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Resistance Management: We know why, but do we know how?

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ABSTRACT

The high efficacy and target specificity of modern single-site fungicides exert strong selection for resistance in pathogen populations. Strategies to prevent or delay the development of resistance are an essential part of product stewardship. Technological advances have provided more rapid, sensitive, and accurate methods for the detection and quantification of resistance in pathogen populations. There has also been good progess in understanding the genetics and mechanisms of resistance to specific fungicide classes. But devising effective ways to reduce selection for resistance while maintaining disease control remains a challenge. There is still no foolproof way to predict when and where resistance will emerge, how quickly it might increase to affect field efficacy, and what management tactics will be most effective in preventing it. Resistance risk assessments based on multiple agricultural, biological, chemical and molecular parameters influencing pathogen evolution are now available, together with mathematical models evaluating the likely outcomes of different resistance management strategies. Despite these advances resistance management remains an inexact science. Delaying or reducing directional selection through use of fungicide mixtures and improved integration with cultivar resistance and agronomic measures, where available, is currently the main strategy, supplemented in future by the new tools emerging from genomics and biotechnology.

INTRODUCTION

Modern, single-site fungicides have played an important role in crop protection by safe-guarding yield and quality as well as stability of production. Growers have had access to a diversity of effective and affordable products that in most circumstances have given an economic return. Hence these fungicides are widely used in intensive production systems. The desirable properties of single-site fungicides, their specific mode of action and high efficacy at relatively low dose rates, have an unfortunate negative consequence. Resistance can occur in the pathogen population, and continuous use of the chemical exerts strong selection pressure

for such resistant biotypes. The rapid evolution of target fungi in response to fungicides and antifungal drugs has become a fact of life both in agricultural systems and the disease clinic. Given the probability that resistance to a single-site fungicide will, sooner or later, occur, strategies to delay the emergence of resistance and to prevent its spread in pathogen populations are vital to prolong the effective life of these chemicals. Resistance management has become an integral part of fungicide use and product stewardship.

KEY COMPONENTS OF RESISTANCE MANAGEMENT

Ideally, resistance management should include three elements at key stages in the development and use of a new fungicide; prediction of the likelihood of resistance occurring, early detection of resistance should it occur, and tactics to reduce the rate of selection of resistance in the field once it has emerged (Figure 1). In reality, much of the emphasis to date has been on how to manage resistance after it has happened, during what has been described as the selection phase as opposed to the emergence phase of resistance evolution (Hobbelen et *al.* 2014). Management tactics are deployed in reaction to events rather than pre-empting them. While some progress has been made in assessing the relative risk of resistance developing in a particular pathogen to a specific class of chemistry, there is as yet no precise way to determine when or where resistance might occur, and resistance management therefore often becomes a fire-fighting exercise.

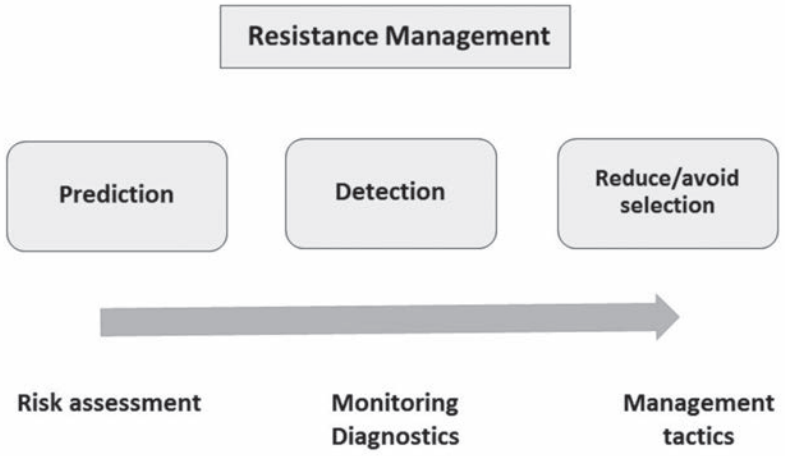

Figure 1 Components of resistance management

Conceptual framework for resistance risk assessment

Early experience with single-site as opposed to multi-site fungicides led to the development of the well-known resistance risk matrix based on fungicide type and pathogen biology. This provided a general framework for estimating risk, and has been updated over the years as more

examples of resistance development became available, and refined to include agronomic risk factors (Figure 2). A recent evaluation of the matrix, based on sixty-seven published cases of fungicide resistance in Europe, with estimates of time from introduction of a fungicide to first detection of resistance, found that while the scheme had useful predictive power when all fungicides were compared, this decreased considerably when only single-site fungicides were included (Grimmer et *al.* 2014). This limitation is important as single-site fungicides are now the predominant type both in practical use and commercial development.

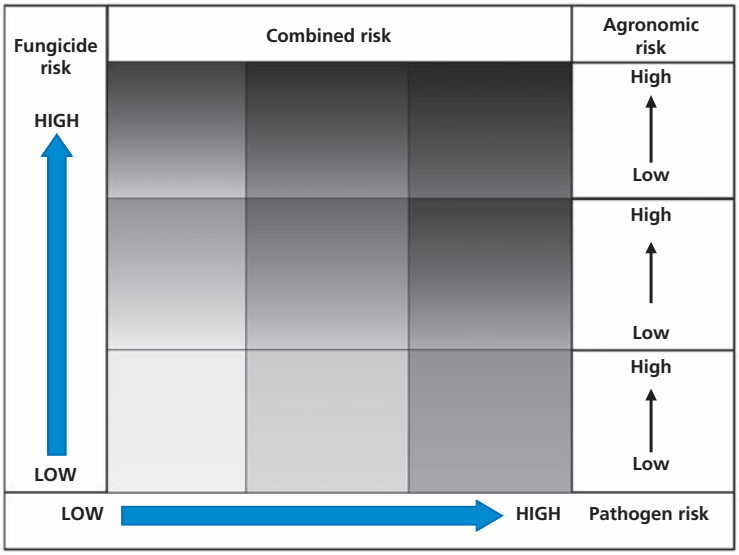

Figure 2 Diagrammatic representation of a fungicide resistance risk matrix. Initial risk is determined by fungicide type and mode of action (for instance multisite versus single-site) and pathogen biology (for instance monocyclic versus polycyclic reproduction). Within each combined group the final risk is determined by agronomic factors such as protected versus open cultivation and frequency of fungicide use. The darker the shading the greater the risk of resistance (based on Kuck & Russell 2006; Brent & Hollomon 2007).

A confounding factor in this general approach to resistance prediction is gaps in our knowledge of pathogen biology and epidemiology. One case in point was the development of resistance to methyl-benzimidazole carbamate (MBC) fungicides in the cereal eyespot fungi *Oculimacula yallundae* and *O. acuformis*. These pathogens (classified at the time as pathotypes of a single species) were considered to be asexual with a single generation of spores per year (monocyclic) that are splash-dispersed over short distances, and hence posed a relatively low risk of resistance. Nonetheless, control failures in UK wheat crops treated with MBCs were first reported in 1981, with highly resistant isolates obtained from two such sites (Brown et *al.* 1984). Within two years, resistance was widespread and even found in untreated fields, indicating a rapid invasion of resistant strains of the fungus (King & Griffin 1985). A

similar increase in highly resistant strains was also reported in other European countries (Leroux et *al.* 2013). Subsequent analysis of initially sensitive isolates showed that strains with a range of resistant phenotypes could be easily selected from spore populations in the laboratory, indicating that mutations affecting MBC sensitivity were commonplace (Hocart et *al.* 1990). Not long after the emergence and spread of MBC resistance in *Oculimacula spp* was first detected, the perfect stage of these fungi was discovered. While the contribution of sexual ascospores to eyespot epidemics is still not fully defined, it seems likely that they played a part in the unexpectedly rapid dispersal of resistance in the field (Lucas et *al.* 2000).

Recently a new risk model based on pathogen, fungicide and agronomic system traits associated with the rate of evolution of resistance has been proposed (Grimmer et *al*. 2015). This integrates a large number of pathogen and fungicide properties along with agronomic factors such as crop species, treated area, fungicide use intensity, and outdoor versus protected cultivation. The time from introduction of a fungicide to first detection of resistance (FDR) was quantified for 61 European cases involving single-site fungicides, and traits associated with FDR time were identified and incorporated in the model. Use of the model explained 61% of the variation in FDR time, with a good correlation between observed and predicted values. One potential advantage of this approach is that it can also be applied to new modes of action with no prior knowledge of resistance development.

Experimental approaches to resistance risk assessment

Laboratory studies aimed at predicting the probability of resistance occurring, as well as the types of resistance that might occur, have been in use for many years. A common approach is to select insensitive strains of the fungus *in vitro,* with or without a mutagenic agent. While this has proved to be of value in demonstrating the biological potential for resistance to occur, it has several limitations. As mutation rates are usually low, one needs very large numbers of propagules to test. In the case of non-culturable fungi such as powdery mildews and rusts the probability of recovering any resistant mutants on host plants is several orders of magnitude lower. But the main issue has been whether laboratory selected strains can accurately reproduce what might happen in the field. This question is also relevant to the use of experimental models, such as yeast, that otherwise have many advantages in terms of their tractability and genetics.

Early examples of such limitations concerned acylalanine fungicides and the Oomycete pathogens *Phytophthora infestans* and *P. capsici*. Prolonged exposure of isolates of *P. infestans* to sub-lethal concentrations of the compounds *in vitro* produced several strains with reduced sensitivity, but these had either lost their virulence or were fully controlled on fungicide-treated plants (Staub et *al.* 1979). The authors concluded that resistance risk assessments should always include *in vivo* tests. In practice, resistance to these fungicides quickly developed in field populations of *P. infestans* (Davidse et *al.* 1981; Carter et *al.* 1982). Subsequent laboratory studies on the soilborne *Phytophthora* species *P. capsici* showed that it was relatively easy to select metalaxyl resistant strains from some isolates on fungicide amended agar, and

that such strains retained full virulence and were able to compete with sensitive wild-types in mixed inoculation tests (Lucas et *al.* 1990). In this species, however, field resistance to acylalanine fungicides emerged much more slowly (Parra & Ristaino 2001).

Laboratory studies on the potential risk of resistance have now been refined with the advent of molecular genetic techniques to detect the specific changes associated with resistant phenotypes (Table 1). These include target site mutations, target over-expression, and transporters responsible for fungicide efflux. It is also now possible to introduce particular mutations through site-directed mutagenesis or gene editing, and then assess their effects on fungicide sensitivity and pathogen fitness. High throughput methods such as RNAseq can be used to simultaneously analyse changes in the expression of multiple genes. With the increasing speed and declining cost of genome sequencing whole genome comparisons between resistant and sensitive biotypes are now feasible (Cools & Hammond-Kosack 2013). Where accurate protein models of the fungicide target site are available, the potential impact of specific changes on fungicide docking can be assessed. But there is still no precise way of predicting which changes will occur in the field, or will persist and invade the pathogen population to the extent that practical control of the disease is compromised.

Table 1 Laboratory approaches for analysing resistance risk.

In vitro studies
• Selection on fungicide-amended agar
• Mutant libraries
• Site-directed mutagenesis and gene editing
• *In vitro* evolution
Molecular modelling
Heterologous expression/ homologous gene replacement
DNA microarrays to analyse gene expression
RNA seq of fungicide-adapted strains
Parallel sequencing of S and R biotypes

Recent risk assessments with the new generation Succinate Dehydrogenase Inhibitor (SDHI) fungicides serve as an example. As single-site respiration inhibitors they are considered to be at moderate to high risk of resistance, analogous to the QoI fungicides. A series of different mutations in genes encoding the molecular target that affect fungicide sensitivity have been reported from field populations or laboratory studies with several pathogens (Sierotzki & Scalliet 2013). The wheat leaf blotch pathogen *Zymoseptoria tritici* has already developed resistance to MBC and QoI fungicides and progressively adapted to azoles, and continued efficacy of SDHIs is therefore important for control of the disease. A mutant library was created by UV mutagenesis of spores from two field isolates and selection on fungicide-amended agar (Fraaije et *al.* 2012). One hundred and twenty-four mutants with reduced sensitivity were recovered and further analysed. A range of mutations affecting sensitivity were found in three of the four subunits of the enzyme (Table 2). A subset of mutants was

tested for pathogenicity and sporulation and shown to be as fit as wild type isolates. Molecular modelling was also used to predict potential effects of individual mutations on fungicide docking. To date, only a few of the mutations found in the *in vitro* library have been detected in field populations of *Z. tritici* (Table 2). UV mutagenesis can therefore show the range of possible variation in sensitivity to a fungicide, but not which of the possible mutations may be selected under field conditions (Lucas et *al.* 2015; Hawkins & Fraaije 2016).

Table 2 Succinate dehydrogenase amino acid substitutions found in mutant lab strains compared with those detected in field isolates of *Zymoseptoria tritici*. Mutations found in both shown in bold. (Source: Fraaije et *al.* 2012; Scalliet et *al.* 2012; Dooley et *al.* 2016).

Lab mutants
SdhB-C137R, S218F, P220T/L, N225H/I/T, R265P, H267F/L/R/Y, I269V & N271K
SdhC-T79I, S83G, A84I/V, L85P, N86K/S, R87C, V88D, I127V, H145R & H152R
SdhD-I127V & D129E/G/S/T
Field strains
SdhB-**N225T** & T268I
SdhC-T79N, W80S, **N86S/K**, R151S/T, **H152R** & I161S
SdhD-**D129E** R47W
Also in combination for example field strain with **SdhC-N86S + SdhD-D129E**

A further question highlighted by recent experience with the SDHI fungicides is why certain pathogens with apparently similar lifestyles, host plants and exposure to fungicides differ in the rate of resistance development. For instance, resistance to SDHIs, as well as the number of mutations involved, has emerged more rapidly in the barley net blotch barley pathogen *Pyrenophora teres* (Rehfus et *al.* 2016) than in *Z. tritici* on wheat. Extrapolation from other pathosystems is useful in terms of predicting what mutations might occur, and also their potential phenotypes, but again is not a precise way of estimating risk.

In some individual cases a molecular rationale for resistance risk might be available. The best documented example is the rust fungi and QoI fungicides where presence of an intron in the mitochondrial cytochrome b target gene leading to incorrect splicing in the presence of the G143A resistance mutation is lethal, and hence survival of this mutation, the most significant change affecting QoI resistance in other pathogens, cannot occur (Grasso et *al.* 2006).

Alternative mutations are however possible, and the overall classification of rust fungi as at low risk of resistance to other MoAs has recently been questioned (Oliver 2014).

DETECTION OF RESISTANCE

While the prediction of resistance remains an inexact science, spectacular progress has been made in the development of sensitive and precise methods for detecting resistance, mainly through the use of molecular diagnostics (Ma & Michailides 2005). Whereas before, detection relied largely on bioassays to identify less sensitive isolates of pathogens, either *in vitro* or, in the case of non-culturable species, *in planta*, there are now a range of rapid molecular methods based on PCR and related technologies that can not only detect mutations responsible for target-site resistance, but also quantify the incidence of resistant-alleles in field populations of pathogens. When coupled with methods for trapping inoculum the spatial and temporal spread of resistance can also be measured (Fraaije et *al.* 2005). Accurate estimates of the proportions of resistance genotypes can be made and mapped on a continental scale (Stammler et *al.* 2008). This information can clarify key steps in the emergence and evolution of resistance under fungicide selection (Cools & Fraaije 2012; Lucas et *al.* 2015), and is also of practical value to inform fungicide treatment regimes and alternative control measures in affected regions.

The advent of genomics and next generation sequencing methods has provided new ways to detect other resistance mechanisms based for instance on over-expression of genes encoding fungicide target proteins or efflux pumps (Cools & Hammond-Kosack 2013). Comparative genomics of different fungal species and lineages has turned up some surprises, such as the existence of paralogues of well-known fungicide target genes such as *CYP51*, that might account for the intrinsic resistance of some species to azoles. Genome-wide expression profiling has been used successfully to identify genes encoding ABC transporters and transcription factors which may be involved in other mechanisms reducing sensitivity to fungicides (Becher et *al.* 2011).

RESISTANCE MANAGEMENT TACTICS

The key challenge in resistance management is to slow the rate of emergence and spread of resistant genotypes by reducing what has been described as the selection coefficient (Rate of increase of R versus S strains; Milgroom & Fry 1988; van den Bosch et *al.* 2014). This will be influenced by the competitiveness of R versus S strains with and without the fungicide, and any fitness costs of the resistance mechanism. The conundrum in practical terms is how to reduce the selection coefficient whilst maintaining an acceptable level of disease control. The main options available are listed in Table 3.

There has been a long running debate about the effects of dose rate (concentration and amount of fungicide applied) on the development of resistance. In evolutionary terms it seems obvious that higher doses will impose a greater selection pressure. But when resistance emerges in a stepwise fashion with continuous rather than discrete sensitivity shifts, it might be argued that using a high dose will be beneficial in controlling all the resistant individuals in a population. In a medical context the “hit early and hit hard” philosophy dates back to the dawn of anti-

microbial chemotherapy. This has recently been questioned, as while high doses might reduce the chances of resistance arising *de novo* during an infection, if resistant genotypes are already present it confers the greatest evolutionary advantage for such individuals (Read et *al.* 2011).

Table 3 Resistance management strategies

- Reduce dose rate
- Reduce number of sprays
- Use fungicide mixtures
- Use fungicide alternations
- Use multisite inhibitors
- Cultivar resistance (conventional and GM)
- Plant defence activators
- RNAi and host-induced gene-silencing (HIGS)
- Agronomic measures
- Biocontrol agents (BCAs)

One problem is the relative lack of experimental evidence for particular pathogens and scenarios. Comparison of published experimental studies on the effects of fungicide dose, and related mathematical models, however, showed that the large majority support the idea that high doses increase selection for resistance (van den Bosch et *al.* 2011; 2014). A specific example is shown in Figure 2. High level resistance to QoI fungicides is conferred by a single amino acid substitution G143A in the mitochondrial cytochrome b target site. Resistance emerged quickly in field populations of cereal powdery mildews including *Blumeria graminis* f.sp.*hordei* on barley. A quantitative allele-specific PCR assay was developed to measure the frequency of R and S alleles in field populations of the pathogen. This was then used to estimate the proportion of R alleles in mildew-infected field plots of spring barley receiving different numbers and concentrations of sprays of azoxystrobin at the start of the season and after one, two or three sprays. Results showed higher levels of R alleles in plots receiving two or three sprays of higher doses of fungicide than those receiving one or no spray (Figure 3). Subsequently a model was derived to predict selection of resistance in foliar pathogens of cereals, and this was tested against data from the four field sites used in the mildew experiment. This accurately predicted selection of R alleles at three out of the four sites; at the fourth the epidemic developed too late in the season to give significant results (Hobbelen *et al.* 2011a). The most commonly advised tactic aimed at reducing selection and prolonging the effective life of a high risk fungicide is to use mixtures or alternations with a multi-site low risk fungicide with a different mode of action. Field experiments as well as models suggest that mixing is usually effective in reducing selection, although the extent of the effect depends on dose rates. A modelling analysis by Hobbelen et *al.* (2011b) suggested that greatest benefit is obtained by using the full recommended dose of the low risk partner and adjusting the dose of the high risk

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fungicide to a level required to give effective disease control. Alternation is generally a less effective tactic, especially when the decay rate of the low risk fungicide limits any overlap between treatments, but experimental evidence for this is limited (van den Bosch et *al.* 2014).

To date the majority of studies on resistance management have focused on the selection phase of resistance evolution rather than the emergence phase in which a resistant mutant occurs for the first time, and subsequently invades the sensitive population. The ideal scenario would be to prevent resistance from occurring in the first place. Two recent modelling studies have addressed this issue. The model devised by Hobbelen et *al.* (2014) predicts that the emergence time of a resistant strain might be affected little by dose rate, but mixing the high risk fungicide with a low risk partner is likely to delay emergence. An alternative model that also factored in some fitness cost of resistance (Mikaberidze et *al.* 2014) found that an optimal ratio of fungicides in the mixture might be found that actually prevents the emergence of resistance. Hence resistance management strategies used to reduce selection may also be of value in delaying the initial development of resistance. These studies highlight the importance of introducing anti-resistance strategies from the moment of first use of a new fungicide.

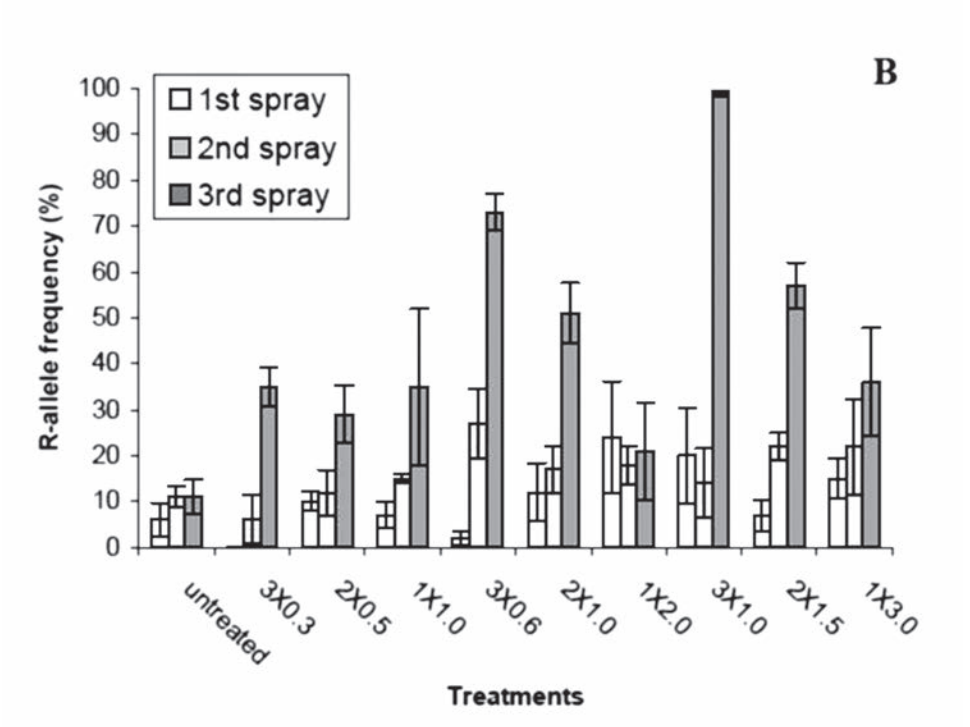

Figure 3 Effect of fungicide application on R-allele frequencies in barley mildew populations at Inverness in 2002 at GS41-3, GS70 and GS 90 after one two or three sprays of azoxstrobin at different doses (l/ha) (from Fraaije et *al*. 2006).

CONCLUSIONS

Overall, fungicides have been a success story in crop protection, but over-reliance on chemistry as a quick fix for disease control has created problems for the sustainable use of these compounds. Integration of alternative control measures (Table 3) will bring obvious benefits in reducing selection pressure in the crop environment, and prolong the effective life of chemicals. For instance, relatively modest improvements in cultivar resistance can help to reduce the amount of fungicide required to control a disease. Comparison of the recommended lists of wheat varieties for growers in the UK over a 15-year period shows that while the numbers of varieties (https://cereals.ahdb.org.uk/varieties/ahdb-recommended-lists.aspx) rated as fully susceptible to Septoria leaf blotch has fallen, the number with higher levels of resistance to this pathogen has remained static. For some other diseases, such as Fusarium ear blight, genetic resistance is currently very limited. There is a need therefore to expand the range of alternative control measures available, including the use of biotechnological and biological approaches.

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