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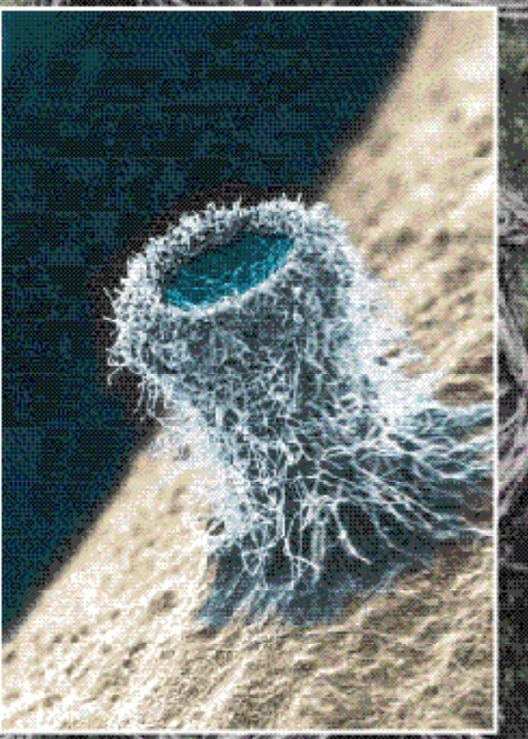
FUNGICIDE RESISTANCE: THE ASSESSMENT OF RISK

2nd revised edition



KEITH J BRENT AND DEREK W HOLLOMON

CropLife INTERNATIONAL

FRAC
FUNGICIDE RESISTANCE ACTION COMMITTEE

Cover:
Scanning electron micrograph of mycelium of *Oculimacula yallundae* (eyespot fungus) growing on a wheat coleoptile. The insert shows a young apothecium on wheat straw from which ascospores are ejected. This is a relatively 'low-risk' pathogen because of its long generation period, but resistance to fungicides has eventually developed. (Bayer CropScience)

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FUNGICIDE RESISTANCE: THE ASSESSMENT OF RISK

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CONTENTS

SUMMARY

- Fungicides are essential for the maintenance of healthy crops and reliable yields of high-quality produce. However, their effectiveness has been seriously affected in some situations by the development of resistance in target pathogens. It is necessary to assess as accurately as possible the risk of resistance arising, in order to guide selection of candidate chemicals for development and the establishment of strategies to ensure their durability, and to support the official registration of new products.
- This monograph reviews current knowledge of factors that determine the probability of resistance developing against a new fungicide, or to a new use of an existing fungicide. It discusses how these factors can be assessed as risk indicators, and the extent to which they can be combined into an overall estimation of resistance risk.
- Some chemical classes of fungicide are known to be more prone to resistance problems than others. New fungicides may belong to an existing class, whose resistance risk, if known, is likely to extend to the new candidate. If pathogen populations resistant to this class have already developed, these are likely to be cross-resistant to the new member. However cross-resistance can be partial, absent or even negatively correlated, and can occur between apparently unrelated chemicals. Therefore cross-resistance tests are a key component of all risk assessments.
- Resistance to fungicides usually results from an alteration at the site of fungicidal action in the target pathogen. Thus knowledge of the mode of action can indicate risk. A single rather than a multiple site of action, and a site of action known to have become resistant to other fungicides, are both positive indicators of risk.
- Resistance mechanisms reflect mutations in the pathogen genome. The ability to generate resistant mutants can be revealed by laboratory experiments in which mutants in pathogen populations are selected by exposure to the fungicide, sometimes in the presence of mutagenic agents. In certain pathogens, it is evident that genetic properties such as diploidy throughout most of the life cycle, or presence of introns adjacent to target site mutations, can suppress expression of resistance. The resistance risk is intensified if the resistant mutants have normal fitness with regard to growth, reproduction and pathogenicity. Genetic recombination tests can indicate whether single-gene or polygenic mutations are likely to occur.

- Monitoring, through bioassay of field isolates, is unlikely to detect major-gene resistance early enough to be useful in risk assessment, unless it is done in field experiments in which multiple fungicide applications are sustained over several years against large pathogen populations (with due precautions to prevent possible resistance spread). Monitoring can detect the early stages of polygenic resistance before this becomes severe enough to cause practical problems. The breadth of the range of sensitivity values (e.g. ED50 values) found in base-line monitoring studies appears not to correlate with risk of practical resistance. In the case of QoI fungicides, highly sensitive and selective DNA probes are being used to detect resistant major-gene mutations in field populations, in larger and more numerous samples, and at lower frequencies, compared with bioassay methods.
- Several epidemiological factors, characteristic of each target disease, affect the rate of resistance development. Short generation time, abundant sporulation, widespread spore dispersal and isolation of pathogen populations tend to increase resistance risk.
- The practical impact of the combined inherent chemical, biochemical, genetic, and epidemiological risk factors, with regard to causing actual resistance development in diseased crops, depends greatly on the conditions of fungicide use. Small amounts and infrequent occurrence of the pathogen, due for example to adverse climatic conditions, application of the fungicide infrequently or in rotation or mixture with other types of fungicide, and concurrent use of non-chemical disease-control measures, will all lower the risks of practical resistance development and consequent damage to crop yield and quality.
- A number of mathematical models defining rates of resistance build-up in relation to different strategies of fungicide use and amounts of disease have been proposed. Whilst they provide a valuable theoretical background, verification requires data that are difficult to obtain, and the models have as yet found little practical use in risk evaluation.
- Systematic assessment of all the inherent risk factors and the conditions of fungicide use allows overall judgements of degree of resistance risk to be made, and appropriate strategies of use to be established. These procedures are now a normal part of fungicide development programmes, and are required to be reported in applications for registration in many countries. With present experience and knowledge assessments must be approximate, at best indicating low, medium and high risk in particular situations.

- More precise prediction, particularly with regard to the time-scale and severity of any resistance build-up, is highly desirable. However, this must await the results of further studies on the biochemistry, genetics and population biology of resistant variants, and on their relationships to the onset of practical resistance problems.

INTRODUCTION

Success in combating crop diseases, and in reducing the damage they cause to yields and produce quality, depends greatly on the timely application of fungicides. Sometimes, however, target pathogens have acquired resistance against certain of the fungicides that normally control them well, and some serious difficulties in disease management have ensued. The Fungicide Resistance Action Committee (FRAC), an inter-company organisation affiliated to CropLife International, has as one of its main aims the communication of information on the problems of fungicide resistance, and on countermeasures, to all who are concerned professionally with crop protection, whether as researchers, advisers, teachers, students, registration officials, marketing managers or distributors. Therefore, FRAC published a monograph entitled ‘Fungicide Resistance in Crop Pathogens: How can it be Managed?’ (Brent, 1995), which gave a general overview of fungicide resistance management. A fully revised edition has been published (Brent and Hollomon, 2007)

One of the key components of fungicide resistance management is the assessment of the risk of the development of resistance, and of course this was one of the topics discussed in the first monograph. However, in view of the importance, and the difficulties, of risk assessment, FRAC commissioned a second monograph to deal specifically with this subject: Fungicide Resistance: the Assessment of Risk (Brent and Hollomon, 1998). Again this was written for a broad readership rather than for specialists, and did not attempt to give an exhaustive review of the very large amount of relevant literature. General reviews of this subject published prior to the First Edition of this Monograph, were by Gisi and Staehle-Csech (1988a, b) and by Brent, Hollomon and Shaw (1990), and we drew freely on these. Subsequently, fungicide resistance risk has been addressed in a Guideline of the European and Mediterranean Plant Protection Organisation (EPPO, 2002), and in a paper by Kuck (2005). This Second Edition retains the general structure and most of the information and discussion given in the first edition, which remain valid and relevant now. It also incorporates new data and comment that reflect the many further developments, over the past eight years, in fungicide research and application, and in the assessment of resistance risks.

In this publication, the term 'fungicide' will be used in a broad sense, covering all agents used to control plant diseases caused by fungi. These now include compounds that act by interfering with specific infection processes, or activating plant defences, rather than by killing the pathogen.

Unless otherwise indicated in the text, 'risk of resistance' will mean the likelihood of resistance developing to an extent that causes failure or significant diminution of disease control in commercial crop protection, and not merely the probability of detecting resistant forms at low levels or of resistance being inducible in experimental situations.

Defined in this way, the evaluation of resistance risk is a matter of great significance for the fungicide manufacturer. It influences decisions on whether a product candidate will be worth developing and marketing, on what use strategies are adopted in order to ensure sustained performance, and on how much and what kind of resistance monitoring should be done. It is also increasingly recognised by registration officials as an important element of efficacy assessment, and by agricultural advisers and farmers as a guide to selecting and scheduling treatments and to the need for vigilance.

In this monograph our approach is to describe in turn the different types of risk indicators and their potential value and limitations for practical use. Then we discuss how the range of indications obtained can be integrated into overall assessments of risk and can be used to determine resistance management strategies and the need for monitoring under different conditions of fungicide use. Finally we consider the use, communication and reliability of existing expertise, speculate on future prospects and identify requirements for further research.

FUNGICIDE-ASSOCIATED RISK

Structural class

Experience of practical problems of fungicide resistance, which now extends well over three decades, indicates clearly that the risk of resistance development can often be judged initially by considering the chemical class to which a fungicide belongs. Each chemical class is characterised by a typical resistance behaviour pattern. Thus certain major classes of fungicide, notably those based on copper (e.g. copper oxychloride and cuprous oxide;) phthalimides (e.g. captan and folpet); and dithiocarbamates (e.g. mancozeb, maneb, zineb and thiram), have very rarely if ever been known to encounter practical resistance, even after many years of use. By

contrast, in some other classes, such as benzimidazoles (e.g. benomyl, carbendazim, thiabendazole), phenylamides (e.g. metalaxyl, oxadixyl), dicarboximides (e.g. iprodione, procymidone, vinclozolin), and strobilurin analogues (e.g. azoxystrobin, kresoxim-methyl, pyraclostrobin), all the members met serious resistance problems that arose in most of their target pathogens, within 2-10 years of the commercial introduction of each class. Resistance to azoles (e.g. triadimefon, flutriafol, epoxiconazole) has developed more gradually, and only in certain pathogens.

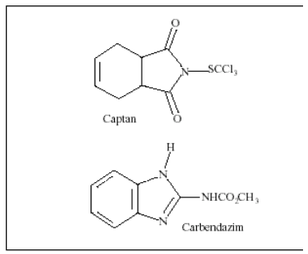
Non-class-specific resistance, that affects members of more than one chemical class, arises commonly against insecticides and herbicides. It results mainly from the development by the target organism of a capacity to inactivate certain pesticides through degradation or conjugation. Fortunately, this type of resistance is insignificant with regard to fungicides. Therefore if a candidate fungicide belongs to a known chemical class, much can be clearly predicted about the risk of resistance to it arising in existing target pathogens for the class, and also in new target pathogens.

Table 1 gives estimates of the liability of different chemical classes of fungicides to select resistant populations of target pathogens. In most cases these estimates are based on performance records and on results of resistance monitoring during the years of commercial use. The estimates for the newest classes are more tentative because of their short periods of commercial use. It is debatable whether the morpholines and related amine fungicides should be included in the low-risk or medium-risk category. Over many years of use, their overall performance has remained very good, but some changes in sensitivity have been detected, and occasionally there has been some loss of disease control.

Risks of resistance development in a particular target pathogen may not be entirely uniform between individual members of a fungicide class. Variation occurs in intrinsic activity and in resistance factors (as shown for azole fungicides in Table 2), both of which could affect selection pressure, degree of resistance encountered and overall effectiveness against partially resistant populations. However, such within-class variation has not proved sufficient to affect the overall categorisation of risk for any fungicide class

A fuller list of chemical classes of fungicides, together with common names of members of each class, and with estimates of degree of resistance risk, and target sites of action is published by FRAC on its website (www.frac.info), and is updated annually.

There are a few cases where fungicides are known to share resistance risks, through cross-resistance, and yet they apparently belong to very different structural classes. Strains of *Botrytis cinerea* resistant to the dicarboximides are also resistant to



Captan + Carbendazim
Captan and carbendazim represent two structural classes of fungicide, phthalimides and benzimidazoles, that have been proved by long experience to carry widely differing resistance risks. Nowadays, risk assessments can give advance warning of such large differences in liability to resistance.
(Bayer CropScience)

Table 1 Estimates of the inherent risk of resistance attached to different chemical classes of fungicides. The actual risk during commercial use may differ, depending on the target disease, its intensity, and the regime of use.

Relative Resistance risk*	**Chemical Class (some are represented by a single compound)**
High	Benzimidazoles, dicarboximides, phenylamides strobilurin analogues (e.g methoxyacrylates, oximino acetates)
Moderate	2-Amino-pyrimidines, amines (including morpholines), anilinopyrimidines, aromatic hydrocarbons, azoles, carboxanilides, carboxylic acid amides, carpropamid, cymoxanil, fenhexamid, kasugamycin, phenylpyrroles, phosphorothiolates, quinoxyfen
Low	Chlorothalonil, coppers, dithiocarbamates, fosetyl-Al, pyroquilon, phthalimides, probenazole, sulphurs, tricyclazole.

*High: widespread and severe decrease of effectiveness due to resistance development occurred in one or more target pathogens, in certain regions, within a few years of introduction.
Medium: decrease of effectiveness detected in a few situations, or to a limited extent, and/or resistant isolates obtained from field samples of target pathogens.
Low: decrease in effectiveness or occurrence of resistant isolates not detected or very rare after many years of use.

aromatic hydrocarbon fungicides, such as dichloran, quintozene and biphenyl (Leroux *et al.*, 1977; Georgopoulos, 1982). The reason for this is not fully understood, but there is evidence for a common mechanism of action, histidine kinase being a possible target site (Leroux *et al*., 2002). Triforine, a piperazine, and fenarimol, a pyrimidine carbinol, are positively correlated for cross-resistance with each other and with the azole fungicides (Georgopoulos, 1982). In this case the cross-resistance was not surprising, because it was well known that these structurally diverse fungicides are all sterol demethylation inhibitors (DMIs). Strobilurin

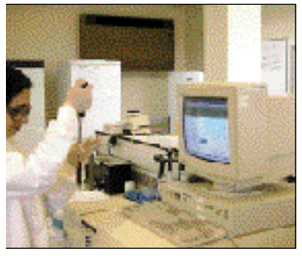
analogues share cross-resistance with the oxazolidinedione fungicide famoxadone, and the imidazolinone fungicide fenamidone. They have closely related target sites within the Qo centre of the ubiquinol-cytochrome c oxidoreductase , which allow a common mechanism of resistance (see below).

Mechanisms of Action

In those cases where they have been elucidated, the mechanisms that underlie the development of resistance often involve some modification of the biochemical target site in the pathogen, which decreases the affinity of the fungicide for its target. Many authors have pointed out that such modifications will occur more readily in a single-site-specific target, which is typical of the more recently introduced fungicides, rather than the multiplicity of targets which tend to be a characteristic of the older fungicides. A single target site can be rendered resistant through one mutation changing a single DNA-base in the target gene and, consequently, just one amino acid in the target protein. Several mutations must occur simultaneously to confer resistance at multiple target-sites, so this will be a much rarer event. If the chance occurrence of a single mutation that affects a target site is 10^{-8}, then the chance of two such mutations, independently affecting two target sites, occurring together is 10^{-16}.

In general, systemic fungicides have been associated with resistance problems to a much greater extent than have non-systemic (‘protectant’) fungicides. However, there are some exceptions. For example, the dicarboximides vinclozolin and iprodione have little or no systemic action, but they have encountered major resistance problems. QoI fungicides vary greatly in their systemicity (e.g. azoxystrobin is highly systemic and trifloxystrobin almost non-systemic), yet all have encountered resistance problems in certain pathogens. Also, there is no mechanistic reason why systemicity *per se* should confer a likelihood of resistance development. Cases of resistance to systemic fungicides can generally be explained through other properties which accompany their ability to be translocated in plants. These are more persistent protective action, eradicant action, and specific biochemical mechanisms of action. These performance attributes will tend to increase the selection pressure favouring resistant mutants, although such effects are very difficult to single out and quantify. The influence of a specific biochemical mechanism of action, so called single-site action, is probably the dominant factor that determines the greater risk of resistance attached to systemic fungicides.

In general, the occurrence of cross-resistance of pathogen strains to a range of different fungicides correlates with the existence of a common mode of action which is shared by these particular fungicides. For example, cross-resistance is shown by all those fungicides known to act at the Qo centre within ubiquinol-cytochrome c oxidoreductase of mitochondrial complex III (QoI fungicides). This again indicates that the mechanism of fungicide resistance involves changes within either the site of action, or some closely linked metabolic pathway. Hence knowledge of the biochemical target site, and also alternative metabolic pathways bypassing the target site, are very useful indicators of risk. If a fungicide acts at a single site, then there is a higher risk of resistance than if it acts at multiple sites. Also if the mode of action is found to be identical with that of an existing fungicide, or fungicide class, then it is likely that the risk of resistance is similar to that of the existing fungicide(s), and that any populations of target pathogens already resistant to the existing fungicide(s), will also be resistant to the new fungicide.



Determining the biochemical mode of action can give valuable clues as to the likelihood of resistance developing, providing it is done in good time.
(Bayer CropScience)

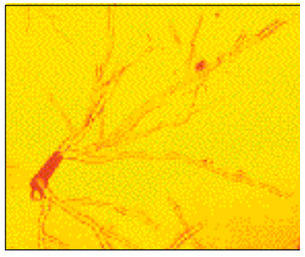
However, these associations between resistance and site-specificity, and between a particular mechanism of action and a particular risk of resistance are not absolute. Amine fungicides (morpholines, piperidines and spiroketalamines) are systemic fungicides which have biochemically specific actions on sterol biosynthesis. However, reductions in sensitivity have been notably smaller, and slower to develop than those encountered in other classes of systemic fungicides, despite their widespread use in cereals, bananas and other crops. It is known that they can act at more than one biochemical site (Ziogas *et al*., 1991), and this may account for their more durable action.

There is one well-known case where two fungicide classes, benzimidazoles and phenylcarbamates, are known to share the same cellular site of action (β-tubulin) but are diametrically opposite in their resistance behaviour. This so-called negative cross-resistance is discussed later.

Thus mode-of-action information must be taken as very useful, although by no means a certain guide to resistance risk. Given the availability of modern molecular and genomic techniques, and the substantial detailed knowledge of biochemistry, much effort is now devoted during early stages of development to mode-of-action studies to identify whether or not a new chemical acts in a different way from existing fungicides. Formerly it took a long time to identify a mode-of-action; only recently has the mode-of-action of dicarboximides been identified (Leroux *et al*., 2002), 30 years after the introduction of this important class of fungicides into commercial use. It is now usual for a new fungicide group to be introduced with

some information on its mode-of-action (e.g. benzophenones, which include metrafenone, Schmitt *et al*., 2006; Opalski *et al*., 2006).

Much research has been done on the mode of action of azole (DMI) fungicides since resistance to anti-fungal drugs has become a significant problem in human medicine. Azoles interact with the haem of cytochrome P450s, and although features of azole chemistry favour binding at the sterol substrate site of the 14 α-demethylase (CYP51), another cytochrome P450, sterol C-22-desaturase (CYP61) may also be involved (Kelly *et al*., 1995). As a consequence of blocking 14 α-demethylation, the later 5-6-desaturase step in the sterol biosynthesis pathway will not accept a 14-methyl sterol substrate, and it is the accumulation of a 5-hydroxy sterol which is the toxic component of azole action (Watson *et al*., 1989). In addition to target site changes, other mechanisms contribute to azole resistance, including changes in the sterol 5-6-desaturase, over-expression of the target sterol 14 α-demethylase (Schnabel and Jones, 2001), and increased expulsion mediated through increased activity of ABC transporters (De Waard *et al*., 2006). It seems that accumulation of several mechanisms may be needed before practical disease control difficulties emerge, which probably accounts for the slow stepwise development of azole resistance in many plant pathogens.



Pyrenophora teres spores germinating on agar. Inhibition of germ tube elongation can be used to assess fungicide sensitivity for appropriate fungicides.
(Syngenta)

There are a few 'fungicides' (more strictly termed disease control agents) in agricultural use that do not affect the viability, growth or reproduction of the target pathogen directly. Tricyclazole and pyroquilon, used to control rice blast disease, specifically affect the penetration of the pathogen (*Magnaporthe grisea*) into the host plant through inhibition of reductase steps in melanin biosynthesis needed for the normal function of appressoria. So far, no resistance problems have arisen with these melanin biosynthesis inhibitor-reductase (MBI-R) fungicides, but there is no obvious reason why not; resistance to carpropamid (melanin biosynthesis inhibitor–dehydratase or MBI-D fungicide), occurred soon after its introduction into Japan (Kaku *et al*., 2003). Probenazole, which also is used against rice blast, acts primarily on the plant, and is known to induce a set of defence reactions known as systemic acquired resistance (SAR). Whilst the commercial use of MBI-R fungicides and probenazole over some 30 years has not led to the development of resistance problems, several other rice blast fungicides have encountered widespread resistance. Lack of resistance to SAR inducers can possibly be explained on the basis that these compounds are known to induce the synthesis of a number of different pathogenesis related proteins (PR proteins), that act against the pathogen.

CROSS-RESISTANCE

Obviously knowledge of whether or not a new fungicide can control strains of the target pathogen that are known to resist other fungicides is a key component of resistance risk assessment. Hence it has now become a routine step in the development of a new fungicide to test it in bio-assays against a representative collection of target-pathogen isolates that are known to resist any of the existing fungicide treatments, including those that do not appear to be closely related to the new product in chemical structure or mode of action. If such strains are not controlled, then it is clear that resistant populations already exist. It may or may not be wise then to proceed with development and marketing, depending on how severe and widespread are the existing resistance problems, what avoidance or delaying strategies of use are already practised, and whether these are appropriate to, and acceptable for, the new product. On the other hand, if such strains are controlled, and if field experiments are regularly successful, then it can reasonably be assumed that the existing pathogen populations which resist other fungicides will not cause problems for the new fungicide. Any resistance that might possibly develop would be of a new type, arising from selection of initially rare mutants.

Usually if a new fungicide has a similar structure and/or mode of action to existing fungicides against which resistance has developed, then cross-resistance is found. Indeed, the several carboxylic acid amide (CAA) fungicides are grouped together by FRAC solely on the basis of their cross-resistance (FRAC, 2005). Sometimes the cross-resistance is only partial. Whilst cross-resistance extends to all DMIs (Gisi *et al*., 2005), Resistance Factors (RF) have decreased as new azole chemistry has been introduced (Table 2), and so the latest, prothioconazole, is effective at dose rates at which the first azole, triadimefon, no longer controls cereal powdery mildew. The degree of cross-resistance often varies between isolates (Gisi *et al*., 1997), although differences are generally not large enough to cause problems in risk assessment. In *M. graminicola* sensitivities to cyproconazole and epoxiconazole are well correlated ($r^2 = 0.67$), but less so for cyproconazole and prothioconazole ($r^2 = 0.45$; Figure 1). For a much smaller set of *M. graminicola* isolates, sensitivities to flutriafol and flusilazole were not significantly related ($r^2 = 0.02$; see Brent and Hollomon, 1998).

Table 2 Resistance factors for DMI fungicides in wheat powdery mildew *(Blumeria graminis f.sp. tritici).*

DMI fungicide	Year of Introduction	Resistance Factor*
Triadimefon	1976	100
Flutriafol	1983	85
Hexaconazole	1986	89
Tebuconazole	1988	30
Epoxiconazole	1993	35
Prothioconazole	2004	5

* Ratio of ED_{50} values between resistant and sensitive isolates

Data from Senior *et al*., (1993); Hollomon (unpublished) and Kuck and Mehl, (2004)

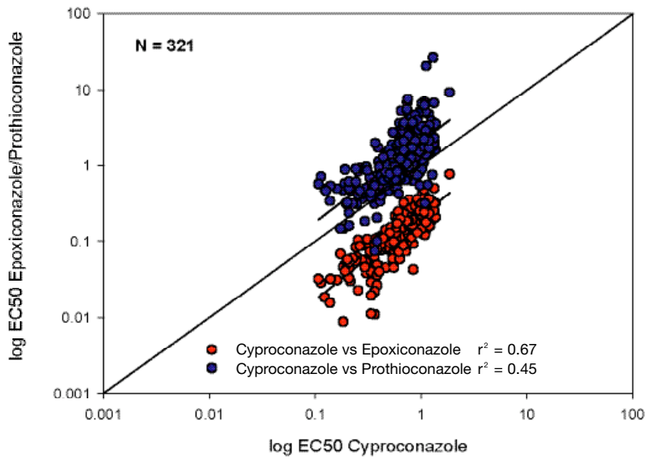


Fig.1. Sensitivities of isolates of *Mycosphaerella graminicola* towards two different pairs of azole (DMI) fungicides. (Syngenta)

Negative cross-resistance is a potentially important factor in risk assessment. Exposure of pathogens to two fungicides that exhibit this negative cross-resistance, should greatly reduce any resistance risk associated with either component, because a shift to resistance against one automatically confers sensitivity against the other. A single amino acid change at amino acid codon 198 in the target β-tubulin causing resistance to benzimidazoles automatically confers sensitivity to phenylcarbamates, and *vice versa*. Mixtures of carbendazim and diethofencarb have been used commercially with some success against *Botrytis cinerea* on grapevines in situations where benzimidazole-resistant strains were already widespread. Unfortunately, another amino acid change at nearby codon 200 confers resistance to both fungicides, with the result that carbendazim / diethofencarb mixtures soon became ineffective in many vineyards (Leroux *et al*., 2002).

Other examples of negative cross-resistance are known in laboratory mutants of *Magnaporthe oryzae* between phosphorothiolates and some experimental phosphoramidate compounds (Uesugi, 1982); in field isolates of *Penicillium expansum* between benzimidazoles and diphenylamine (Rosenberger and Meyer, 1985); in both laboratory mutants and field isolates of *Ustilago nuda* between different carboxamides; in various pathogens between DMI fungicides (see review by Leroux, *et al*., 2002). The not uncommon occurrence of negative cross-resistance between fungicides with a similar mode of action illustrates the crucial importance of backing up mode of action studies, and any conclusions drawn, by conducting cross-resistance tests.

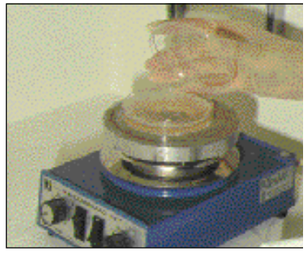
GENETIC STUDIES

Artificial Mutagenesis

The potential in the target pathogen for mutations conferring resistance is the basic cause of a resistance risk for a new fungicide. The key question of whether such a potential exists can be tested directly in the laboratory, by selecting spores or mycelium on a culture medium containing the new fungicide at a concentration known to inhibit the growth of the wild-type. The likelihood of a resistant mutation occurring can be increased by exposure before selection to a chemical mutagen or ultra-violet light. Resistant survivors form colonies, and spores from these can be examined for their degree of resistance by exposure to different concentrations of the fungicide.

If stable resistant forms are produced in such selection experiments, it is essential that they should then be tested for their potential fitness as crop pathogens. Often, the induction of mutation to resistance also causes damage to the pathogen so that it grows, multiplies and/or infects less well than the wild-type, to such a degree that it does not offer any practical threat to fungicide performance in the field. Even when resistance factors are small (5 – 10 fold), as in the case of flumorph resistant mutants of *Phytophthora infestans* (Yuan *et al*., 2006), sporulation and growth may be severely reduced. Testing for fitness must be restricted to the laboratory, and must be carefully controlled, because there is a danger that an artificially produced mutant could spread in the field and itself cause resistance problems.

Testing for fitness in the laboratory should involve testing for rate of growth and degree of sporulation *in vitro* and on host plants. Failures or severe reductions in these activities in all mutants suggest that the type of mutation induced in the laboratory will not cause practical problems. Competition experiments, using mixed inocula of spores from sensitive and resistant strains, also can indicate fitness differences. If the mutants are normal (or better than normal) in their growth, pathogenicity and sporulation, then a positive indication of risk is given.



Inducing mutations in *Rhynchosporium secalis* spores by exposure to UV light as part of a fungicide resistance risk appraisal exercise. Similar tests are now often done for new fungicides. (Syngenta)

It is highly desirable that the experimental fungus should be a plant pathogen which is sensitive to the fungicide under study. Sometimes saprophytic fungi are used, which can be more convenient to handle in the laboratory but do not permit the submission of resistant mutants to pathogenicity tests. Ideally, mutagenic tests should be done on all major target pathogens for the particular fungicide, but this will seldom be feasible because of cost constraints. Another advantage of using target pathogens in mutagenic tests is that resistant mutants can be checked for their degree of resistance to fungicide treatment after inoculation onto host plants.

Mutation frequency is decreased by the action of DNA repair mechanisms. These are better for nuclear DNA than for mitochondrial DNA, so that the latter is more liable to mutation. In addition, the frequency of DNA base changes in mitochondrial DNA is further increased by its close proximity to reactive oxygen species generated during respiration. The risk of resistance, therefore, seems likely to be higher for target sites encoded in the mitochondrial genome (as in the special case of QoI and QiI fungicides), than for targets encoded in nuclear DNA. However, in practice both benzimidazole and phenylamide resistance, which result from nuclear mutations, developed equally quickly.

Experience indicates that the capacity for a target pathogen to produce resistant mutants with normal fitness in laboratory experiments is generally associated with a

potential for the development of resistant populations in crops during commercial use of the fungicide. It was relatively easy to generate mutants resistant to benzimidazoles and the phenylamides in many target pathogens, but for low-risk fungicides, such as copper compounds or dithiocarbamates, laboratory mutants occur rarely, have a low degree of resistance, and show poor growth and pathogenicity.

If mutant spores survive and produce colonies on fungicide amended agar, a genetic and biochemical potential for resistance is indicated. (Syngenta)

However, in some cases the relationships between response to mutagens and risk of practical resistance have been less clear-cut. Thus mutants highly resistant to amine (morpholine) fungicides are readily obtained in the laboratory, but development of field resistance has been slight, and product performance in practice has been maintained. Laboratory mutants of several fungi that were resistant to DMI fungicides had reduced growth and sporulation, and their pathogenicity was in inverse proportion to the degree of resistance (Fuchs and Drandarevski, 1976). The investigators concluded that practical resistance to DMIs would be unlikely to arise. DMI resistance problems have in fact arisen, although relatively slowly. Presumably this discrepancy between lack of fitness in laboratory mutants and fitness in field mutants reflected a selection for fitness in resistant mutants which occurred under field conditions, but would not occur in mutant production and screening experiments in the laboratory.

If a number of mutant isolates are produced by mutagenic treatment, then it is very informative to compare them for their degree of resistance and their fitness parameters, and if possible to cross them or genetically analyse them in other ways, to reveal whether they are identical, or whether they include different allelic forms or mutations in different genes.

A non-target type of QoI-resistant mutation in a laboratory mutant of *Mycosphaerella graminicola*, involved a nuclear gene and the enhanced production of an alternative oxidase, allowing respiration to bypass the QoI target site (Ziogas *et al*., 1997). It was considered unlikely to cause practical resistance because this laboratory mutant proved more sensitive than the wild-type to azoxystrobin *in vivo*. Subsequently, partially resistant isolates of *M. graminicola* with increased alternative oxidase activity have been obtained from QoI treated wheat crops (Miguez *et al*., 2004) and it has been suggested that this change rescues the pathogen sufficiently from the effects of QoI fungicides to allow further selection of more highly resistant target-site mutations (Avilla-Adme and Köller, 2002, Wood and Hollomon, 2003).

Overall, the reliability of the results of mutagenic experiments as indicators of resistance risk is still debated. The consensus view is that they have given useful

information on the basic potential for resistance, and on the genetic and biochemical nature of such resistance, and are well worth doing. Any resulting availability of resistant mutants can also aid biochemical mode of action studies. However, mutagenic testing must be regarded as one component of a much broader risk assessment exercise, and the results certainly cannot be relied upon as a total or infallible guide to the subsequent response of pathogen populations in the field, especially where laboratory mutants express reduced fitness.

Gene Characterisation

Most plant pathogens (ascomycetes, basidiomycetes) are haploid during most of their life cycle, so it would make no difference whether a resistance mutation was dominant or recessive. However, in diploid pathogens, such as oomycetes, resistance would spread more slowly if it was inherited in a recessive manner, as for example with zoxamide (Young and Slawecki, 2005; Gisi *et al*., 2007).

The production of laboratory mutants resistant to QoI fungicides was reported in yeast, and other micro-organisms, before these fungicides were commercialised (Colson, 1993; Ziogas *et al*., 1997). Eleven different point mutations in the target cytochrome b gene were identified (Esposti *et al*., 1993; Brasseur *et al*. 1996), but some of these were linked to impaired growth *in vitro*, due to respiratory deficiency. Uniquely, the QoI target is coded by a mitochondrial gene rather than a nuclear gene, so the significance of these molecular studies with regard to the risk of practical resistance could not be fully assessed on the basis of past experience. Indeed, a prediction that any practical resistance would arise gradually in a step-wise manner (Godwin *et al*., 1999) was soon proved incorrect. Resistance developed in field populations of several pathogen species within two to four years of introduction. In all cases only two of the target-site point mutations (G143A; F129L) identified in earlier laboratory studies were detected in resistant isolates, even though these two mutations generate very different levels of resistance (Gisi *et al*., 2002).

DNA sequencing permits exploration of the structure of a gene encoding a target protein, and its influence on resistance risk. In some pathogens, such as rusts, *Pyrenophora teres* and *Alternaria solani*, the amino acid codon 143 in the mitochondrial cytochrome b gene is followed immediately by an intron. The point mutation at codon 143 that confers QoI resistance is likely to adversely affect the intron splicing process, with the result that the b-type cytochrome no longer functions, and the mutation is lethal (Grasso *et al*., 2006; Sierotzki *et al*., 2007). This may well explain why QoI resistance has not been a problem in the control of these diseases, despite widespread use of QoIs against them.

Genetic Recombination

Along with mutation and migration, recombination provides an opportunity to introduce novel genotypes into a population. In many plant pathogens re-assortment of genes can be achieved not only through sexual recombination, but also through anastomosis followed by recombination at mitosis (the parasexual cycle), and this latter process again can produce new genotypes. Resistance genes may recombine with better fitness characteristics, to give phenotypes that will spread under practical conditions. Furthermore, sexual reproduction can produce wind-dispersed spores, so where the dispersal of asexual spores is limited to rain-splash events, the operation of a sexual stage increases population size and the speed at which resistance can spread (e.g. in *Mycosphaerella graminicola*).

Sexual or parasexual recombination could equally well break up resistant combinations of genes in situations of polygenically determined resistance. Felsenstein (1994) suggested that the more frequent occurrence of sexual reproduction and associated redistribution of genes in wheat powdery mildew compared with barley powdery mildew may be the main cause of the generally slower development of DMI resistance in the former pathogen. Consequently it is difficult to predict the likely impact of recombination in field populations on the build-up of resistance.

Where the sexual stage exists and can be manipulated in the laboratory, or where recombination through the parasexual cycle or protoplast fusion can be induced, crossing experiments can be done to determine whether differences in fungicide sensitivity between pathogen isolates are under monogenic or polygenic control. Such knowledge can influence considerably resistance risk analysis, and also the establishment of use strategies and planning of monitoring programmes. Some examples of recombination studies are those reported by Butters *et al*., (1986) and Brown *et al*., (1992) for ethirimol and triadimenol resistance in barley powdery mildew, by Faretra and Pollastro, (1993), Hilber *et al*., (1994) and Eberle and Schauz, (1996) for fludioxonil resistance in *Botrytis cinerea* and *Ustilago maydis*, and by Shattock, (2002) for metalaxyl resistance in *Phytophthora infestans*. Recombination studies involving resistant mutants or resistant field isolates are now being undertaken increasingly as a part of the risk evaluation for a new product. The most recent example are the studies made for CAA fungicides in *Plasmopara viticola*, in which resistance was shown to be inherited by one or two recessive nuclear genes (Gisi *et al*., 2007).

FORCED SELECTION

Laboratory and glasshouse studies

Attempts have been made to demonstrate the capacity for a fungicide to select resistant mutants by exposing successive generations of pathogens taken from sensitive field populations to repeated fungicide treatments, either *in vitro* or on plants in a glass-house or controlled-environment chambers. This can be done either with a fixed fungicide concentration, which is likely to induce the selection of a discrete resistant population based on major gene mutation, or with increasing concentrations of fungicide, which could favour a stepwise build-up based on polygenic mutation.

Early studies on the possible selection of resistance to phenylamide fungicides *de novo* in *Phytophthora* spp. by serial transfers on fungicide-amended agar or fungicide-treated plants, were summarised by Davidse (1982). Taken overall, the results indicated that resistant strains were obtained more readily by *in vitro* treatments than by passage through fungicide-treated plants, that isolates showing *in vitro* resistance were often non-pathogenic or displayed normal sensitivity on treated plants, and that in comparison with serial transfer, mutagenic treatments produced more highly resistant isolates with a greater proportion also displaying resistance *in vivo* and normal virulence. Phenylamide-resistant field populations of *Phytophthora infestans* arose within two years from the first commercial use of these fungicides. Thus, in this case serial transfer experiments were less useful than mutagenic experiments as an indicator of practical risk

Strains of *Botrytis cinerea* resistant to both dicarboximide fungicides (Beever and Byrde, 1982) and the phenylpyrrole fungicide fludioxonil (Hilber, 1994) are easily obtained in the laboratory, by inoculating conidia or mycelium from wild-type cultures onto fungicide-amended agar plates. However these resistant strains are less fit than wild-type strains in tests for growth competition *in vitro*, osmotic sensitivity and pathogenicity. In practice, resistance to the dicarboximides gradually built up in vineyards in regions of intensive use. The dicarboximide-resistant field isolates lack resistance to fludioxonil, and show a greater degree of fitness and a lower degree of dicarboximide resistance than the doubly resistant laboratory strains. Also they were not selected by fludioxonil application in field experiments. Possibly, the greater fitness of the dicarboximide resistant field strains evolved gradually through sustained selection pressure from repeated and widespread use of dicarboximides under field conditions.

To avoid cross contamination between different pathogen isolates, plants and pathogens can be contained as shown for vines and *Erysiphe necator.* (Syngenta)

Thus selection of mutants through exposure of initially sensitive cultures to fungicides in the laboratory can give an indication of the genetic and biochemical potential for evolution of resistant variants, but fitness is often impaired. However, more comparisons of forced selection experiments with mutagenic experiments involving artificial mutagenesis, and with field monitoring, are needed in order to judge more clearly their value in resistance risk evaluation. It is possible that repeated selection by exposure to increasing fungicide concentrations could be particularly useful as an indicator of polygenic resistance, where stepwise development of resistance is thought to occur. Surprisingly, little attention appears to have been given to 'training' experiments with fungicides against which polygenic resistance is known to develop.

Forced selection experiments also have been done starting from inocula of prepared mixtures of resistant and sensitive spores at set ratios (e.g. 0.1 or 1.0% resistant spores), rather than from wholly sensitive inocula as described above. Whilst not designed to indicate potential risk from an initially sensitive situation, such experiments can give valuable information about the competitiveness of resistant strains, and the selective effects of different fungicide treatments (e.g. Hunter *et al*., 1987; Gisi, 1988, 1991).

Field studies

Forced selection experiments in the field have the advantage of exposing the fungicide to a much wider genetic diversity within a pathogen population than where just a few selected isolates are used in growth room or greenhouse experiments, and a range of environmental conditions that cannot easily be reproduced elsewhere. Repeated, sole applications of the fungicide are made, generally over a number of years, to plots containing plants susceptible to the target pathogen. Disease development may be encouraged by providing inoculum, or by spraying or misting with water. Samples are tested for sensitivity at appropriate intervals. Because of the inherent dangers in this approach, it should be taken only after careful assessment of the risk of resistant strains arising and spreading to commercial crops, and after appropriate precautions are taken.

A field study of the possible selection of strains of the cereal eyespot pathogen (*Oculimacula* spp.) resistant to benzimidazole fungicides failed to reveal the occurrence of resistance over a five-year period (Fehrmann *et al*., 1982), although during the period of this study major problems of benzimidazole-resistant eyespot arose in several countries. This pathogen generally produces only one generation per

year, so that with an initial mutant frequency of say 10^{-8} and with a 10% pathogen survival after each annual fungicide treatment, it would then take seven years for a 10% proportion of resistant mutants, that would be readily detectable by the sampling and testing procedures used, to be reached. In this experiment, benzimidazole-resistant strains were in fact detected after seven years - too late to be of practical use as a risk indicator.

Another long-term field study over eleven years also involving the cereal eyespot pathogen (*Oculimacula* spp.), examined the effects of intensive selection on the development of resistance to the anilinopyrimidine fungicide, cyprodinil (Babij *et al*., 2000). Up to 8% of isolates were resistant at any one sampling, but their frequency fluctuated from year to year. Only after eight years of continuous and intensive treatment was a small, but significant decline in sensitivity detectable, although overall disease control was not affected. Similar results were obtained for field populations of *Botrytis cinerea* (Chapeland *et al*., 1999). This slow and fluctuating decline in sensitivity to cyprodinil contrasts with the sudden increase in benzimidazole resistance, and might be considered to indicate polygenic changes, as well as a moderate resistance risk for anilinopyrimidines as compared to a high risk for benzimidazoles. However, in both pathogens resistance to anilinopyrimidines in field isolates is in fact controlled by a single major gene.

Testing the sensitivity of *Erysiphe necator* to azoxystrobin. Fungicide is applied to vine seedlings which are then inoculated with specific pathogen isolates. The test is used to establish base line sensitivity and for product monitoring. (Syngenta)

In situations where a high frequency of selection opportunities can be achieved, as for example with a pathogen producing many generations per season (e.g. *Venturia inaequalis*, *Mycosphaerella fijiensis* var *difformis*), and where the frequency of applications of the fungicide per season can be very high (e.g. up to 20), then there will be a reasonable chance of 'forcing' the development of major-gene resistance in field populations (if the basic potential for resistance exists). The gradual development of a polygenic resistance can also be demonstrated in field experiments, as in the cases of ethirimol and triadimefon (Brent *et al*., 1989). The appearance of resistance in such experiments must be taken as a serious warning of possible resistance problems. However, the limited size of pathogen populations in experimental plots, compared with those in commercial fields, and the chance that experimental plots may be invaded by sensitive populations from other sites, imply that a negative result cannot be fully relied on to indicate low risk.

Such field experiments require a high resource allocation, not only to manage the field work, but also to sample and bioassay isolates. Where a molecular resistance mechanism has been identified, rapid PCR methods to detect resistant alleles (discussed below) greatly increase the number of samples, especially of obligate

pathogens, that can be tested, and improves the ability to quantify the evolution of fungicide resistance at the population level by directly monitoring the genotype. Using these techniques it was easy to see the effects of selection within one growing season on the development of QoI resistance (G143A) in two wheat pathogens, *Blumeria graminis* f.sp *tritici* (Fraaije *et al*., 2002) and *Mycosphaerella graminicola* (Fraaije *et al*., 2005).

DETECTION AND MONITORING

Agrochemical companies make, or commission, surveys of the sensitivity of field isolates of the main target pathogens, prior to the introduction of any new fungicide into commercial use. Such surveys are often, and aptly, referred to as 'base-line' studies. In addition to satisfying requirements for registration, there are three good scientific reasons for undertaking them:

- To develop and test an accurate, rapid, reproducible method for determining the degree of sensitivity of large numbers of field samples of major target pathogens, so that such a method is readily available for any future monitoring that may be required.
- To obtain initial data regarding the range of sensitivity that exists in major target pathogens and major areas of use, to serve as a base-line against which any future measurements of sensitivity can be compared in order to reveal any possible shifts in sensitivity.
- To detect any differences in sensitivity between samples that might, through the build-up of the less sensitive components, lead to future resistance problems.

The importance of achieving the first two requirements and the methodology are discussed in the third monograph in this series (Russell, 2003). The last requirement is particularly relevant to the assessment of resistance risk. It would be very valuable to know whether or not any initially rare, resistant variants, and any early increases in their proportion in response to fungicide treatment, could be detected in field populations of target pathogens. A knowledge of the fitness of such variants, and whether this subsequently changes through selection, would also be valuable.

Unfortunately, it is generally not feasible using commonly employed bio-assay procedures to detect major-gene mutants in samples from field populations until frequencies of 1% or more are reached. At these levels, an obvious loss of disease

control may well result after only one or two more fungicide treatments. A warning that is sufficiently early to use in risk assessment cannot be obtained unless an impractically large number of samples are tested. It can be calculated that 300 samples must be tested to give a 95% chance of detecting resistance even at a 1% level. The problem of detecting rare resistant mutants of *Blumeria graminis* in field populations of barley mildew is shown in Table 3.

Table 3 Sample size needed to detect (with 95% confidence) rare resistant mutants in populations of *Blumeria graminis*

Mutant frequency	**Sample size (number of pustules)**	**Area of crop sampled (ha) ***
1×10^{-4}	3×10^{4}	0.0001
1×10^{-6}	3×10^{6}	0.01
1×10^{-8}	3×10^{8}	1
1×10^{-10}	3×10^{10}	100
1×10^{-12}	3×10^{12}	10,000

* assumes 10% leaf area infection and every pustule tested separately

Source: Brent *et al.*, 1990

With multi-step (polygenic) resistance, however, monitoring can give a useful indication of the presence or absence of risk. Multi-step resistance arises through a gradual shift in the range of sensitivity, and is considered to involve a series of mutations in different genes. The early stages of this process, whilst not obvious in the field, can be detected by successive sensitivity surveys over several years because a substantial proportion of the population is involved (e.g. Heaney *et al*., 1986).

Results of mutagenic or sexual-crossing tests may give some early evidence as to whether major-gene or multi-step resistance can be expected, but only field experience can give a reliable indication.

Wind impaction spore trap on a car roof. This is used, especially for *Blumeria graminis*, to conduct surveys to monitor the development and status of fungicide resistance. (Syngenta)

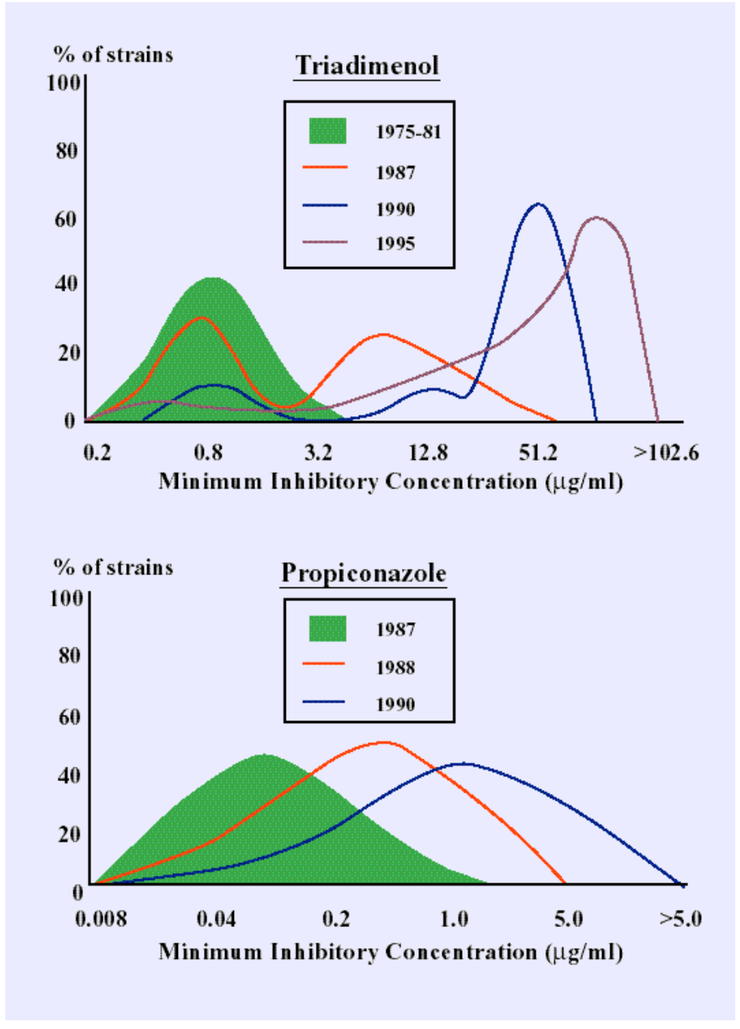
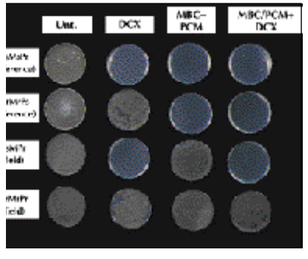


Fig.2.
Triadimenol vs. Propiconazole
Different patterns of sensitivity shifts shown by *Rhynchosporium secalis* towards two azole fungicides. The data were obtained from tests on more than 3000 samples from UK barley crops. Source: Kendall *et al.*, (1993), with additional 1995 data.



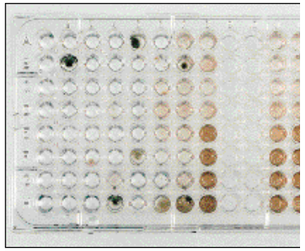
Agar plate tests are used to determine fungicide sensitivity. This test shows *Botrytis cinerea* and three fungicides. (Syngenta)

A gradual, unimodal shift in sensitivity will result from multi-step or polygenic resistance, whereas a bimodal development of distinct sensitive and resistant populations will reflect the selection of a single, major resistance gene. The data in Figure 2 illustrate how the pattern of resistance development in the field can vary between individual fungicides within a class, and cannot always be clearly categorised. *Rhynchosporium secalis* populations on barley in the UK underwent a gradual, unimodal shift towards lower sensitivity to propiconazole, typical of multi-step resistance. However, at the same time there was an irregular change in sensitivity to triadimenol, which could be interpreted as a skewed unimodal change, or could be partially bimodal, possibly involving the effect of a major gene mutation modified by polygenic mutations.

Even for multi-step resistance, however, the first sensitivity surveys in commercial crops made prior to new fungicide introductions are unlikely *per se* to aid initial risk assessment. Shifts in sensitivity will only occur in response to the use of the fungicide in these crops. Successive sensitivity surveys done in field trial plots might give initial indications of sensitivity shifts for certain pathogens, particularly if repetitive or persistent treatments are applied. However, invasion from other sites may well confuse the results in the case of highly mobile pathogens. Subsequent monitoring for sensitivity changes in commercial crops treated and untreated with the new fungicide can give useful warning of any future difficulties of control caused by polygenic resistance, so that, if necessary, use strategies can be modified and monitoring sustained or intensified.

Whenever base-line studies are done, some variation in sensitivity between isolates is found. The range of sensitivity encountered differs according to the particular fungicide-pathogen combination under study. For example, isolates of *Mycosphaerella graminicola* obtained in France showed a relatively narrow, ten-fold, range of sensitivity to azoxystrobin, when tested *in vivo*, (Godwin *et al*., 1999), whereas isolates of barley (Hollomon *et al*., 1996) and grape (Green and Gustafson, 2006) powdery mildews showed a much broader, 100 to 1,000-fold, range of sensitivity against quinoxyfen. All such isolates are easily controlled by application of the fungicide at concentrations well below the recommended rate of application.

Can the range of sensitivities found in base-line tests act as an indicator of the risk of future resistance problems? Possibly the broader the base-line range the greater could be the propensity for subsequent shifts to much lower levels of sensitivity under fungicide selection pressure; perhaps this would be more likely to apply if polygenic resistance is involved. Although base-line studies have been made for several years, there is little evidence for any such relationship. Certainly QoI



Procedures using microtitre plate techniques allow the testing of many isolates more quickly and with less effort. Results are read automatically and transferred directly into computer data bases. (Bayer CropScience)

Real-time PCR diagnostics (Q-PCR) allow the detection of point mutations causing resistance. (Syngenta)

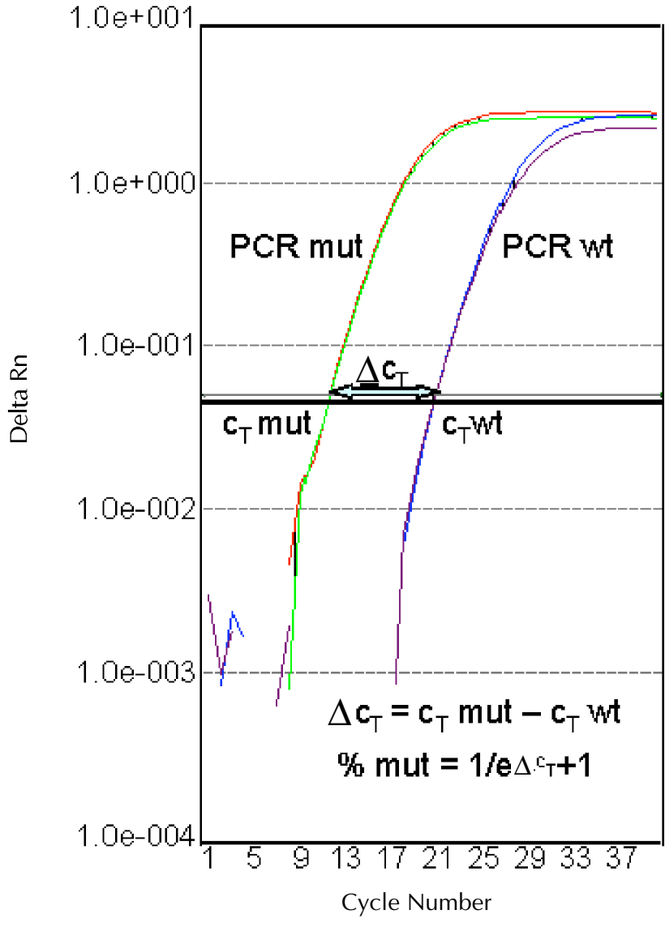


Fig.3.
Q-PCR for G143A mutation in cyt b gene. DNA was extracted from sporangia of wild-type (wt) and resistant (mut) Plasmopara viticola. The ΔcT indicates 99.8% of mutated DNA in the sample. The two lines each for wt and mut are repetitions
Source: Syngenta CP, Stein, Switzerland

resistance is now common in some *Mycosphaerella graminicola* populations (with a narrow base-line range), whereas the occurrence of quinoxyfen resistance in powdery mildews (with a broader range) is less widespread. However, the question remains an open one, and it is only when base-line results can be correlated with long-term records of the subsequent development or absence of practical resistance that an answer may be found.

Our discussion of the value of sensitivity surveys for assessing risk has assumed that conventional bio-assay procedures, involving the submission of isolates to fungicide treatment *in vitro* or *in vivo*, will have been used. These are frequently time consuming and resource-intensive, and often fail to detect resistance early enough to assist initial risk assessments. Adapting bio-assays to micro-titre plate formats increases the number of isolates that can be tested in a given time and may reduce the cost, but is restricted to pathogens that can be manipulated in this way.

The development of molecular detection technologies is a rapidly advancing field (McCartney *et al*., 2002), allowing detection of rare fungicide resistant mutations in plant pathogens at frequencies as low as 1 in 10,000 (Windass *et al*., 2000). To detect a point mutation causing resistance, PCR (Polymerase Chain Reaction) assays can be devised using either allele-specific primers or appropriate probes or restriction digests to interrogate amplified DNA fragments. The recent development of quantitative real-time PCR (Q-PCR) allows the frequency of a resistant allele in a DNA sample to be determined (Fig 3). Coupled with 384-well micro-titre plate formats, real-time PCR systems have become affordable for commercial use, and are being used to follow the response of plant pathogen populations to selection by different fungicides (Fraaije *et al*., 2002; Gisi *et al*., 2005). They do not require the pathogen to be isolated and cultivated *in vitro* but can use diseased plant material, from which DNA is extracted and analysed with pathogen specific primers. The Q-PCR method can quantify resistance in a bulk sample and therefore gives some information on the fitness of different resistance alleles under field conditions, a key parameter for prediction of resistance risk.

Understanding the molecular basis of resistance, and the DNA changes that accompany it, is an essential prerequisite to PCR monitoring. Genetic studies during early stages of discovery, should allow molecular diagnostics to be designed prior to commercialisation of a new fungicide group. However, as pointed out earlier, resistant mutations generated artificially in the laboratory are not necessarily found in field populations. For example, eleven point mutations in the mitochondrial cytochrome b gene causing QoI resistance, were identified in “model organisms” (e.g. yeast) prior to the commercial introduction of QoI fungicides. Of these, only

Applying fungicide droplets to banana plants to test for sensitivity of *Mycosphaerella fijiensis* var. *difformis*, a high-risk pathogen that causes black Sigatoka disease. (Syngenta)

two have subsequently emerged as practically important for plant pathogens. QoI resistance caused by a G143A mutation first appeared in wheat powdery mildew in Northern Germany in 1998, and the same resistant mutation has since been identified in other resistant populations of this and other pathogens around the world. PCR-based diagnostics were designed and used in industrial laboratories (co-ordinated by the FRAC QoI Working Group) to test for the presence of the G143A mutation, and hence the risk of resistance developing, in many important pathogens prior to the possible appearance of disease control failures. Similar diagnostic methods are available for detection of target-site resistance to benzimidazole and to a limited degree also for DMI fungicides, although in the latter case, a direct correlation between resistance and occurrence of specific mutations in the target site is not so obvious due to the polygenic nature of resistance.

The extent to which these molecular detection methods will prove useful, with regard to risk assessment remains to be seen, although experience so far is encouraging. They certainly allow the rapid and definitive detection of mutants with known resistance mechanisms, at low frequencies, but it is not always possible to correlate frequency with any subsequent decrease in field performance. At present, these molecular techniques must be supported by monitoring for field performance. Bioassays are still needed to preclude the possibility of the existence in the field, of other resistant variants with a slightly or completely different resistance mechanism that would not be detected by the applied molecular test.

PATHOGEN RISK

As already discussed, different classes of fungicides, whether defined by chemical structure or by mechanism of action can differ greatly in their overall liability to lose effectiveness through resistance arising in target pathogens. This liability can best be termed the ‘inherent (or intrinsic) fungicide-associated risk’, but more briefly ‘fungicide risk’.

There are also marked differences between pathogens in their overall tendencies to become resistant to fungicides applied against them. This can best be termed the ‘inherent (or intrinsic) pathogen-associated risk’, but more briefly the ‘pathogen risk’. Large differences in pathogen risk can be found between certain classes, genera and species of plant pathogens.

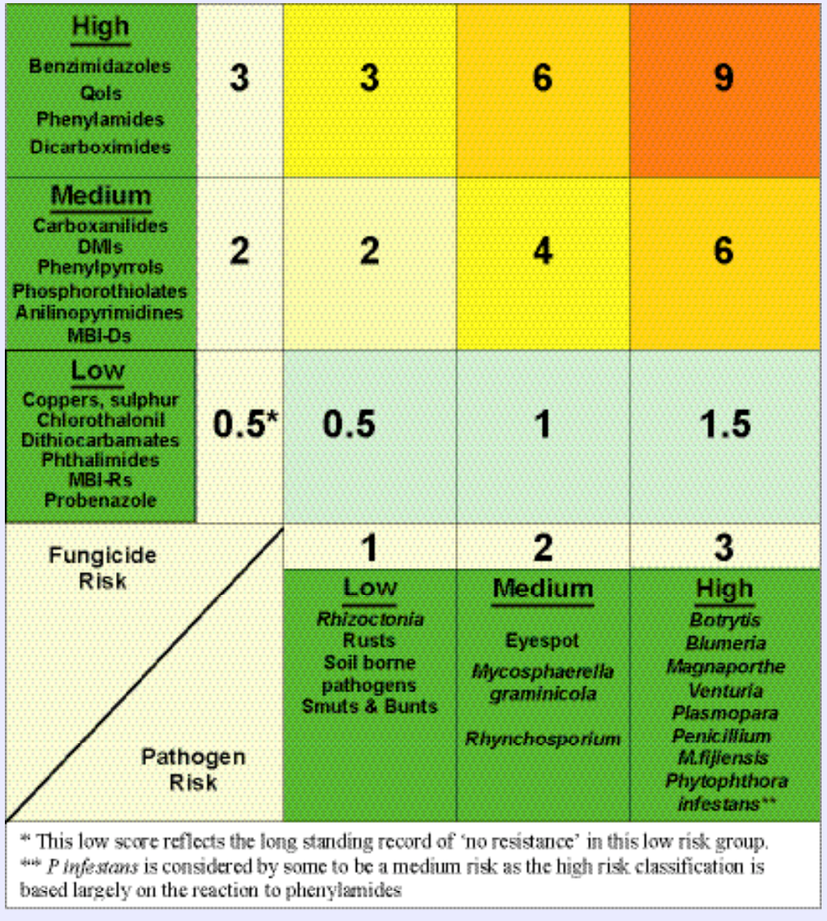
Factors relating directly to disease epidemiology, and indirectly to disease management, combine with genetic factors to form the pathogen risk. The most important factors determining pathogen risk appear to be:

- life cycle of the pathogen; the shorter the generation time, the more frequent the need for exposure to the fungicide and the faster the build-up of resistance.
- abundance of sporulation; the more spores that are released in the crop the greater the availability of individual genomes for mutation and selection, and the faster the spread of resistant mutants.
- ability of spores to spread between plants, crops and regions.
- ability to infect at all crop stages, requiring repeated fungicide treatment.
- occurrence of a sexual stage in the life cycle; this could either favour or hinder resistance development.
- ability to mutate or to express mutant genes: certain pathogens seem to produce fit mutants more readily than others; diploidy may suppress expression of recessive mutations; gene structure may render mutations lethal.

Figure 4 shows how the pathogen risk combines with the fungicide risk to give an overall inherent or basic risk of resistance for a number of combinations of leading fungicides and important target pathogens. In any assessment of the risk of fungicide resistance, the general influence of each of the inherent risk factors can be forecast, in semi-quantitative terms, to a reasonable degree of confidence. However, the degree of impact which each will have on the rate and severity of resistance development is much harder to assess, as is the way in which the factors interact. The simplest approach is to assume that each factor has a similar impact, and that the factors interact in a multiplicative way. The overall inherent resistance risk can then be determined for a disease-fungicide combination, to a high, medium or low level. Fuller lists of fungicides and pathogens categorised according to estimates of their inherent resistance risks are shown on the FRAC web-site (www.frac.info).

This simple and useful concept of degrees of fungicide-associated risk and of pathogen-associated risk combining to give a degree of overall inherent risk for each fungicide/target-pathogen combination fits generally with world-wide experience. Attachment of a characteristic degree of risk (at low, medium or high level) to each fungicide and to each pathogen can usually be done with reasonable confidence and consensus. However, difficulties of judgement can arise. For example, *Phytophthora*

Combined risk: 0.5 – 1.5 = low, 2-6 = medium, 9 = high



* This low score reflects the long standing record of 'no resistance' in this low risk group.
** *P infestans* is considered by some to be a medium risk as the high risk classification is based largely on the reaction to phenylamides

Fig 4.
This diagram exemplifies interactions between inherent (or intrinsic) fungicide and pathogen risks of resistance development. The risk categorisation is approximate and the scores are arbitrary. Nevertheless, these are probably the best estimates that can be made in the light of current knowledge. They represent risks under conditions of unrestricted fungicide use and severe, sustained disease pressure. Estimates of actual risk in a country or region must also take into account a range of conditions of fungicide use (see below).

infestans (cause of potato/tomato late blight) rapidly developed serious resistance towards phenylamide fungicides, and was therefore rated as a high risk pathogen. However, in more recent years it has not developed resistance to other widely used site-specific fungicides such as QoIs, carboxylic acid amides or cymoxanil. In contrast the related pathogen *Plasmopara viticola* (grape downy mildew) has developed resistance to all these fungicides. It is debatable whether the two pathogens should both still be rated overall as high-risk, or as medium- and high-risk for *P. infestans* and *P. viticola* respectively. Reasons for this difference in behaviour are unknown, one possible factor being the different degree of sexual recombination in the life cycle.

A more complex scheme of risk interactions, involving 'agronomic risk' (comprising effects of locally variable factors such as disease pressure, climate, complexity of cultivars) as well as inherent fungicide and pathogen risks, has been presented by Kuck (2005, reproduced on FRAC web site, Pathogen Risk List). This usefully emphasises the importance of taking local conditions into account when assessing actual risk and the associated need for monitoring (discussed in next section). However, it introduces an increased and arguably unobtainable degree of precision, with fourteen different risk scores, and the complexity tends to obscure the simple concept of inherent, interacting fungicide and pathogen risks which Figure 4 aims to illustrate.

CONDITIONS OF FUNGICIDE USE

Much practical experience, together with some experimentation, indicates that the actual risk of resistance depends not only on the inherent risk of a particular fungicide-pathogen combination, as indicated in Figure 4, but also on the conditions of fungicide use. These are sometimes referred to as risk modifiers, but in fact they are direct and important determinants of resistance risk in practice, and must always be included as an integral part of risk assessment.

The most important conditions of use that affect resistance risk are considered to be:

- number of repeated applications of the at-risk fungicide; the more frequent the treatment is applied to selectable populations of the pathogen, the more rapid the selection of mutants.
- exclusivity of treatment; the more exclusive the treatment with the same, at-risk fungicide, the more sustained the selection pressure; alternation or combined application with other types of fungicide with different mechanisms of action and/or resistance, and preferably with lower inherent risk, can reduce risk.

- the amount, or 'dose', of fungicide used for each application may influence risk; relationships of dose to resistance development are discussed below.
- amounts of pathogen exposed to the fungicide; if disease incidence is relatively low, sporadic or irregular from season to season, within a particular region, then occurrence and selection of possible resistant mutants is reduced.
- fragmentation of the area of use and predominance of use of the fungicide; the greater the area that requires treatment, locally or regionally, and the greater the uniformity of use, the more widespread the selection and build-up of resistant variants.
- concurrent use of integrated disease management; the greater the use of non-chemical methods, such as disease-resistant varieties, rotation of crops, or hygienic practices that lower the disease pressure and thus fungicide selection pressure.
- isolation of pathogen populations (e.g. in greenhouses or polythene tunnels, isolated agronomic regions), preventing re-entry of sensitive forms, can favour development of resistant populations

It is a common practice for farmers to economise by applying fungicides at rates lower than those recommended by the manufacturer, whilst retaining the normal frequency of applications. In some circumstances, for example where the crop variety has a degree of disease resistance, or where conditions permit only light disease development, the use of reduced rates can give satisfactory results, and is supported by some advisory services. Sometimes the manufacturer will indicate a range of application rates which can be used according to conditions. The question of whether and how the dose rate affects the risk of resistance development has been debated for many years. Unfortunately the experimental data concerning this issue remain few and somewhat conflicting (Brent, 1995; Metcalfe *et al*., 2000; Brent and Hollomon, 2007).

There is a consensus view, supported by the mathematical models considered in the next section, that the risk of major gene resistance increases as the dose increases, just as the effectiveness of disease control increases with dose. This is because the degree of disease control is proportional to selection pressure in favour of high-level resistant mutants. There is also a widely held view that the risk of development of polygenic resistance, which appears to be a stepwise process, will be low at very low dose rates, because these will exert little or no selection pressure, will rise to a maximum at an intermediate rate, which will select low-level mutants, and will decline at higher rates because the low-level mutants will be killed or stopped from

growing and multiplying. Of the two mathematical models that apply to polygenic resistance, one (Shaw, 1989) supports the above hypothesis, and the other (Josepovits, 1989) indicates that dose rate will have little if any effect on resistance development. It should be stressed that the dose-resistance relationships outlined above, and illustrated in Figure 5, are not firmly established, even qualitatively. Much more experimental evidence needs to be produced and analysed before the effects of dose can be considered as a part of the risk assessment procedure.

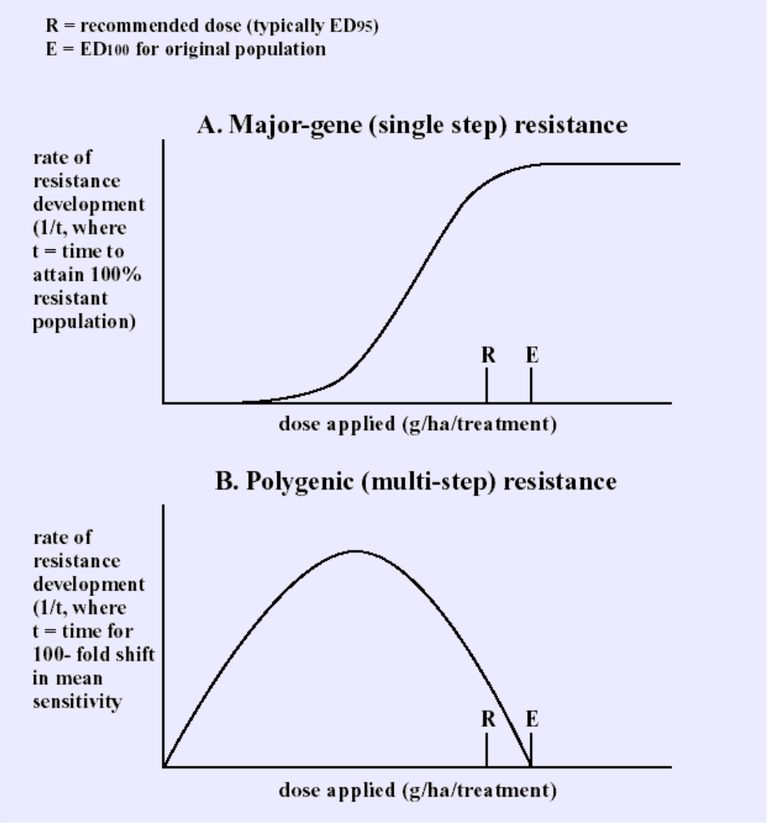


Fig.5.
Hypothetical relationships of the rate of development of resistance to the dose of fungicide applied. It is assumed that the different doses are applied in an identical number and timings of treatments. Experimental data relating to such relationships are few, and more research is needed.

The above discussion of effects of altering dose rates assumes that the frequency of application is unchanged. If frequency is increased in order to compensate for any reduced performance from a lowered dose, then selection pressure and resistance risk will also increase, possibly to higher levels than those exerted by the standard schedule.

MATHEMATICAL MODELS

A number of mathematical models were proposed some years ago, for the prediction of the rate of development of resistance in relation to different regimes of fungicide use (for earlier references see Chin, 1987; Milgroom and Fry, 1988). These relate to single-step resistance, assuming that two distinct biotypes, differing widely in sensitivity due to one major-gene mutation, occur in different proportions according to the degree of selection exerted by fungicide treatments.

The general conclusions from these models are similar and accord with conclusions drawn earlier from existing knowledge of population genetics and epidemiology. They predict, for example, that rapidity of resistance development will be associated with frequent pathogen reproduction, highly effective and persistent action of the at-risk fungicide, greater initial frequency and fitness of resistant mutants, and the sole use of the at-risk fungicide. Rotation or mixture with another fungicide to which mutants remain sensitive are both predicted to delay, but not totally prevent, resistance development. Indications of the relative value of using mixtures or rotations of single fungicides vary between models and according to the assumptions made within some of the models. For example in the model of Kable and Jeffery (1980) complete spray coverage, not allowing escape of any part of the pathogen population, favours the use of alternating fungicides, whereas as coverage decreases the use of mixtures becomes more effective.

The predicted time-scales of resistance development seem to be of the same order of magnitude as those encountered in practice (Skylakakis, 1982), and some examples for one model are given in Table 4. However, verification of the accuracy of each model under a range of conditions has not been attempted. This would be very difficult because of the inaccessibility of data on key aspects such as the relative frequency of mutants at the time of first treatment, the fitness of mutants in the field, and the uniformity of fungicide exposure.

The models considered so far do not apply to the multi-step or polygenically based development of resistance. Models proposed by Shaw (1989) and Josepovits (1989)

Table 4 Predicted and observed duration of selection pressure required for practical resistance to occur

Pathogen	Fungicide	Standard selection time* (days)	Duration of selection pressure	
			Observed	Predicted
Cercospora beticola	Benomyl	9.5 - 14.3	130 - 263 d	140 - 200 d
Phytophthora infestans	Metalaxyl	3.7 - 3.8	57 - 70 d	200 - 400 d
Sphaerotheca fuliginea	Dimethirimol	8.5 - 16. 5	98 - 236 d	112 - 224 d
Ustilago nuda	Carboxin	158	5 - 7 y	11 y

*Time for proportion of resistant sub-population to increase by e (2.7 times)

Source: Skylakakis, 1982

relate specifically to this type of resistance. Again parameters such as rapid pathogen growth and reproduction and the repetitive use of one fungicide tend to favour resistance development. The mean level and the spread of fungicide resistance that are ultimately attained in response to a particular fungicide regime will be determined largely by the extent to which fitness is affected as the number of mutations towards resistance increases. Unfortunately relationships of this type are not at present measurable, and verification of these models has not been achieved. A further model, which relates to pesticide resistance generally, incorporates effects of pesticide dose and indicates factors that determine the suitability of pesticides for use in mixtures (Birch and Shaw, 1997). Attempts to experimentally validate this model using azole and QoI fungicides, and the wheat pathogen *Mycosphaerella graminicola*, have been reported (Metcalfe *et al*., 2000).

A recent modelling study (Parnell *et al*., 2006) has predicted that the regional spread of single gene resistance over large distances will depend on the proportion of fields of a particular crop that are sprayed, and not only on within-field use strategies. The extent of any loss in fitness caused by the resistant mutation, and the effectiveness of the fungicide against the wild-type sensitive pathogen, also influence the speed that

resistance will spread. It is suggested that some fields should be left untreated, or treated with different, non-cross-resistant fungicides. Both verification of the model and systematic commercialisation of such a 'patchwork' strategy will probably be difficult to achieve, although the authors point out that analogous non-Bt-treated refugia for Bt-sensitive insect populations have been established in Arizona through legislation.

Overall, the range of mathematical models that have been published have provided a valuable theoretical background to resistance studies. However, they have not, to our knowledge, been used in the practical assessment of resistance risk because of the lack of verification and the difficulties of getting the data required both to verify and to work the models. Improvements in the detection of resistance alleles, especially at low frequencies, using molecular diagnostic techniques, and more reliable estimates of sensitivity (ED_{50}) measurements (Godet *et al*., 2000), may be useful in overcoming these difficulties.

INTEGRATION OF RISK FACTORS

The study of case histories of resistance development in practice, and consideration of the underlying genetic, biochemical and epidemiological processes, indicate that a very complex, interacting and continually changing set of factors determine the rate and severity of development of fungicide resistance, with regard to a particular fungicide, pathogen, crop type, and region. It is a daunting task to attempt to fit together all available data, and to identify and find further data, in order to make a reasonably reliable assessment. However, it is necessary to do this, not only to guide the manufacturer in decision-making on product introduction and label recommendations, but also many registration authorities who now regard the assessment of resistance risk and establishment of appropriate use strategies and monitoring programmes as key components of the efficacy protocols required in submissions for pesticide approval.

Each main usage of a new fungicide requires a separate risk assessment, which must draw together the fungicide risk factors, the pathogens risk factors, and the likely conditions of use in different regions or countries. This should be done in a systematic way. It is possible to draw up a checklist of different factors. An example is given in Table 5. Another example was presented by Gisi and Staehle-Csech, (1988a). It is also possible to allocate risk categories or scores to each factor, and to add them up to give an overall risk assessment.

Whilst such a scheme gives a useful framework for reviewing available information, any effort to quantify each risk factor, or to produce an overall numerical score for risk, is beset by problems. Not all the factors are at present measurable with any degree of precision; the 'fitness' of resistant mutants under field conditions is probably the most critical and difficult factor to measure. Nor are they equally important, and it is virtually impossible to ascribe weightings to each factor other than by personal judgement. At our present state of knowledge, probably the best that can be done is to note information relevant to each factor and to make a high, medium or low risk rating accordingly. The resulting risk profile can be used as a basis for assessing the prospects of obtaining durable performance under a range of possible use strategies, and of the need for monitoring in the different use situations.

If the overall risk assessment for a particular pattern of use of a new fungicide is anything other than 'low' then it becomes very desirable of course to attach a reliable time-scale with regard to the speed of build-up of resistance under different circumstances of use. This cannot be done at present. Studies of case histories of resistance development, which have similar fungicide-associated or disease-associated characteristics to those considered to apply to the test fungicide, may give some idea of how many years it may take for problems to arise. It remains vitally necessary, however, to maintain a very close watch for any sign of deterioration of product performance under practical conditions, and if possible also to monitor for the sensitivity of representative samples of the target pathogen taken from treated crops.

The framework in Table 5 applies specifically to the assessment of risk for a new fungicide. If a fungicide that is already in commercial use is submitted to assessment for the risk of resistance arising during use in a new region or against a new target disease, then the record-to-date of the fungicide in established uses or locations, regarding either the build-up or the absence of resistance, of course becomes a major additional factor, particularly if the fungicide has been in commercial use for a considerable time.

Extensive programmes of resistance monitoring, coupled with estimates of fungicide efficacy in the field, usually indicate geographic variations in the occurrence of resistance problems. Regions with a high incidence of resistance ('hot spots'), are generally regions of high disease pressure, induced by disease-favourable climatic conditions, and hence of the most intensive use of the at-risk fungicide class. For example, resistance of *Blumeria graminis* to a wide range of fungicides has tended to arise quickly and frequently in Northern France, Germany and the UK, whereas in Italy and Spain where infection pressure (and consequently fungicide use) are

Table 5. A framework for the assessment of risk of the development of resistance during commercial use of a new fungicide.

Factor	Positive indication of resistance risk
Fungicide -associated (inherent)	
Fungicide class	When the test fungicide is a member of a chemical class which has a record of resistance problems
Site of action in target pathogen	If there is a single site of action; or if the site is known to be capable of change to a form that is unaffected or less affected by other fungicides
Cross-resistance	If there are target pathogen strains resistant to existing fungicides which also resist the test fungicide; if the resistance factor for the test fungicide is relatively high
Response to mutagenic agents	If treatment with mutagenic agents causes the target pathogen to produce resistant, fit mutants
Response in sexual crossing	If sexual crossing cause the target pathogen experiments to produce resistant, fit recombinants
Response to repetitive fungicide application	If repeated exposure of the target pathogen to the test fungicide, in the laboratory or in field plots, causes the appearance of resistant, fit strains at detectable levels; the distribution of sensitive and resistant isolates (bi-modal or uni-modal) can indicate whether major- gene or polygenic resistance is likely to occur
Pathogen - associated (inherent)	
Generation time	If multiplication cycles of the target pathogen, and hence fungicide applications, are frequent
Amount of sporulation	If sporulation of the pathogen is abundant
Spore dispersal	If spores spread readily between plants, crops and regions
Genetic adaptability	If the pathogen is haploid, has a gene structure that allows expression of mutations to resistance, has an obligatory sequence of sexual and asexual reproduction in the disease cycle or shows other signs of genetic adaptability
History of resistance	If the pathogen has a record of developing resistance to fungicides (of any kind)
Conditions of use (locally determined)	
Application of the fungicide	If fungicide applications will be repetitive, if the fungicide (or fungicides related to it by cross-resistance) will be used continually and/or widely throughout crops in the region
Complementary measures	If other types of fungicide (as mixtures or in rotation) or if non-chemical disease-suppressant measures (e.g. crop rotation, resistant varieties, hygienic precautions) are not to be used
Pathogen incidence (‘infection pressure’)	If the pathogen is present in large amounts and/or large areas, and/or is multiplying over long time periods
Pathogen isolation	If the pathogen is confined (e.g. in greenhouse or polythene tunnels) preventing re-entry of sensitive forms

relatively low, the sensitivity of powdery mildew populations is still near to that of untreated base-line populations. Other examples of regional focus for resistance in various pathogens in Europe are listed by Kuck (2005). When such 'hot spot' regions can be identified, they can be considered to offer the highest risk to new fungicides, and should receive priority in allocation of resources to precautionary measures such as base-line sensitivity surveys and subsequent resistance monitoring. On the other hand, regions with usually low disease levels and more limited fungicide use will not merit such priority, even with fungicide-pathogen combinations of relatively high inherent risk.

CURRENT TRENDS AND FUTURE DEVELOPMENTS

Risk assessment and fungicide development

It is encouraging that, over the past twenty years, the assessment of the risk of resistance has become a routine part of the development of a new fungicide by most if not all the companies concerned. The amount of attention given, and the procedures adopted vary to some extent between companies, and for commercial reasons disclosure of the methods used, and of the results, are often restricted or delayed. In general, however, consideration of the factors presented in Table 5 is used as the basis of risk assessment. This work may be done entirely in-house by the industrial developer of the fungicide, or it may be contracted to a public-sector or private-sector laboratory.

When two or more companies are concurrently developing and/or marketing fungicides which have the same mode of action or are subject to cross-resistance, and hence share a common risk, then there is much to be gained by collaborating in risk assessment. This approach is fostered by FRAC, which in such situations endeavours to set up Working Groups as early during the development or commercial use of a new class of fungicide as possible. These groups make risk assessments, share information from base-line and other monitoring activities, and when necessary devise and promote agreed strategies of fungicide application that offer the best prospects for durability of product performance. At present FRAC has anilinopyrimidine, SBI, QoI and carboxylic acid amide (CAA) working groups. There is also a special working group that focuses on the effective use of several classes of fungicide against Black Sigatoka disease of bananas. Former working groups for benzimidazole, dicarboximide and phenylamide fungicides have been

converted to 'Expert Fora', to provide information and review annually resistance status and management guidelines.

As an example, a set of recommendations that has recently been issued by the FRAC QoI Working Group for the use of QoI based fungicides on cereals is shown in Figure 6. The establishment of such recommendations, which are reviewed annually, automatically involves the shared assessment of risk under a range of use scenarios, as well as the sharing of results of observations on performance and of sensitivity monitoring in order to verify the risk assessments and the effectiveness of the adopted strategy of use. The latest recommendations from all Working Groups are published on the FRAC website at www.frac.info.

Registration requirements

Pesticide registration authorities world-wide increasingly require information relevant to the assessment of resistance risk, the development of anti-resistance use strategies, and the establishment of base-line data. Consideration of such information is now a necessary part of the assessment of efficacy and of the information and instructions given on product labels. However, it is not an easy matter to specify what data should be provided, and how such data should be judged in relation to approval and to the conditions attached thereto.

It has to be fully understood by those concerned with pesticide registration that resistance risk assessment, like weather forecasting, is a useful process but an imprecise one, and that any improvement in its accuracy will be very gradual.

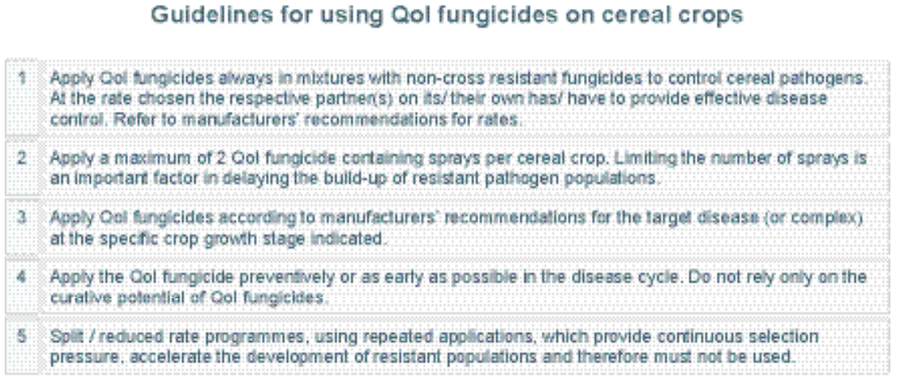


Fig. 6.
FRAC Guidelines for the use of QoI fungicides on cereals, produced by the FRAC QoI Working Group. Further crop and pathogen specific guidelines are included on the FRAC website (www.frac.info).

Because of the complexity of the interacting factors that determine resistance development, and because our knowledge and skills in this area are still very limited, it is difficult to attribute precise figures of resistance risk arising in target pathogens in different countries to a new fungicide. Any overall judgements of resistance risk that go beyond low, moderate or high would at present require a degree of predictive accuracy and confidence which has not been achieved.

EU Registration Directives (91/414/EEC and 93/717/EEC) stipulate that registration data for new active ingredients should include: 'information on the actual or possible occurrence of resistance and on proposed avoidance or management strategies'. Working in collaboration with representatives of FRAC, other Resistance Action Committees and registration authorities, the European Plant Protection Organisation (EPPO or OEPP) has produced a Standard for Resistance Risk Analysis (EPPO, 2002). This recommends the submission of a resistance risk assessment, and if necessary sensitivity base-line data, a proposed resistance management strategy, plans for monitoring, and an example product label with appropriate information on resistance management. Although this Standard has been accepted by registration authorities throughout the European Union as a general basis for relevant requirements in their registration procedures, individual member countries still handle the question of resistance risk in their own ways. Attempts to establish uniformity of requirements across Europe must await the outcome of the review of Directive 91/414 currently underway. Outside Europe, registration requirements regarding resistance risk vary widely from country to country; in some, for example the USA, there are at present no requirements in this respect.

So far, the trend has been for acceptance of company information and plans. Some general views of the agrochemical industry on this topic have been presented by Kuck, (2005). As in the EPPO Standard, and in this Monograph, it is emphasised that assessment of risk and the need for sensitivity surveys and special use strategies must take into account not only inherent fungicide and pathogen properties but also conditions of use within each country.

Research priorities and support

Discovery and exploitation of new modes of action remain a high priority in efforts to combat resistance. It is encouraging, therefore, that agrochemical companies devote considerable resources towards risk assessment and other resistance studies relevant to the development and use of new products. As discussed earlier this involves inter-company collaboration, fostered by FRAC. To some extent, industrial results are now published in journals and conference proceedings, and are shared

with public-sector researchers through regional joint action groups, such as the UK Fungicide Resistance Action Group (FRAG -UK). It is important that such results should be published as soon as available since, in conjunction with knowledge of subsequent product use and performance, they give valuable guidelines for future risk assessment methodology.

It becomes very clear from the foregoing sections that there are huge gaps in our knowledge of the many interacting factors that determine resistance development and of their relative importance, and that consequently our ability to predict the severity and the time-scale of practical resistance development, in relation to options for use strategies, is at present very limited indeed. Improvement requires the identification of key research projects, and financial and institutional support for their completion.

The genetic, biochemical or biophysical changes that underlie resistance development are reasonably well understood for three fungicide classes, the benzimidazoles, carboxanilides and QoIs. Knowledge is growing, but it is still far from complete regarding the complex of mutations and mechanisms that appear to give rise to resistance in other classes, including DMI fungicides. Equally important is understanding why some pathogens (e.g. powdery and downy mildews) have a high resistance risk to a particular fungicide class, whereas others (e.g. rusts) have not developed resistance in practice to these same fungicides. A point mutation in the target protein may not always give the same resistance level in different pathogen isolates, and it is important to understand why this is so. Developments in genomics and molecular diagnostics provide a framework to compare the structure of 'target genes' in different pathogens, and perhaps identify mutant 'hot spots' which may be linked to resistance. Clearly there are now new opportunities to explore molecular and genetic aspects of resistance in pathogens which are difficult to analyse using conventional approaches. Inroads into knowledge of the behaviour of mutant alleles in field populations of crop pathogens, particularly at very early stages of resistance development, are becoming achievable as very sensitive and specific detection methods are being developed and used (Wille *et al*., 2002).

The influence of different strategies of fungicide use on the rate of development of resistant populations is often discussed, and views are expressed and prescriptions recommended which, through necessity, are out of proportion to the small amount of relevant experimental data that is available. Many more long-term field studies still need to be done, especially for newer fungicide groups such as QoIs and CAAs, on the effects of application factors such as dose rate, mixture or rotation of fungicides, timing of sprays in relation to stage of the disease, and the persistence of

action, and on stability of resistance. The full recording, publication and up-dating of case histories must be strongly encouraged. If possible these should describe risk assessment and base-line studies, then the selection and implementation of use strategies adopted, and finally the outcome in terms of practical resistance development or non-development, including results of sensitivity and performance monitoring. Valuable guidelines for the assessment of risk, and also the formulation of avoidance measures, have emerged from records of past experience, and this should be a continuing process.

The main limiting factors with regard to the progress of basic research relevant to fungicide resistance risk assessment are lack of funding and limited capacity in agrochemical companies, and low prioritising by official research policy-makers world-wide. Industrial organisations do fund a number of projects in public-sector laboratories concerned with risk assessment. Often these are short-term, typically being relatively routine sensitivity tests, cross-resistance or mutagenesis assays. Longer-term research on resistance mechanisms and genetics, and on field behaviour of mutant populations, must mainly depend on government funding. This is well justified and should be increased, because of its basic scientific thrust, which is considerable, and also because of its importance with regard to registration of pesticides and to their most economic and environmentally safe use.

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After graduating at London University in Botany and Microbiology, Keith Brent worked for over twenty years in ICI, now Syngenta. At first he studied the biochemistry of filamentous fungi, at the Akers Research Laboratories, Welwyn. In 1964 he moved to Jealott's Hill Research Station where he led research on fungicides discovery and development and tackled some of the initial problems of fungicide resistance. In 1979 he was appointed Head of the Crop Protection Division at Long Ashton Research Station, University of Bristol, where he also became Deputy Director. During this period he continued to be involved in fungicide research, and also taught in international courses on fungicide resistance in seven countries world-wide. Since 1992 he has worked as an international consultant in crop protection and agricultural research management and in 1995 he authored the first FRAC Monograph.

Derek W Hollomon PhD

Having gained a degree in Agricultural Botany at Reading University, Derek Hollomon started his plant pathology research at Hull University and was awarded a doctorate in 1965. After several years of post-doctoral research in Canada, Australia and the USA, he returned to the UK to initiate research at Rothamsted on the mode of action of the systemic fungicides that were then beginning to be used for cereal disease control. His research soon extended to resistance problems, and these have continued to be a major interest since he moved to the Long Ashton Research Station in 1985. His work has involved much collaboration with the agrochemical industry, and also has kept him in close contact with the growers. He was awarded the British Crop Protection Council Medal in 1995. Since 2002, he has been a visiting fellow in the Biochemistry Department of the University of Bristol researching the pathway of respiration in pathogenic fungi. He is technical editor of the journal Pest Management Science.

FUNGICIDE RESISTANCE :
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