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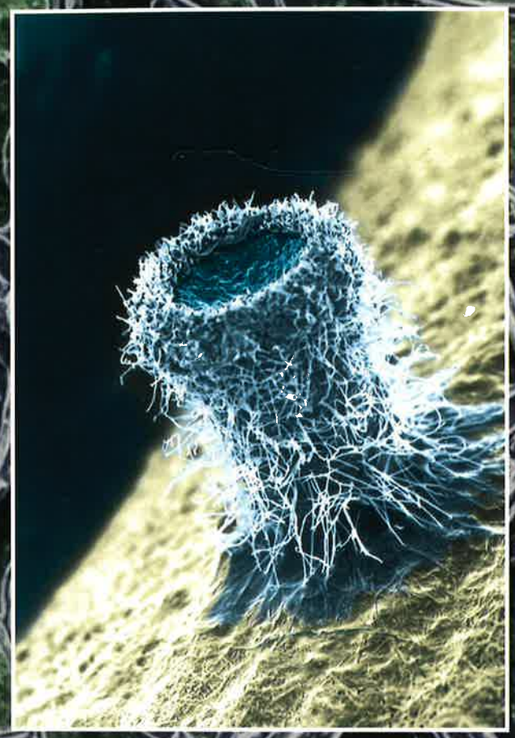
# FUNGICIDE RESISTANCE: THE ASSESSMENT OF RISK



KEITH J BRENT AND DEREK W HOLLOMON



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FUNGICIDE RESISTANCE
FRAC
ACTION COMMITTEE

Cover:
Scanning electron micrograph of mycelium of *Tapesia 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. (AgrEvo)





# FUNGICIDE RESISTANCE: THE ASSESSMENT OF RISK

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Inserts:
The page inserts show a spore mass (cirrhus) exuding from a pycnidium of *Septoria tritici (Mycosphaerella graminicola)*, a major foliar pathogen of wheat.
(AgrEvo)



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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 fungi. An ability to determine the risk of resistance arising would help greatly both the selection of candidate chemicals for development and the establishment of strategies to ensure their durability.

- 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 genetic mutations in the target pathogen. The ability to generate resistant mutations can be revealed by laboratory experiments in which mutants in fungus populations are selected by exposure to the fungicide, sometimes in the presence of mutagenic agents. The 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 more readily detect the early stages of polygenic resistance before this becomes severe enough to cause practical problems. The significance for risk evaluation of the breadth of the range of sensitivity values (e.g. ED50 values) found in base-line monitoring studies is not yet clear. For the future, it is possible that highly sensitive and selective DNA probes could be used to detect resistant major-gene mutations in field populations, in larger and more numerous samples, and at much lower frequencies, compared with current bio-assay methods.

- Several epidemiological factors, specific to each target disease, affect the rate of resistance development. Short generation time, abundant sporulation and isolation of pathogen populations tend to increase resistance risk.
- The influence of inherent chemical, biochemical, genetic and epidemiological risk factors can be modified by effects of different disease management methods. Reduced application frequency, rotation or mixture with other types of fungicide, and concurrent use of non-chemical disease-control measures, will tend to lower the risk of resistance development.
- A number of mathematical models defining rates of resistance build-up in relation to different strategies of fungicide use 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 and modifying risk factors 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. With present experience and knowledge assessments must be approximate, at best indicating low, medium and high risk.
- 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 fungi 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 the Global Crop Protection Federation (GCPF), 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 has published a monograph entitled 'Fungicide Resistance in Crop Pathogens: How can it be Managed?' (Brent,1995), which gives a general overview of fungicide resistance management.

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 earlier monograph. However, in view of the importance, and the difficulties, of risk assessment, FRAC decided to commission a second monograph to deal specifically with this subject. Again this is written for a broad readership rather than for specialists, and it does not attempt to give an exhaustive review of the very large amount of relevant literature. The only previous general review of this subject, known to us, is that by Brent, Hollomon and Shaw (1990), and we have drawn freely on this.

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 farm 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. Finally we consider the usefulness and reliability of our current expertise, speculate on future prospects and identify requirements for further research.

## KNOWLEDGE OF THE CHEMICAL STRUCTURES AND MECHANISMS OF ACTION OF FUNGICIDES

### Structural class

Experience of practical problems of fungicide resistance, which now extends over three decades, indicates clearly that the risk of resistance development depends greatly upon 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, captafol and folpet, and dithiocarbamates e.g. mancozeb, maneb, zineb and thiram, have never 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, and dicarboximides e.g. iprodione, procymidone, vinclozolin, 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 the azoles, e.g. triadimefon, flutriafol, flusilazole etc, has developed more gradually, and only in certain pathogens.



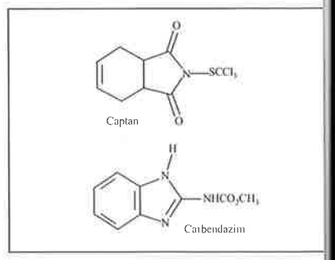


Non-class-specific resistance, that affects members of more than one class, arises commonly against insecticides and herbicides. It results mainly from the development by the target organism of a capacity to inactivate certain pesticides through degradation or conjugation. Fortunately, this type of resistance is not known to arise against fungicides. Therefore if a candidate fungicide belongs to a known chemical class, much can be clearly predicted about the risk of resistance arising in existing target fungi for the class, and also in new target fungi.

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, phenylpyrroles, anilinopyrimidines and strobilurins, are more tentative because of their short periods of commercial use. Published data from mode of action studies, mutagenesis tests and/or monitoring of pathogen populations (see for example Hilber et al, 1994; Forster et al, 1996; Godwin et al, 1997; Ziogas et al, 1997) suggest that these classes carry a moderate resistance risk, and appropriate use and surveillance strategies have been established for each class.

It is debatable whether the morpholine 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.

Despite this overall link between fungicide chemistry and resistance, structural differences that occur within a chemical class can influence resistance risk. Between members of the azole class, for example, resistance factors differ considerably, and often consistently (Kendall et al, 1993; Senior et al, 1993). The presence of a methylene group in tebuconazole confers resistance factors much lower than those shown against triadimenol. This has been reflected by good field performance by this compound in situations where triadimenol has failed. In *Rhynchosporium secalis*, replacement of a triazole group by an imidazole reduces resistance factors still further. Consequently, in assessing the risk of resistance for a new fungicide that falls within a known chemical class, it is always necessary to do cross-resistance tests that compare it with other members of the same class.




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. (AgrEvo)

Table 1 Estimates of the inherent risk of resistance attached to different chemical classes of fungicides. The actual risk during commercial use may be reduced or increased according to the target disease and the regime of use.

| Resistance risk | Chemical Class or Compound |
|---|---|
| High | Benzimidazoles, dicarboximides, phenylamides |
| Moderate | 2-Amino-pyrimidines, anilinopyrimidines, aromatic hydrocarbons, azoles, carboxanilides, cymoxanil, dimethomorph, fentins, phenylpyrroles, phosphorothiolates, pyrimidinecarbinols, strobilurins |
| Low | Acibenzolar-S-methyl, chlorothalonil, coppers, dithiocarbamates, fluazinam, phthalimides, probenazole, quinoxyfen, sulphurs, tricyclazole |

There are a few cases where fungicides are known to share resistance risks, through cross-resistance, and yet they apparently belong to different structural classes. Strains of *Botrytis cinerea* resistant to the dicarboximides are also resistant to aromatic hydrocarbon fungicides, such as dichloran, quintozene and biphenyl (Leroux et al, 1977; Georgopoulos, 1982). The reason for this is not clear, but presumably there is some similarity in the mechanisms of action, which are not well understood for either group. It may be relevant that the dicarboximides contain an aromatic (chlorinated benzene) residue, and that members of both groups can increase the frequency of mitotic segregation in *Aspergillus nidulans* (Georgopoulos et al, 1979). Triforine, a piperazine, and fenarimol, a pyrimidine carbinol, are positively correlated for cross-resistance with each other and with the azole fungicides (Sherald et al, 1973; Fuchs et al, 1977; Barug and Kerkenaar, 1979, 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).





The development of DMI resistance seems to involve a number of different mechanisms (see review by Joseph-Horne and Hollomon, 1997), which include alterations of the sterol demethylase site of fungitoxic action.

**Mechanisms of action**

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 vinclozolin, iprodione, dodine and the fentin fungicides have little or no systemic action but they have encountered major resistance problems. There is no theoretical reason why systemicity *per se* should confer a likelihood of resistance development, and all cases of resistance to systemic fungicides can be explained readily through other properties which accompany the ability to translocate in plants. These are the more powerful and persistent protective action, the eradicant action, and the selective biochemical mechanism of action, which are typical of systemic fungicides. These performance attributes will tend to increase the selection pressure favouring resistant mutants in pathogen populations in crops, although such effects are very difficult to single out and quantify. The influence of the selective biochemical mechanism of action is probably the predominant factor that determines the greater risk of resistance attached to systemic fungicides, and the relative lack of resistance in non-systemic fungicides. However, our knowledge of mechanisms of fungicide resistance is still too limited to permit a clear generalisation about this.

In the few cases where they have been elucidated, the mechanisms that underlie the development of fungicide resistance have mainly proved to be some kind of modification of the biochemical target site in the pathogen, which render the site less sensitive to damage by the fungicide. Many authors have pointed out that such modifications will occur much more readily in a single, specific target site, which is typical of a systemic fungicide, rather than in the multiplicity of target sites which tend to be characteristic of the 'protectant' fungicides. A single target site can be overcome by one mutation, whereas several changes simultaneously are needed to overcome multiple sites, and this will be a much rarer event. If the chance of occurrence of a single mutation that affects one target site is $10^{-8}$, then the chance of two such mutations, affecting two target sites, occurring together is $10^{-16}$.

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

As a rule, the occurrence of cross-resistance of pathogen strains to a range of fungicides correlates with the existence of a common mode of action that is shared by these particular fungicides. This again indicates that the mechanism of fungicide resistance usually involves changes within, or closely linked to, the site of fungicidal action. Hence a knowledge of the biochemical action of a fungicide can be a very useful indicator of resistance risk. If the 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.

However, these associations between resistance and site-specificity, and between a particular mechanism of action and a particular risk of resistance, are not absolute. For example, the organo-mercurials and organo-tin fungicides are considered to be multi-site inhibitors, and yet practical resistance problems arose eventually (after some 13 years of use in the case of the organo-tins, and after about 40 years with the organo-mercurials). The mechanisms of resistance in these cases are not well understood, although there is limited evidence suggesting that resistance to the organo-mercurials may be associated with decreased uptake by the pathogen. The morpholines are systemic fungicides which have biochemically specific actions on sterol biosynthesis. However, reductions in sensitivity have been notably less, and slower to develop than those encountered by other classes of systemic fungicide, despite many years of widespread use of the morpholines in cereals, bananas and other crops (Hollomon, 1994). It is known that they act at more than one biochemical site, 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 (page 13).

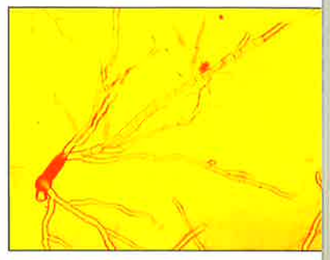
Thus mode-of-action information must be taken as a very useful but by no means certain guide to resistance risk. Unfortunately, the mode of action of a new fungicide may not be known by the time decisions on its commercial introduction and use are required. Indeed, even with the availability of modern techniques, and a now substantial knowledge of this area of biochemistry, it remains





exceptional for any mode of action to be fully explored or understood for many years. The mode of action of the dicarboximides is still far from understood, almost 30 years after the introduction of this important class of fungicides into commercial use.

Although much is known about the mode of action of the DMI and morpholine fungicides, understanding is still growing and secondary mechanisms of action that could affect resistance reactions are still being found. The morpholines are now known to act against at least three different stages in sterol biosynthesis (Ziogas et al,1991). Azoles interact with the haem of cytochrome P450s, and although features of azole fungicide chemistry may favour binding at the sterol substrate site of the 14-α–demethylase, CYP 51, another P450, sterol C-22-desaturase, CYP 61, may also be involved (Kelly et al, 1995). The relative importance of these different mechanisms to the action of the different fungicides that affect sterol biosynthesis could well affect their relative resistance risks. It is notable that mode of action and resistance mechanisms in the azole fungicides are now receiving increased attention because resistance to azole antifungals has become a significant problem in human medicine.



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

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 fungi directly. Tricyclazole and pyroquilon, used to control rice blast disease, specifically affect the penetration of the pathogen (*Magnaporthe oryzae*) into the host plant, through inhibiting melanin biosynthesis in appressoria. Probenazole, which also is used against rice blast, acts primarily on the plant, and is known to induce a set of defence reactions against fungal pathogens, known as systemic activated resistance or systemic acquired resistance (SAR). It is notable that the commercial use of these fungicides over some 15-20 years has not led to the development of resistance problems, whereas several other rice blast fungicides have encountered widespread resistance. There is no obvious reason why mutants resistant to the melanin biosynthesis inhibitors should not arise. Lack of resistance to SAR inducers can be explained on the basis that these compounds are known to induce the production of a number of different plant proteins, known as pathogenesis related proteins (PR proteins), that act against the pathogen. Hence the inducer in effect acts as a multi-site fungicide. The durability of the effectiveness of the recently introduced SAR inducer acibenzolar-S-methyl (CGA 245704) will be watched with interest; the

manufacturers have expressed the view that resistance will be very unlikely to develop (Ruess et al, 1996).

## CROSS-RESISTANCE

Obviously a 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 initially rare mutants.

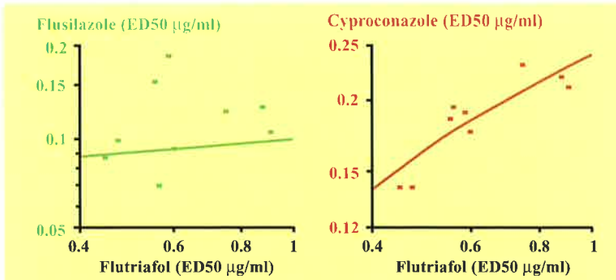
Usually if a new fungicide has a similar structure and/or mode of action to existing fungicides against which resistance has developed, then cross-resistance is found. Sometimes the cross-resistance is only partial, involving lower resistance factors than those shown against existing fungicides. The degree of cross-resistance, or cross-sensitivity, often varies from isolate to isolate. Generally such variation is not large enough to cause problems in risk assessment, but it can differ considerably according to the particular fungicides that are tested, as illustrated in Figure 1, and also in a study by Gisi et al (1997) on the same pathogen species but with different azole fungicides.

It is known for a new fungicide to exhibit negative cross-resistance, i.e. it acts solely on strains that are resistant to certain existing fungicides and does not





affect wild type strains. The detection of cross-resistance, partial cross-resistance or negative cross-resistance can greatly influence, in different ways, the assessment of risk and the planning of use strategies.




To obtain these data nine isolates were assayed for their sensitivity to flutriafol, flusilazole and cyproconazole

(Source: Kendall, 1996, personal observation)

**Fig.1.**

Sensitivities of a set of nine isolates of *Septoria tritici* towards two different pairs of azole fungicides. Sensitivities to flutriafol and cyproconazole are closely related (correlation coefficient 0.77) in contrast to sensitivities to flutriafol and flusilazole which are not significantly related (correlation coefficient 0.02). Source: S J Kendall and D W Hollomon, unpublished data.

In one case of negative cross-resistance the biochemical cause is known. Wild-type biotypes of target pathogens are sensitive to benzimidazoles and unaffected by phenylcarbamates. When benzimidazole-resistant strains arise these usually prove to have become sensitive to phenylcarbamates. It is now known that the target-site change that prevents the binding of carbendazim to the β-tubulin target site allows binding by diethofencarb. The molecular mechanism involves just a single amino acid change in the β–tubulin (Wheeler et al, 1995). 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. Mixtures of carbendazim and diethofencarb have been used commercially with some success against *Botrytis cinerea* on grape-vines in situations where benzimidazole-resistant strains were already widespread. Unfortunately double-resistant populations, unaffected by carbendazim and diethofencarb, or by a mixture of these fungicides, have developed in some situations and have necessitated the use of alternative treatments (Leroux and Moncomble, 1994).

Several other cases of negative cross-resistance are known, for example 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, and in both laboratory mutants and field isolates of *Ustilago nuda* between different carboxanilides and of various pathogens between DMI fungicides (see review by Leroux, 1992). 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 therefrom, by conducting cross-resistance tests.

## GENETIC STUDIES

### Mutagenesis

The potential in the target pathogen for resistance-conferring gene mutations 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 treating cultures of target fungi with mutagenic agents. Either chemical agents, such as nitrosoguanidine, or ultra-violet light are used. Spores from the treated cultures are placed on a culture medium containing the new fungicide at a concentration known to inhibit the growth of wild-type spores. Resistant survivors form colonies, and the 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 mutagenic 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. 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, infectivity and sporulation, then a positive indication of risk is given.

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.

There is now much experience which 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. With both the benzimidazoles and the phenylamides, the classes that have encountered the most rapid and severe resistance development in practice, it is relatively easy to produce fully fit, highly resistant mutants of target organisms (e.g. van Tuyl, 1977; Davidse, 1981). In contrast, with 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 (Dekker, 1981).

However, in some cases the relationships between response to mutagens and risk of practical resistance have been less clear-cut. Thus mutants that are highly resistant to morpholine fungicides are readily obtained in the laboratory, but in practice good performance has been maintained over many years and development of field resistance has been slight (Hollomon, 1994). 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. Major 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

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. (Zeneca)

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

occurred under field conditions but would not occur in mutant production and screening experiments in the laboratory.

The production of laboratory mutants resistant to fungicides belonging to some of the newer classes has been reported (strobilurins, Colson, 1993, Ziogas et al, 1997, Godwin et al, 1998; quinoxyfen, Hollomon et al, 1996, 1997). It is too early to tell what the practical experience will be. However, use strategies aimed at avoiding resistance have been established so that if practical resistance does not arise this will not necessarily mean that the indications of risk from mutation studies were false ones!

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.

Mutants resistant to strobilurins have been produced by laboratory selection in yeast, *Septoria tritici* and other micro-organisms, and have been shown to arise through several different point mutations in the cytochrome b gene (Bennoun et al, 1991, Colson, 1993, Gennis et al, 1993, Godwin et al, 1998). These mutants tend to have impaired growth *in vitro*, due to respiratory deficiency. Their pathogenicity and response to stobilurin treatment *in vivo* are not yet reported. The cytochrome b gene is known to be mitochondrial. This is the first case where a fungicide target site has proved to be coded by a mitochondrial gene rather than a nuclear gene. Hence the significance of these mutagenic studies with regard to the risk of practical resistance cannot be assessed on the basis of past experience. Clearly, target-site resistance has been shown to be biochemically and genetically possible. It has been predicted that any practical resistance is likely to arise in a step-wise manner, through a gradual increase in the proportion of resistant mitochondria, and that it is important for this reason to avoid sub-optimal doses (Godwin et al, 1998). It is notable that weed resistance to triazine herbicides, which is a widespread problem, is caused by a mutation in a non-nuclear (chloroplastic) gene (Jasieniuk et al, 1996).

A different, non-target type of strobilurin-resistant mutation, involving a nuclear gene and the enhanced production of an alternative oxidase with a reduced sensitivity to strobilurins, has been reported recently; however, the resistant





mutant proved more sensitive than the wild-type to azoxystrobin *in vivo* (Ziogas et al, 1997). The future performance of the strobilurins, and the nature of any resistance that may occur, will be watched with much scientific as well as commercial interest!

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 muchbroader 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.

## Genetic recombination

Along with mutation and migration, recombination provides an opportunity to introduce novel genotypes into a population. In many plant pathogens reassortment 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 be recombined in this way with better fitness characteristics, to give phenotypes that will spread under practical conditions. Furthermore, sexual reproduction usually produces wind-dispersed spores, so that in populations of pathogens, such as *Septoria* spp., where the dispersal of the 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.

Sexual or parasexual recombination could equally well break up highly 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 greater development of DMI resistance in the latter 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 by Brown et al (1992) for ethirimol and triadimenol resistance in barley powdery mildew, by Shattock (1986) for metalaxyl resistance in *Phytophthora infestans* and by Faretra and Pollastro (1993), Hilber et al (1994) and Eberle and Schauz (1996) for fludioxonil resistance in *Botrytis cinerea* and *Ustilago maydis*. Recombination studies involving resistant mutants or isolates are now being undertaken increasingly as a part of the risk evaluation for a new product.

## Intensive selection

Attempts have been made to demonstrate the capacity for a fungicide to select resistant mutants through conducting experiments in which successive generations of pathogens are exposed 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 will favour a stepwise build-up based on polygenic mutation.

A number of studies were done on the selection of resistance to phenylamide fungicides in *Phytophthora* spp. by serial transfers on fungicide-amended agar or fungicide-treated plants, and/or by treatment with mutagenic agents (Staub et al, 1979; Bruin, 1980; Davidse, 1981), and were reviewed 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 either 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.





To judge from these studies with the phenylamides, the use of mutagenic agents seems to give a better indication of the potential for practical resistance development than does selection by repeated exposure to the fungicide. This is not surprising, because mutagenic treatment is likely to increase greatly the proportion and the range of mutants, both of which are otherwise likely to be very limited in the small populations (relative to field populations) used in laboratory experiments.

Strains of *Botrytis cinerea* resistant to both dicarboximide fungicides (see Beever and Byrde, 1983), and the phenylpyrrole fungicide fludioxonil (Hilber et al, 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 did gradually build 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 through sustained selection pressure from repeated and widespread use of dicarboximides under field conditions. It remains to be seen whether any practical resistance will build up against fludioxonil, which has recently come into commercial use. The manufacturer's strategy of marketing this fungicide as a mixture with cyprodinil, with a maximum of two applications per season on grapes, should hinder any such development.

Thus selection of mutants through exposure of 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. Possibly, as in the case of the studies on phenylamides discussed above, mutagenic experiments can better indicate the potential for retention or restoration of fitness. However, more comparisons of results of mutagenic and selection experiments, and of 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.

To avoid cross contamination between different fungal isolates, plants and fungi can be contained as shown for vines and *Uncinula necator*. (Zeneca)

Surprisingly, no attention appears to have been given to 'training' experiments with fungicides against which polygenic resistance is known to develop.

Intensive selection experiments have also been done in the field. 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 (*Pseudocercosporella herpotrichoides*) resistant to benzimidazole fungicides failed to reveal the occurrence of resistance over a five-year period (Fehrman 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, which 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.

In situations where an abnormally high frequency of selection opportunities can be achieved, as for example with a pathogen producing many generations per season, and where the frequency of application of the test fungicide can be abnormally high, then there will be a reasonable chance of 'forcing' the development of major-gene resistance in the field (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.

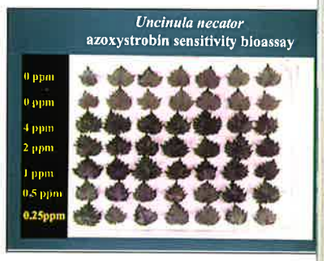




## DETECTION AND MONITORING

It has now become usual for agrochemical companies to make, or to 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, There are three 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 fungi, 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.




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

The importance of achieving the first two requirements, and the methodology involved are discussed elsewhere (Brent, 1992). The third 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 at present 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 *Erysiphe graminis* in field populations of barley mildew is shown in Table 2.

Table 2. Sample size needed to detect (with 95% confidence) rare resistant mutants in populations of *Erysiphe graminis*

| **Mutant frequency** | **Sample size (number of pustules)** | **Area of crop sampled (ha) *** |
|---|---|---|
| $1 \times 10^{-4}$ | $3 \times 10^{4}$ | 0.0001 |
| $1 \times 10^{-6}$ | $3 \times 10^{6}$ | 0.01 |
| $1 \times 10^{-8}$ | $3 \times 10^{8}$ | 1 |
| $1 \times 10^{-10}$ | $3 \times 10^{10}$ | 100 |
| $1 \times 10^{-12}$ | $3 \times 10^{12}$ | 10000 |

* 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 (as described by Brent, 1995). The early stages of this process, whilst not obvious in the field, can be detected by successive sensitivity surveys because a substantial proportion of the population is involved and relatively few samples are necessary (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.





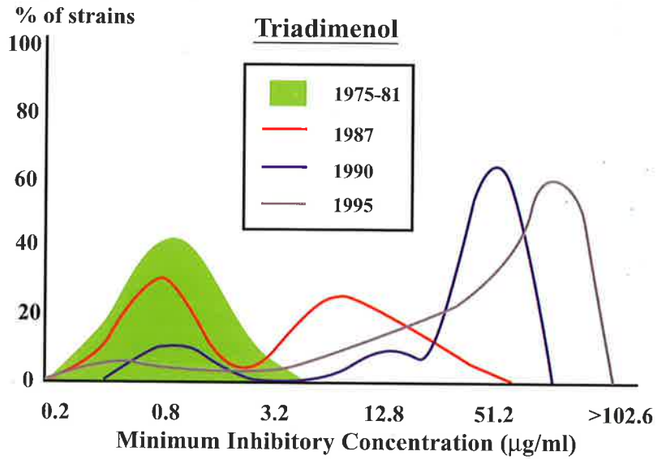
A gradual, unimodal shift in sensitivity will result from multi-step or polygenic resistance, whereas a bimodal development of distinct sensitive and resistant populations will reflect the selection of a single, major resistance gene. The data in Figure 2 illustrate how the pattern of resistance development 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.

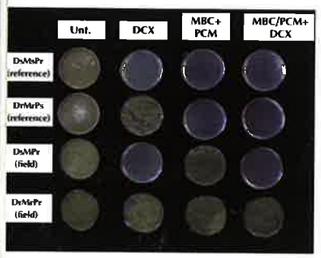
Even for multi-step resistance, however, the first sensitivity surveys in commercial crops made prior to new fungicide introduction are unlikely *per se* to aid initial risk assessment. Shifts in sensitivity will only occur in response to the use of the fungicide in these crops. Successive sensitivity 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. To take two recent examples, isolates of *Septoria tritici* obtained in France showed a relatively narrow, ten-fold, range of sensitivity to azoxystrobin, when tested *in vivo*, with ED50 values between 0.1 and 1.0 mg/l. (Godwin et al, 1998). Isolates of barley powdery mildew obtained in the UK, showed a much broader, thousand-fold range of sensitivity against quinoxyfen, when tested *in vivo*, with ED50 values between 0.0005 and 0.5 mg/l. (Hollomon et al, 1996). All such isolates are easily controlled by application of the fungicide at concentrations well below the recommended rate of application.

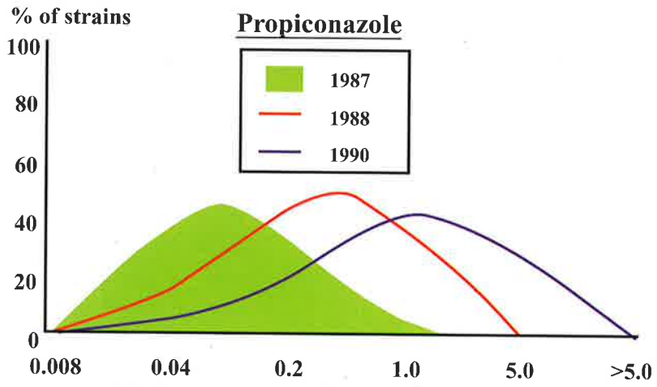
Wind impaction spore trap on a car roof. This is used, especially for *Erysiphe graminis*, to conduct surveys to monitor the development and status of fungicide resistance (Zeneca)

**Fig.2.**
Different patterns of resistance development shown by *Rhynchosporium secalis* towards two triazole 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.




In the 1980's agar plate tests were typically used to determine fungicide sensitivity. This test shows *Botrytis cinerea* and 3 fungicides. (Zeneca)







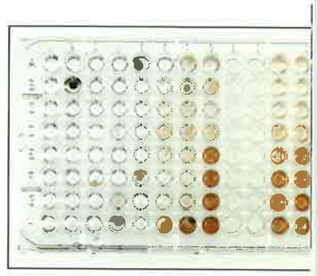

The question arises as to whether the range of sensitivities found in base-line tests can act as an indicator of the risk of future resistance problems. Possibly the broader the base-line range the greater could be the propensity for subsequent shift to much lower levels of sensitivity under fungicide selection pressure; perhaps this would be more likely to apply if polygenic resistance is involved. However, there is no evidence for or against any such relationship, and the question remains an entirely open one. Unfortunately, base-line studies have been made only over the past few years, 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 often time-consuming and resource-intensive, and generally fail to detect resistance early enough to assist initial risk assessments. Adapting bioassays 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 fungi that can be manipulated in this way.

The development of molecular detection technologies is a rapidly advancing field with respect to the detection of human genetic disorders, and is enabling the detection of rare mutants in populations (Mei et al, 1997). They could become applicable to the detection and identification of fungicide-resistant mutant genes of plant pathogens at very low frequencies. This information could re-vitalise the role of modelling and allow the effects of selection to be predicted more accurately. It could also allow the direct measurement of 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, opens the way to developing highly specific and potentially very sensitive tests that directly detect and identify resistant gene mutations. Efforts to achieve this through the use of immunological methods such as ELISA have proved unsuccessful, but the ability to isolate resistance genes and to clone them through polymerase chain reactions (PCR) has exposed many new opportunities for diagnosis.

Using PCR, coupled in some cases with DNA hybridisation technology, together



Recent procedures use microtitre plate techniques to test many isolates more quickly and with less effort. Results are read automatically and transferred directly into computer data bases. In future, use of DNA probes may bring further improvements. (AgrEvo)

with a simple dot blot format, has allowed detection of benzimidazole-resistant alleles in several pathogens (Koenraadt and Jones, 1992; Wheeler et al, 1995). Use of this detection method in field studies on resistance development has already begun. It is notable that 11 sites of point mutation to benzimidazole resistance have been detected in β-tubulin from laboratory mutants, but resistant field isolates of *Venturia inaequalis*, *Botrytis cinerea* and *Penicillium digitatum* have all shown mutation at amino acid loci 198 or 200. Possibly mutation at other sites occurs in the field but is associated with loss of fitness. Research is now proceeding with regard to DMI resistance in grape powdery mildew (Delye et al, 1997), and sufficient information is probably available to permit work on the molecular detection of strobilurin resistance.

The extent to which these molecular detection methods will prove useful, with regard to risk assessment (or more generally to resistance monitoring), remains to be seen. They certainly offer prospects of the rapid and definitive detection of mutants with known resistance mechanisms, at relatively low frequencies. However, there will still be a need to collect and test large numbers of independent samples from diverse pathogen populations, in order to determine how general is the occurrence, or the absence, of the resistant mutant. Also one cannot preclude the possibility of the existence in the field of resistant variants with a slightly or completely different resistance mechanism that would not be detected by the applied molecular test.

## DISEASE-ASSOCIATED RISK

The approaches that we have considered so far have referred to the assessment of the basic capacity of target pathogens to become resistant to fungicide treatments through mutation. This capacity for genetic change towards resistance varies greatly with respect to the different fungicide classes, as we have discussed, but varies relatively little between different genera or species of target fungi. Hence it is sometimes referred to as the 'fungicide risk', although the term 'fungicide-associated risk' seems preferable .

The risk of the subsequent selection of the resistant mutants, leading to their build-up to commercially serious proportions, does depend greatly on the target





disease and on the way in which it must be treated. This has been called the'disease risk', although again 'disease-associated risk' seems more apt.

A number of factors relating directly to disease epidemiology, and indirectly to disease management combine to form the 'disease-associated risk' for each combination of crop disease and fungicide treatment. The most important of the epidemiological factors are:

- 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.
- isolation of pathogen populations; the more isolated the crop, through distance from other crops or through protection in glass-houses or plastic tunnels, the less the chance of ingress of sensitive forms or loss of resistant forms.
- occurrence of a sexual stage in the life cycle; this could either favour or hinder resistance development, as discussed on page 17

Figure 3 shows how the disease-associated risk combines with the fungicide-associated risk to give an overall inherent or basic risk of resistance for a number of combinations of leading fungicides and important target diseases. 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 an additive way. In this way an overall disease risk can be determined for a disease-fungicide combination, to a high, medium or low level.

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