doi: 10.17221/42/2015-PPS

Fungicide Resistance: Facing the Challenge

Derek W. HOLLOMON

Orchard House, Bristol, United Kingdom

Abstract

Hollomon D.W. (2015): **Fungicide resistance: facing the challenge**. Plant Protect. Sci., 51: 170–176

Fungicide resistance continues to generate disease control problems in many crops. Experience amassed over the past fifty years has emphasised the importance of diversity in modes of action in anti-resistance strategies. Because of losses, not only from resistance, but increasingly from environmental and health concerns, the number of modes of action has become dangerously small. This paper considers three challenges facing crop protection in the search for durable disease control systems. Greater understanding of the biochemistry surrounding fungal development and pathogenicity provides opportunities for the discovery and development of novel modes of action, and some recent advances in this area are discussed. To ensure sufficient resources available to take a novel discovery forward to a commercial product, a second challenge facing manufacturers involves early assessment of resistance risk. A third challenge facing researchers, manufacturers and growers requires translation of resistance risk into effective and durable disease control strategies in actual crops. At the core of this challenge is using resistance risk evidence obtained in laboratory and glasshouse studies using individual isolates of target pathogens, to evaluate the fitness cost of resistance in pathogen populations in field crops. Increasingly, management of resistance is seen as the integration of fungicides with non-chemical disease control methods. But success of any Integrated Disease Management (IDM) strategy ultimately depends on the ability of growers to maintain production and profitability.

Keywords: disease control; mode of action; risk analysis; fitness cost

Since Greek and Roman times chemistry has been used to reduce crop damage. By the early 19th century sulphur and copper compounds were being used, both as foliar sprays and seed treatments, to control a number of diseases. Following extensive field work, the introduction of Bordeaux mixture to control grape downy mildew (*Plasmopara viticola* Millardet, 1885) marked the beginning of the agrochemical industry, and prompted further research which identified several organic compounds active against a wide range of diseases. Many, including dithiocarbamates (thiram, Mancozeb), phthalimides (captafol), and chlorothalonil, are still used widely as foliar sprays or seed treatments. Despite their extensive use in some cases for over sixty years, resistance has not evolved to these largely non-systemic protectant fungicides, because of their multi-site modes of action.

Protectant fungicides are generally non-systemic and do not control established infections. Foliar treatments must be frequent, often at weekly intervals, because of losses from the leaf surface through weathering, and the need to protect new foliage. The concept of systemic fungicides, which are taken up and redistributed within plants, had its origin in the discovery that the antifungal antibiotic griseofulvin, isolated from *Penicillium griseofulvum,* was translocated within plants, controlled established infections, and protected new growth (Brian *et al.* 1951). Its high cost meant that griseofulvin never became a commercial fungicide, although it is used today in medicine. This provided the impetus for successful research programmes which has resulted in the many novel systemic fungicides we have today. Because systemic fungicides inevitably have a close association with the biochemistry and physiology of their hosts, modes of action are specific and seek out a biochemical target-site lethal in the pathogen but not in its host. Currently, at least forty-five different modes of action (www.FRAC.info 2015) are identified for fungicides.

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Finding new modes of action

The diversity of modes of action is a core component of anti-resistance strategies. The introduction of phenylamides (Metalaxyl; Benalaxyl) in the late 1970s offered a new era in the control of potato late blight (*Phytophthora infestans*) and other oomycete pathogens. But in 1981 resistance blunted this advance in blight control, at least in regions where phenylamides were used alone and not in mixtures with Mancozeb. Despite this, phenylamides (and especially Metalaxyl) still occupy a large market share for oomycete fungicides (Phillips McDoughall 2012). A core property of fungicide resistance is that pathogens show cross resistance to compounds with the same mode of action, but not to other modes of action. At least nine other modes of action (Table 1), many of which have been developed since the 1980s, are available for oomycete control, which ensures that effective anti-resistance strategies involving different modes of action are available to growers. This has contributed significantly to the durability of phenylamides, and emphasises the importance of searching for new modes of action.

Control of the major ascomycete and basidiomycete diseases in many major crops relies on fungicides that inhibit either steps in sterol biosynthesis, respiration, methionine biosynthesis, tubulin function or signal transduction. Resistance to all these modes of action has already existed in many pathogen populations, and a lack of alternative modes of action seriously limits the scope for durable anti-resistance strategies. Indeed, control strategies frequently depend on the use of multi-site inhibitors, such as chlorothalonil or Mancozeb, in mixtures with at-risk fungicides.

Clearly, finding and exploiting novel modes of action is a key challenge.

Detailed biochemical and molecular analyses of a mode of action can reveal significant differences between related compounds, which can be exploited in the development of novel chemistry (Hollomon 2012). Prothioconazole, which is a pro-fungicide and is metabolised to the active desthio form, generates lower resistance levels than other azoles in many pathogens, and this is linked to a somewhat different mode of action (Price *et al*. 2015), When bound to the target sterol, 14α sterol demethylase (CYP51), the active form generates a novel spectrum which indicates it interacts differently with the target enzyme than do other azoles.

Although it is well established that QoI (strobilurin) fungicides bind within the Qo pocket of the cytochrome bc-1 of mitochondrial complex III, it

Table 1. Fungicide groups and key active ingredients available for oomycete control

Fungicide groups and key active ingredients	FRAC mode of action codes	Resistance risk
Phenylamides metalaxyl mefenoxam, oxadixyl, benalaxyl, kiralaxyl	4(A1)	high
QoIs azoxystrobin, fenamidone, famoxadone	11(C3)	high
QiIs cyazofamid, amisulbrom	21(C4)	high
Benzamides/carboxamides ethaboxam, zoxamide	22(B3)	low
Cyano-acetamide oximes cymoxanil	27(U)	moderate
Dinitroanilines fluazinam	29(C5)	moderate
Phosphonates fosetyl-Al	33(U)	low
CAAs dimethomorph, flumorph, iprovalicarb, benthiavalicarb, mandipropamid	40(H5)	moderate
Benzamides fluopicolide	43(B5)	moderate
Multisites e.g. Mancozeb, chlorothalonil, copper	(M1–M5)	low

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is also recognised that there are two different sites within this pocket at which fungicides can bind (Vallieres *et al*. 2012). QoI fungicides interact with one of these sites in such a way that a single mutation (Gly143Ala) causes resistance, whereas the recently introduced fungicide Ametoctradin (QoSI; FRAC code 45) binds to the second site, and does not show cross resistance with QoIs (Fehr *et al*. 2015). Currently Ametoctradin is only registered for use against oomycete pathogens, but there seems no reason why this important target site could not be exploited to widen the disease control spectrum.

A new fungicide, the isoxazoline oxathiopiprolin, apparently shows no cross resistance with other fungicide groups (Ji & Csinos 2015), suggesting that it may have a novel mode of action, which appears to involve targeting oxysterol binding proteins which regulate sterol biosynthesis (Andreassi *et al*. 2013). But how useful this fungicide might be in augmenting anti-resistance strategies remains to be established. Some biofungicides (e.g. Seranade, PlantShield) provide useful control, especially of soil borne pathogens, although their modes of action and cross-resistance patterns are generally unknown. If a biofungicide can be shown to have a new mode of action, a search for novel chemistry might enhance activity and lead to additional products for resistance management strategies. Both strobilurin and phenylpyrrole fungicides have been developed in this way from natural products.

Assessment of resistance risk

Discovery of a new fungicide, especially where this involves novel chemistry, presents industry with a challenge to determine its resistance risk, and to decide if it is worthwhile investing further resources in its development. Risk assessment requires integration of many factors (Brent & Hollomon 2007a), but a routine initial step involves establishing if cross-resistance exists in target pathogens known to resist existing fungicides. Although resistance factors can differ between analogues in the same mode of action group (e.g. DMIs, QoIs), or between isolates, these differences are generally small and not a problem in risk assessment. Lack of cross resistance points to a novel mode of action.

Resistance is a phenomenon of natural selection, and the potential of pathogens to generate resistant mutants is a key factor in risk assessment. This risk can be addressed by attempts to generate resistant mutants in target pathogens, either by artificial mutagenesis following treatment of spores with chemical mutagens or U/V-irradiation, or exposing successive generations to increasing fungicide concentrations. Stable resistant mutants in genetically tractable pathogens can provide evidence of whether resistance is under single or multiple gene control, which greatly influences risk analysis.

Experience shows that resistance levels that can cause loss of control are often linked to amino acid changes in a protein that affect binding of a fungicide to its target. Ultimately, this must be confirmed by biochemical analysis of the interaction of the fungicide with purified target protein. In the past this information has not emerged until a novel fungicide has been launched commercially. But in recent years a battery of molecular, imaging, and recombinant DNA techniques have been incorporated into risk analysis programmes, and together with protein modelling and crystallography, they can predict the impact of different amino acid change on resistance (Frey *et al*. 2010). Evidence obtained in this way can provide the DNA sequence data to develop rapid molecular diagnostic techniques to monitor the frequency of mutations in pathogen populations.

A final, and perhaps the most difficult, step in risk analysis involves determining, preferably in a target pathogen, the fitness impact of mutations. Measurements of infection efficiency, sporulation, and growth provide a useful guide to fitness, but ideally these should be augmented with competition experiments involving mixtures of resistant and sensitive isolates in order to determine the relative fitness. The effect of resistant mutations on the activity of target enzymes greatly adds to the values of any risk analysis. It is essential that any fitness tests are carried out under controlled conditions in the laboratory or glasshouse, to ensure that artificial mutants are not released into field crops.

Managing resistance in crops

The discussion of resistance risk in the previous section was based around properties of individual resistant mutants. In actual crops resistance does emerge from individual mutations, but the wide genetic diversity within pathogen populations means it is not straightforward to translate risk into effective resistance management and disease control strate-

doi: 10.17221/42/2015-PPS

Table 2. Pathogen (intrinsic) properties influencing evolution and spread of resistance (from Hollomon 2015)

Biochemical	dependence on disruptible biochemical steps availability of resistance mechanisms
Epidemiological	dispersal method e.g. wind, rain splash, soilborne abundance of sporulation pathogen lifecycle: short or long generation time ability to infect all crop stages, requiring repeated treatment isolation of pathogen populations preventing re-entry of more competitive sensitive genotypes
Genetic	relative abundance of genotypes with different sensitivities fitness properties of different genotypes sexual or asexual reproduction: influence of inheritance of resistance mutation rate if relevant, dominance of resistance alleles

gies. Many pathogen properties that contribute to risk (Table 2) are outside the control of growers, whereas many treatment measures (Table 3) provide opportunities to adjust resistance risk for particular pathogen/fungicide combinations (Brent, Hollomon 2007b).

Central to managing resistance in pathogen populations is the knowledge of any fitness cost, which may vary with environmental change, and be influenced by compensatory mechanisms that improve pathogen fitness. Consequently, measuring fitness cost directly in pathogen populations is not easy, and is resource intensive, involving bioassays of many isolates, although molecular diagnostic techniques offer scope to reduce this cost. Experimental designs must allow for measurement of immigration of sensitive or resistant individuals from neighbouring crops. Although monitoring a mixture of azoxystrobin resistant and sensitive isolates of *Magnaporthe oryzae* inoculated on ryegrass *Lolium perenne* (Ma & Uddin 2009), and the frequency of azole resistance in *Cercospora beticola* during an epidemic (Karaoglanidis *et al*. 2001), both indicated a fitness penalty linked to resistance. In general, many similar studies have not found a significant fitness penalty associated with resistance (e.g. Corio-Costet *et al*. 2010; Chapara *et al*. 2011; Karaoglanidis *et al*. 2011).

Linked to the relative fitness cost between sensitive and resistant individuals is the stability of resistance, an important practical issue since it determines if a fungicide can be used again after a period of withdrawal. Resistance levels may be stable for a long time, as in the case of benzimidazoles, but stability can be influenced by other mutations. Monitoring carbendazim resistance in *Botrytis cinerea* in French vineyards over many years encountered the highly resistant and stable *BenR2* phenotype, which was sensitive to diethofencarb. Taking advantage of this negative cross resistance a carendazim diethofencarb mixture was introduced, and *BenR1* was soon replaced by *BenR2* which was resistant to both mixture partners. However, *BenR2* quickly disappeared when other modes of action replaced the mixture partners. Clearly the second β-tubulin mutation (Phe200Tyr) causing diethofencarb resistance carried a fitness penalty not matched by the adjacent Glu198Ala mutation causing carbendazim resistance (Walker *et al*. 2013). In contrast, the stability of QoI

Table 3. Fungicide properties influencing evolution and spread of resistance (from Hollomon 2015)

Biochemical	interaction with target metabolism and its susceptibility (mode of action)
Physico-chemical/toxicological	stability, solubility, volatility, polarity partition and transport properties
Application	initial dose and distribution formulation exclusive and repeated use of "at-risk" mode of action extent of treated area integration with other disease management tools, including biofungicides resistant crop varieties, crop rotation, crop hygiene

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resistance (Gly143Ala) in grape powdery mildew populations (*Erysiphe necator* = *Uncinula necator*) was possibly enhanced after withdrawal of QoIs for several years, the presence of DMI resistance was caused by a mutation in the *cyp51* gene (Tyr136Phe) (Rallos *et al*. 2014).

Stability can also be influenced by environmental changes. DMI resistant isolates of *Cercospora beticola* remained stable after many generations in the absence of flutriafol, but after exposure to cold temperatures resistant levels declined and flutriafol sensitivity increased (Karaoglanidis & Thanassoulopoulos 2002). A more detailed consideration of the impact of stability on resistance management is discussed by Ishii (2015), but it is interesting that where resistance not only to fungicides, but also insecticides and herbicides, is not stable, and products are re-introduced after some years, resistance usually emerges again very rapidly.

Strategies to manage the evolution of resistance must not only reduce the population of the resistant phenotypes relative to sensitive ones, but also overall disease levels. A widely adopted strategy employs the use of mixtures in which partners have different modes of action, and which are often available from manufacturers as formulated pre-packed mixtures. Since the mixture partner controls both resistant and sensitive phenotypes, overall population size is reduced, and selection for resistance slowed down. Both modelling and experimental evidence from many studies show that mixtures do indeed slow the evolution to an at risk partner (Brent & Hollomon 2007b; van den Bosch *et al*. 2014). Multi-site inhibitors (e.g. Mancozeb, Clorothalonil) where possible are favoured mixture partners, but at risk single site fungicides can be used, especially where these mixtures provide good control, not just of the target pathogen. In this case one "at risk" opposes selection against the other. Although a recent modelling exercise showed that a mixture of two high resistance risk partners enhanced the life of both fungicides (Hobbelen *et al*. 2013), experimental evidence supporting use of just "at risk" partners is limited to a few studies ((Brent *et al*. 1989; Lorenz *et al*. 1992; Gisi *et al*. 2005; Thygesen *et al*. 2009).

Alternation or rotation of fungicides with different modes of action is also seen as a strategy to manage resistance. Resistance to the "at risk" fungicide is only selected when it is used, and not when the other fungicide is applied. Evolution of resistance is slowed, but seldom stopped, because the "at risk" fungicide is only used half the time. Some experimental studies support this, although when compared with mixture strategies using the same fungicide/pathogen system, a majority showed slower evolution of resistance following mixture treatments than alternation (van den Bosch *et al*. 2014). In practice, in many crops there are too few spray treatments to allow a useful comparison between mixtures and alternation.

Involvement of dose rate in selection for resistance remains controversial. Certainly the concept that high (= recommended) rates prevent, or slow down resistance to "at risk" fungicides is not supported by experimental evidence (van den Bosch *et al*. 2014), although where resistance is caused by many changes (= polygenic), it may be reduced. Treatment rates are often reduced by growers for economic reasons, and clearly this reduces selection pressure, although where this is achieved by splitting doses and exposing the target pathogen for a longer time, resistance levels may actually be increased. Providing effective disease control can be maintained, lowering the dose rate of the "at risk" partner, or increasing the rate of the partner fungicide, extends the life of the "at risk" fungicide (van den Bosch *et al*. 2014). This is a conclusion supported by a recent modelling study using data from a trial involving a QoI ("at risk") and chlorothalonil ("low risk") mixture to control Septoria leaf spot disease (*Zymoseptoria tritici* = *Mycosphaerella graminicola*) (Hobbelen *et al*. 2013).

Providing resistance does not carry a fitness cost, regardless of whether a mixture or alternation strategy is employed, pathogen populations will eventually become dominated by resistant phenotypes, and the "at risk" mixture partner will no longer be effective. But where a fitness cost is linked to resistance, adjusting the dose of the "at risk" fungicide whilst maintaining the partner at a level that ensures effective disease control, resistance may be kept at a level within the pathogen population which allows the "at risk" fungicide to contribute to disease control (Mikaberidze *et al*. 2014). These authors also suggest that changing the mixture partner and increasing chemical diversity could ensure that resistance to the "at risk" partner is prevented. However, translating predictions from mathematical models into optimum dose rates for mixture partners under field condition is not easy.

Guidance for management strategies discourages curative (= eradicant) in favour of preventative use, although there is no experimental evidence comparing the effect of these two approaches on selection for

doi: 10.17221/42/2015-PPS

resistance. In practice preventative use equates with early treatment when pathogen populations are small, although this is no guarantee that selection for resistance will not occur. But where a non-systemic partner is being used, it needs to be applied preventatively and before the initial inoculum reaches the crop.

Integrated Disease Management (IDM) offers an important contribution to delaying, and possibly avoiding resistance. Resistant crop cultivars, agronomic and crop hygienic measures, and biocontrol agents (although these are not necessarily immune from resistance) reduce disease levels and mean that fungicides are used less frequently and selection pressure reduced. Evaluation and introduction of anti-resistance strategies is the responsibility of manufacturers, but to succeed, strategies must be used over large areas, and their performance monitored. There must be a commitment to implementation from all involved companies, and to achieve this, various working groups exist within FRAC through which relevant data can be exchanged between companies. But the biggest challenge remains with growers who must integrate resistance management within effective and profitable disease control strategies.

CONCLUDING REMARKS

The concept of effective management of resistance using different modes of action or alternation is easily grasped by growers. It relies on maintaining sufficient diversity of modes of action which has become dangerously small, especially for the control of ascomycete and basidiomycete pathogens. This has created challenges for both the agrochemical industry to discover and commercialise novel modes of action, and for growers to successfully incorporate new products into effective disease control strategies without reducing productivity. By integrating fungicide use with other disease control approaches, including agronomic measures and resistant cultivars generated, either by conventional plant breeding or by genetic modification, growers have a key role to play in delaying selection for resistance by reducing the need for fungicides.

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Received June 9, 2015
Accepted after corrections August 18, 2015

Corresponding author:

Dr Derek W. Hollomon, Bristol road, Chew Stoke, Bristol, BS40 8UB, United Kingdom;
E-mail: orchardhse@ukgateway.net