance, then addresses future development possibilities

for resistance management guidance. A section on the complementary roles of experiments and modelling to inform resistance management guidance is also included, followed by an overview of how resistance management guidance can be updated.

Chapter 7 examines the evolution of fungicide resistance and identifies the practical implications of this. The chapter begins by first identifying the evolutionary origins of fungicide resistance, focusing specifically on standing variation and de novo mutation and how this can impact resistance risk and management. The chapter moves on to review the adaptive potential and overall risk of fungicide resistance for pathogens, then describes trait complexity and how this can affect fungicide resistance in certain genotypes. A section on the fitness penalties within fungicide-resistant genotypes is also described, focusing specifically on the costs and trade-offs and the impact on risk levels and management. The predictability of resistance is also reviewed, before the chapter concludes with an overview on how fungicide resistance is still an ongoing challenge and emphasises the importance of improved evolutionary understanding and how this can lead to better decision making.

The next chapter draws attention to the role of extension in fungicide resistance management. Chapter 8 features three case studies that illustrate how the extension service has been involved in (i) identifying resistance-related problems in producer fields, (ii) formulating and implementing solutions, and (iii) continued education of producers to adjust to ever changing resistance landscapes. Extension agents and specialists have two goals associated with resistance management: (1) provision of non-biased, accurate and current information to producers relative to resistance management best practices, and (2) rapid identification of resistance when it occurs, to be quickly followed by new recommendations – the feedback loop. The strong working relationship between extension agents and producers educates both parties, continues to build trust, and generates the foundation for change.

The final chapter of Part 1 focuses on the key challenges in developing new fungicides for effective control of plant diseases. Chapter 9 first provides an overview of past and present fungicide development, focusing on how chemical disease control became a key concept within the nineteenth century and how these methods have developed up to this point. The chapter also highlights the current challenges facing fungicide development and registration, before it moves on to consider fungicide resistance as a driver for innovation. A section on how fungicide development can change in the future as the industry adapts to different fungicide requirements is also provided.

Part 2 Case studies: resistance in key groups of fungicides

Part 2 opens with a chapter that discusses understanding resistance to SBI fungicides. Chapter 10 starts by first highlighting the current market and trends

for this type of fungicide, then goes on to provide a short history of de-methylation inhibitor (DMI) fungicides, one of the four subclasses SBI fungicides can be divided into. This is then followed by an overview of resistance risk and general resistance characteristics of SBI fungicides. A section on mode of action and mechanisms of resistance is also included, followed by a discussion that provides specific examples of resistance to DMIs. The chapter also includes a section that highlights recommended uses for resistance management.

The next chapter draws attention to Qol fungicide resistance, focusing specifically on selection patterns and the current situation. Chapter 11 begins by describing the history of resistance and resistance mechanisms, then goes on to provide three case studies to describe different Qol resistance evolutionary patterns and dynamics. Each case study draws attention to a specific crop and fungicide, the first draws attention to fungicide use in soybean in Brazil, the second focuses on grapes in France and the final discusses sugar beet in Europe. A section on current global sensitivity to Qol groups 11, 11A and 45 is also provided.

The subject of Chapter 12 is understanding resistance to SDHI fungicides. The chapter begins by first describing the mode of action of SDHI fungicides, focusing specifically on succinate dehydrogenase (succinate-ubiquinone oxidoreductase, Complex II) target and the succinate dehydrogenase inhibitor (carboxamide, Group 7) fungicides. It then moves on to describe resistance mechanisms such as target site and non-target site, which is followed by a section on fitness cost and resistance risk assessment. The chapter concludes with a case study on the control of net-form net blotch in barley in South Australia.

Chapter 13 focuses on understanding resistance to AP fungicides. The chapter first describes the mode of action of AP fungicides, drawing attention to enzyme secretion, methionine biosynthesis and mitochondrial function. It goes on to discuss mechanisms of resistance in the target sites and non-target sites of AP fungicides, which is followed by an overview of the fitness cost of AP fungicide resistance. The chapter also highlights the importance of monitoring AP fungicide resistance and general fungicide resistance management practices.

The final chapter of the book discusses understanding resistance to OSBPI fungicides. Chapter 14 first examines the current status of fungicide resistance in oomycetes. It then moves on to review the mode of action and biological activity of OSBPI fungicides. A case study on OSBPI resistance risk assessment is also included, followed by an overview of sensitivity monitoring. The chapter also highlights the characterisation of resistant mutants, discusses molecular monitoring and also provides use recommendations for these fungicides.

Acknowledgements

Cover image acknowledgement:

- Barley plants showing symptoms of spot form net blotch, net form net blotch and smut. 2021. © Francisco J. Lopez-Ruiz.

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Part 1

Understanding and managing resistance

Chapter 1

How pathogens develop resistance to fungicides: an overview

Richard Oliver, University of Nottingham, UK

1 Introduction

Fungicides form one of the four major pillars used by growers/farmers to protect crops from diseases, the others being genetics, agronomy and biosecurity. It is difficult to reliably estimate the contributions of each of these methods to crop protection, but there is no doubting the sustained demand from farmers for fungicides that reliably and economically deliver a healthy crop. The global annual market for fungicides was close to US$20 billion in 2020 and is growing at a steady pace.

Scientific fungicide use goes back to the eighteenth century, pre-dating the controlled use of pure chemicals in clinical therapeutic settings by more than 100 years. Resistance to fungicides was not a significant issue until the 1960s when systemic fungicides with single modes of action (MOA) were introduced. Resistance to single-site MOA is now a dominant factor in the fungicide industry. It impacts all aspects of fungicide development and is a major issue in determining the useful life of the products. I refer here not just to fungi *sensu strictu* but to all eukaryotic pathogenic microorganisms targeted by crop protection products (CPP).

Most fungicides work by inhibiting a key biochemical process that the fungus needs to colonise the plant, induce symptoms and complete its life cycle. As with other cases of 'Natural Selection', evolution to resistance occurs

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when fungicides exert an inhibitory effect on the pathogen population growth. If the pathogen population varies in its sensitivity to the fungicide, the more sensitive isolates will be relatively inhibited, whilst the more resistant ones will grow and reproduce at a higher rate. As a result of this selection pressure, evolution towards resistance will inevitably occur. The speed at which resistance develops, the degree of resistance and the pathogenic fitness of the resistant population in the absence of the fungicide (the fitness penalty) all depend on features of the pathogen, the disease and the fungicide. Resistance may occur and render the fungicide useless in a matter of weeks. Alternatively, the useful economic life of fungicides can realistically be extended indefinitely if its properties are suitable and its use is managed wisely.

2 Detecting and measuring resistance

The fungicide resistance era began with the discovery of resistance to the methyl-benzimidazole carbamate (MBC) fungicides in sugar beet fields in Greece in 1972. Benomyl was introduced to control *Cercospora beticola* and, at first, was a substantial improvement on the organo-tin products then in use. In just the third season of use, catastrophic disease levels were noted, which were substantially worse than in organo-tin-protected controls. At first, it was believed the benomyl was being washed off the leaves by the irrigation equipment. The response was to increase the dose but to no avail. It was noted that fields that had received benomyl in each of the previous seasons were the most and earliest affected. It was only when isolates were tested *in vitro* that it was fully accepted that this was a clear case of evolved resistance to a specific fungicide (Georgopoulos and Dovas, 1973). It was later shown that resistant isolates carried an E198A mutation (i.e. a glutamate (E) at position 198 in the protein was changed to an alanine (A)) in the *b-tubulin* gene and that this genotype fully accounted for the phenotype (Mair et al., 2016). The mutant strains were as pathogenic as the wild type on untreated plants.

This first case of fungicide resistance contains many elements which have become familiar over the ensuing decades; blaming product application failure, repeated use, higher doses, solo use, delays in resistance detection and inappropriate responses.

There are many occasions when fungicides produce disappointing results, and only a small fraction of these cases prove to be due to evolved fungicide resistance. In most cases, the product was inappropriately applied or unsuited to the task. Furthermore, if the first evidence of resistance was a field failure, options for mitigating the outbreak would be limited. It is much better to detect resistance prior to the evolution of populations that create epidemic outbreaks on crops.

The classic way to detect resistance is to make large-scale, random isolations of the pathogen/s of interest and measure the EC50 phenotype – the concentration of fungicide that reduces the growth rate by 50% in an *in vitro* assay. The process of population-level phenotypic assessment is slow and expensive but irreplaceable, at least in the initial phases of releasing a new active. The first requirement is to establish a baseline of naïve and presumably fully sensitive isolates of the pathogen. Such studies typically uncover a surprisingly large range of EC50s covering a factor of 10 or more (Fig. 1). The presence and molecular basis of the hypersensitive isolates is a poorly studied aspect of fungicide resistance research. The EC50 values of the naïve population can be plotted on a cumulative frequency graph and typically forms a sigmoid shape. The EC50 can be defined as the inflexion point of the population cumulative frequency graph. Similar plots of isolate EC50s in successive growing seasons can be readily examined for shifts in the mid-point EC50s.

The methods used to measure EC50s vary between pathogens and labs. Obligate pathogens are particularly difficult, and the results obtained have a low degree of precision. The easiest pathogens are ones that grow in cultures as yeasts such as *Zymoseptoria tritici*. Then, turbidimetric methods can be used reliably to measure growth rates in liquid cultures, typically in a microtiter well plate (Oliver and Beckerman, 2022). As growth proceeds via a sigmoid progression, first slow, then fast and later slow again, estimates of the *maximum growth rate* are preferred but are more time-consuming than growth accrued at a *particular time point*. Filamentous organisms are not well suited to liquid

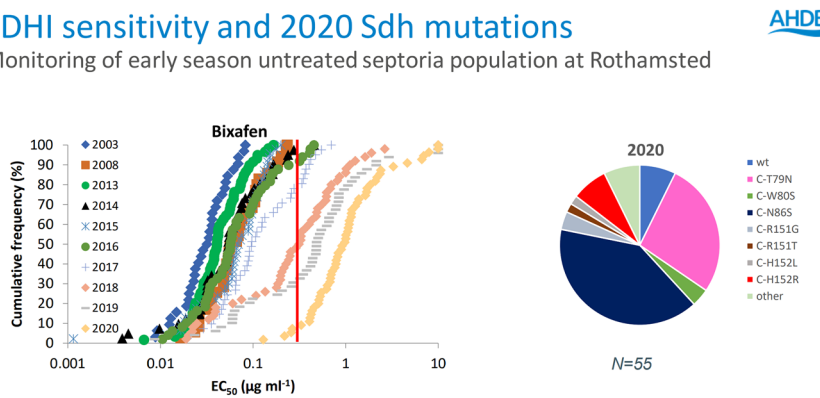

Figure 1 An example of EC50 analyses of a fungal population over time and during the evolution of fungicide resistance. The blue diamonds show the range of EC50 values of a naïve wild-type population prior to the use of a fungicide. The cumulative frequency plot shows a typical sigmoid shape, with the population having EC50 values varying from about 0.01 µg ml^{-1} to 0.1 µg ml^{-1}. Populations sampled in successive years have sigmoid curves shifted to higher EC50 values. The pie chart shows the distribution of genotypes of the SDH-C subunit in the 2020 samples. Figure courtesy of Bart Fraaije and funded by AHDB, with permission.

culture methods; the best results are radial growth rate tests on solid media but involve the use of formidable quantities of petri dishes, media and incubator space (Oliver and Beckerman, 2022). The ratio of the EC50 of the resistant isolate and a naïve one is called the resistance factor (RF). The choice of naïve strain obviously impacts the calculated value of the RF.

It is important to recognise that any measurable and heritable increase in EC50 is evidence of the evolution of resistance. There has been much debate about whether smaller RFs should be called 'tolerance' or 'reduced sensitivity' and that the use of the word 'resistance' should be restricted to cases of field failure. This is a false dichotomy. Even if isolates with small RFs are still well controlled by the label rate of the fungicide, the increase in EC50 indicates that the population is evolving under the selection pressure of the fungicide. This may be a stable situation or may be a prelude to the further increase in EC50s.

Determining EC50s and RFs is costly both in time and money. A cheaper method is to use a single concentration of fungicide and determine whether isolates can grow more than a defined threshold – so-called *discriminatory dose* methods (DD). The choice of dose would normally be equivalent to a full field dose. These are difficult to estimate, so more realistically, the concentration would be two to five times higher than the most resistant naïve isolate. DD tests give significantly less information than full EC50s.

It has been generally assumed that evolved resistance to fungicides detected as increased growth on an infected plant compared to a control isolate can be replicated when testing the isolates *in vitro* via liquid or solid culture methods. Recently there has been a significant case of fungicide failure *in planta* that could not be fully replicated *in vitro* (Van De Wouw et al., 2017). Seedlings grown from treated seeds could be infected by the resistant isolates but not by sensitive ones, but the resistance factor *in vitro* was insufficient to account for the breakdown. The mechanism has not yet been fully documented.

It is well recognised that the ideal scenario is to be able to detect resistance prior to field failure. This is important not just after the first introduction of a new active but also when resistance has been detected in one territory or region but not yet in a neighbouring region. If resistance were present in 1% of isolates, it would be necessary to test 300 isolates to have a 95% chance of detecting at least one. A cheaper way to detect rare cases of resistance in previously reliable fungicides is to set up a 'bait test' (Fig. 2). Bait trials reduce the number of isolates needed to detect resistance but only give a qualitative impression of the resistance frequency. The test is set up in a field treated with the standard fungicide regime. Within the field, a small plot is sprayed with fungicides suspected of resistance development, at concentrations ranging up to two times the max label dose. Any lesions on the higher doses is evidence that resistance is developing. Furthermore, the highest dose on which the

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