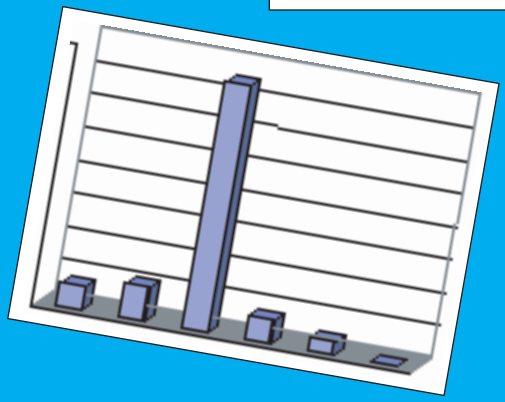
SENSITIVITY BASELINES IN FUNGICIDE RESISTANCE RESEARCH AND MANAGEMENT

P E RUSSELL



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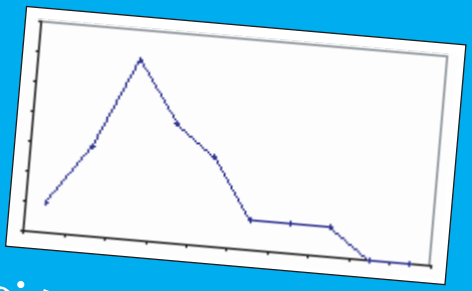
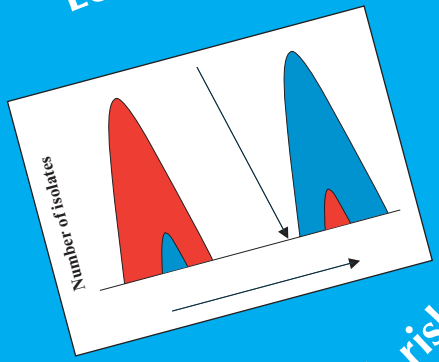
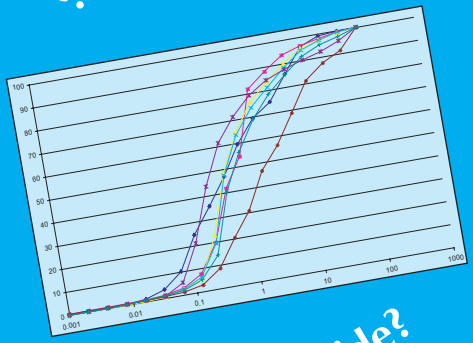


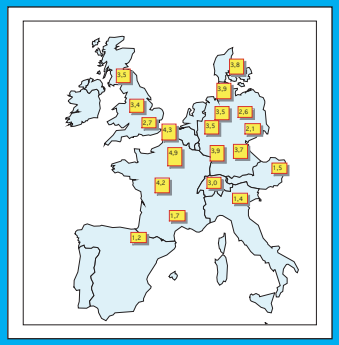
High risk fungicide?

Low risk pathogen?





High risk pathogen?



Low risk fungicide?

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SUMMARY

Baseline construction is now a major task undertaken by scientists working in the crop protection industry and forms a significant part of the registration process for all pesticides. This publication, written with an emphasis on this regulatory process, will be of major value to the commercial sector and to Regulatory Officials concerned with examining the Resistance Risk Assessment component of new product Registration Dossiers. It is, however, equally relevant to scientists in the public sector who wish to embark on resistance management studies.

The document follows the baseline production process through its logical stages:

- A consideration of what a baseline is, when it is necessary and what the alternatives are. The influence of chemistry and the pathogen is highlighted alongside considerations of other products on the market.

- Guidance on what makes a good baseline: the importance of standard methods, the influence of sample number and origin and particularly the geographical area being sampled. Theoretical and real examples are used to illustrate the points discussed.

- Using a baseline in practice: detecting shifts and the use of bridging data for different locations and crops. Detecting shifts from the baseline and use of a ‘discriminatory dose rate’ to declare test samples ‘resistant’ or ‘sensitive’.

- The relevance and use of molecular testing procedures is discussed, highlighting the advantages as well as the current limitations.

- A summary of the regulatory requirements for Europe are given, illustrating the different data types required.

- Appendices provide information on how to establish assay methods, the probability of detection of resistance and the common dangers and pitfalls of baseline construction.

INTRODUCTION

Fungicide resistance and its management is of great importance to all concerned with crop protection. Without effective product management, resistance could arise very quickly, as happened with the methyl benzimidazole carbamates (mbc), dicarboximides and phenylamides in the early 1980's. These compounds were introduced without the benefit of adequate resistance management strategies, largely because the phenomenon of resistance was quite novel and the scientific principles of management not established. The first signs of resistance developing were failures in field control of various diseases, primarily *Botrytis cinerea* on grapevine (mbc and dicarboximides) and *Phytophthora infestans* on potato (phenylamides), (Smith, 1988; Lorenz, 1988; Staub 1984). Resistance management procedures were quickly put in place and have been most successful. Nevertheless, they were all made in retrospect and without full knowledge of the nature of the sensitivity/resistance profile of the target fungi. Had these measures failed, there is every chance that the compounds would have been withdrawn from the market, leading to a financial loss for the manufacturer which in turn would have led to less investment in new crop protection chemicals, and a loss of valuable products to the user such that they would have had to rely on older, less effective and less environmentally friendly products.

Since those days, the costs of research and development for new pesticides have escalated, leading to increased risks for the financial investments made. Resistance development is one of the major risks involved and it is a wise company that now assumes a significant risk of this happening for a new molecule and so plans to manage it from the beginning. But this raises problems. How do you develop anti-resistance strategies? How do you recognise resistance development, or more importantly, are there ways to monitor the effectiveness of a new fungicide on a fungus such that the development of resistance can be detected before it is a major problem and allow extra management procedures to be put in place? The key element in answering these questions is that you must know the response of your target fungus to the fungicide before the fungus has been exposed to it in practice. You thus need to know the sensitivity *baseline* for your fungus/fungicide combination. Only with this information is it possible to monitor the effect of the fungicide on the fungus to see if the response is changing towards resistance.

This aspect has been recognised by the regulatory authorities in Europe (Anon 1994), with the intention of protecting the environment and helping the user, by

ensuring that new products are introduced with an adequate resistance management strategy. Within this scheme, the provision of baseline sensitivity data is a requirement for registration of new molecules and re-registration of established molecules.

DEFINITION OF THE TERM 'BASELINE'

The word 'baseline' has many uses in everyday language but all of them include the concept that it is a point of reference to be used in a decision making process. The 'baseline' in a tennis court, for instance, defines the court area such that balls are deemed 'in' or 'out' of play depending upon which side of the 'base' line they land. For fungicide resistance research and management a 'baseline' can be defined as:

A profile of the sensitivity of the target fungus to the fungicide constructed by using biological or molecular biological techniques to assess the response of previously unexposed fungal individuals or populations to the fungicide.

The primary use of baselines is as a tool for the establishment of, and subsequent monitoring of, fungicide resistance management strategies. The term baseline is universally applied to new compounds from new chemistry but when applied to molecules for which, for whatever reason, it is not possible to find a population that has never been exposed to the type of chemistry associated with the new molecule, the terms *'sensitivity profile'* or even *'pre-market introduction profile'* may often be used. For the purpose of this document, the terms are interchangeable, depending on circumstance.

By implicit definition, the baseline is not a single data point but is constructed by sampling a number of individuals or populations and establishing the variability between them. The baseline, however expressed, visually, mathematically or both, will illustrate this variability.

In practical use, the baseline establishes a reference point for accepted fungal sensitivity to a fungicide. Fungal isolates or populations that are found with a sensitivity profile that falls outside the baseline response are normally considered to be 'less sensitive' or 'resistant' to the fungicide.

It cannot be stressed too strongly that the shape of the baseline distribution gives no information on the absolute risk of resistance developing in practice. However, if:

- the baseline shows any sign of adopting a disruptive pattern (see later)
  or:
- the baseline shows a skew distribution with a long tail for the less sensitive end
  and:
- the outlying portion of the distribution can be associated with a true resistance response

then this is a clear warning that resistance is possible and that strict anti-resistance measures should be taken before market introduction. The baseline produced will still be valid, but the future commercial monitoring emphasis should rest on assessing the development of the proven resistant segment over time, and possibly investigating the impact of alternative resistance management strategies.

WHEN IS A BASELINE NECESSARY?

Fungicides are introduced to market with specific targets to control. Each of those targets, in theory, requires a baseline to be established so that product use strategies can be monitored and possible resistance can be detected. However, experience suggests that some targets are very prone to resistance development, others are less prone and in some cases there has not been any real evidence of resistance development to date. Commercial considerations also have an influence.

Pathogen	Crop	Disease
Botryotinia fuckeliana (Botrytis cinerea)	various especially grapevine	grey mould
Erysiphe graminis	wheat/barley	powdery mildew
Mycosphaerella fijiensis	banana	black sigatoka
Phytophthora infestans	potato	late blight
Plasmopara viticola	grapevine	downy mildew
Pseudoperonospora cubensis and related	cucurbits	downy mildew
Pyricularia oryzae	rice	rice blast
Sphareotheca fuligenea and related	cucurbits	powdery mildew
Venturia spp.	apple, pear	scab

Table 1: Plant pathogens accepted as showing a high risk of development of resistance to fungicides (adapted from EPPO 2002)

High risk and low risk pathogens:
Table 1, adapted from EPPO (2002) lists the plant pathogens from major world markets which have shown themselves to be capable of becoming resistant to fungicides in a time span sufficiently short to be a serious threat to the commercial success and user value of various products.

In contrast, several pathogens are regarded as posing a lower risk because widespread resistance is not a problem or has been slow to develop. In some cases this is undoubtedly due to the pattern of product use. This does not mean that they will never show significant resistance to fungicides in future, but could illustrate a general lack of mechanisms to become resistant in practice either through an inherent biological property of the fungus (for example its epidemiology) or through the use pattern of the products designed to control them.

The EPPO Guideline (EPPO 2002) does not list these and decisions on baseline production must be made on individual case reviews. Examples are given in Table 2. In using this list it should be noted that EPPO includes *Gibberella fujikuroi* and *Uncinula necator* in Table 1, while the FRAC view is that they should be included

Pathogen	Crop	Disease
Bremia lactucae	lettuce	downy mildew
*Gibberella fujikuroi**	rice	bakanae
Leptosphaera nodorum (Stagonospora nodorum)	wheat	leaf spot
Monilia spp.	stone and pome fruit	Monilia rots
Mycosphaerella graminicola (Septoria tritici)	wheat	leaf spot
Mycosphaerella musicola	banana	yellow sigatoka
Peronospora spp.	various	downy mildew
Podosphaera leucotricha	apple	powdery mildew
Puccinia spp	Wheat/barley	rusts
Pyrenophora teres	barley	net blotch
Tapesia spp.	wheat/barley	eyespot
*Uncinula necator**	grapevine	powdery mildew
** The EPPO Guideline lists these pathogens as high risk and baselines are normally requested*		

Table 2: Plant pathogens accepted as showing a medium risk of development of resistance to fungicides

in Table 2. The reason for this difference of approach arises from the fact that there is little hard data to classify pathogens into the groups other than historical experience and opinion as to the risks posed by the pathogens.

Commercial considerations, pathogen and market priorities
Some pathogens are of local importance, but in commercial market terms are considered as minor pathogens. It is doubted if any commercial company would base a modern product development and sales campaign on such uses if there was no other use available for the new product. For this reason it is possible that the costs involved in establishing a baseline and for an ongoing campaign to monitor the year by year sensitivity profile of the target pathogen could not be justified. This does not mean that baselines could not be produced. There is no scientific reason why they could not, but they are more likely to be produced as research tools in situations where the cost of their production is not a major issue e.g. in academia or the public sector. Decisions on baseline production must be made on a case by case basis. Typical pathogens and diseases are given in Table 3.

Pathogen	Crop	Disease
Alternaria spp.	various	Leaf spots
Fusarium spp. and related	various	fusarioses
Hemileia vastatrix	coffee	rust
Phytophthora spp. (soil borne)	various	Damping off
Pythium spp	various	Damping off
Rhizoctonia spp.	various	Foot and root rots
Rhynchosporium secalis	barley	Leaf blotch/scald
Sclerotinia spp.	various	sclerotinia diseases
Tilletia spp.	cereals	bunts
Ustilago spp.	cereals	smuts

Table 3: Plant pathogens of minor commercial importance for which a baseline may not be justified for commercial reasons

WHAT ARE ALTERNATIVES TO A NORMAL BASELINE?

In some cases it may not be possible to establish a baseline using bioassay procedures. This could be for commercial reasons as given above, or due to unexposed samples not being available or maybe even biological difficulties. In such circumstances we may still need a reference point against which to compare product

performance as part of a monitoring campaign. This can be done by using efficacy data:

Using efficacy data as a baseline
During the development phase of new product introduction many field trials are conducted in which the efficacy of the new product is established for the selected use. In many cases it is reasonable to expect that the target pathogen(s) have not been exposed to this mode of action before and so represent a true, natural, non selected population. The efficacy response can therefore be used as an expected response in the absence of resistance. Future monitoring of efficacy can thus be used as an indicator of the possible development of resistance, with any reductions in efficacy below an agreed threshold value raising concerns and generating investigations to examine if resistance could be to blame. In such circumstances it is very important to eliminate all other possible causes of reductions in efficacy e.g. application errors, dose rate variations, intense disease pressures etc before a state of resistance is concluded and further research is carried out.

An example of such data is given in Table 4.

Variety	Sown	Assessed	Abavit UT activity %	Reference activity %
Frisia	15/3/83	29/6/83	100	99.45 Arbosan UT
Frisia	25/3/83	1/7/83	100	100 Arbosan UT
Frisia	19/4/83	11/7/83	100	99.7*
–	27/9/83	29/5/84	100	96.67*
Astrix	30/9/97	29/5/98	100	100 Solitär
Astrix	16/9/97	29/5/98	100	100 Solitär
–	30/9/97	29/5/98	100	100 Solitär

Table 4. Control of *Pyrenophora graminea* by prochloraz + carboxin seed treatment 1983 – 1998 (* reference not recorded)

The data come from efficacy evaluations for a seed treatment based on prochloraz and carboxin in Germany. At the time of introduction of the product (Abavit UT) the risk of resistance developing was considered to be low and no baseline data obtained. Efficacy data were obtained from various development trials and continued to be gathered up to 1998.

The data clearly show that there was no decrease in the activity of the seed treatment over a period of 14 – 15 years, indicating that no resistance had developed. Parallel data were obtained for many more seed-borne pathogens and all showed no decrease in efficacy over the years. These efficacy data are thus an excellent substitute for a baseline.

BASELINE AND FUNGICIDE CHEMISTRY

Once decisions on baseline production have been made based on commercial considerations the next factors to consider are based on the chemistry of the new fungicide.

The clearest situation concerns a new fungicide from a new area of chemistry, but a new fungicide from established chemistry must also be considered. It may also be desirable to construct a baseline for a molecule that has been in use for some time. In both these latter cases it is possible that some change in population sensitivity will already have taken place before the research is carried out and the results must be considered accordingly.

New chemistry, new mode of action
During the discovery, research and development of a new fungicide, a company will attempt to determine the biochemical mode of action of its new molecule. Only in a few rare cases has this been achieved before market introduction, for example strobilurins. It will be normal, however, to establish whether or not the mode of action is novel or already known. If the mode of action is clearly novel, it is quite probable that there are no molecules with a similar mode of action on the market and hence there will be no prior knowledge of suitable baseline determination techniques. In contrast, with a new molecule from a known mode of action, techniques may already be available.

Commercially, this situation raises a curious possibility. Such is the emphasis on baseline construction and resistance management that very often the baseline and management strategies will be defined before the biochemical mode of action is determined. This happened for the molecule quinoxyfen (Hollomon *et al*. 1997). It is fairly certain that other crop protection research companies will have been researching new molecules that could well have had a similar biochemical mode of action to quinoxyfen but because of commercial confidentiality procedures there was no opportunity to check for possible cross resistance/sensitivity between the molecules.

The first priority will therefore be to establish methods for the assessment of the sensitivity of the fungus to the fungicide. This will involve much research and, if the fungus involved is an obligate pathogen, could become very costly in resource and financial terms. Guidance on selection of an appropriate assay method is given in Appendix 1.

New fungicide, established mode of action
For a new molecule from an established area of chemistry it is highly probable that basic research into sensitivity assessment techniques will already have been done and published. It is also possible that baselines have already been published for certain molecule/pathogen combinations. Such information can be invaluable in providing guidance on production of a baseline for the new molecule. However, it would be dangerous to assume that such techniques and established baselines can be adopted for the new molecule. This is because individual molecules have individual dose response curves and will have different physico-chemical properties. The shape of baseline distributions could thus differ between molecules, both in relation to the doses used and the responses obtained. Data provided by Elcock *et al*. (2000) illustrate this well for DMI fungicides and *Mycosphaerella graminicola*. The established techniques will need to be validated for the new molecule and a new, molecule specific baseline prepared. Reference to FRAC could well reveal suitable techniques and information.

It will also be important to consider the resistance status of the pathogen to this chemistry. If the chemistry is well established it is possible that resistance is already known. Care must thus be taken to ensure the baseline is constructed from fungal isolates from the sensitive population. Using other molecules from the area of chemistry can be a great help here, along with use of reference resistant isolates if they are available.

Baseline in retrospect for an established product.
This situation could be similar to that for a new molecule from established chemistry provided that the molecule being considered is from an area of chemistry that has already been researched.

If the molecule being considered is the only representative on the market for that mode of action and there is no early research on sensitivity to refer to, then it may still be possible to construct a baseline. However, commercially it may not be desirable to expend resource on establishing a baseline from bioassay studies and a reference based on efficacy may suffice (see ‘What are alternatives to a normal baseline’ section), especially if resistance is not known. If resistance is known or

suspected the situation is a little more complex. In this case, by definition there must be a means of detecting resistance and quantifying the response of the fungus. It should thus be possible to establish a baseline (often referred to as a *sensitivity profile*) based on this technology, but care must be taken to ensure that it is primarily constructed from isolates deemed to be sensitive, although positioning known resistant isolates on the baseline could help with further decision making.

SAMPLING PROCEDURES, SAMPLE NUMBERS AND DOSE RATES

Various authors have considered how best to survey and sample plant diseases (see Holderness, 2002, for a review). Such procedures are generally designed to establish the incidence of plant diseases and the damage (loss) they cause. Surveys of the incidence may be undertaken more than once per season. Many of the parameters considered in such studies are also applicable to the procedures involved in establishing sensitivity baselines. There are, however, differences in objectives and approach to the problem of how best to conduct the survey. Surveying the incidence of a disease in order to produce information on its spatial distribution requires knowledge of the epidemiology of the pathogen and hence the spatial distribution patterns that may be expected. When establishing a baseline, knowledge of the spatial distribution pattern will be of more importance than the incidence of disease. Sampling procedures and patterns may be common, but with a difference in objectives insomuch that the baseline establishment seeks to sample and assess the *different fungal populations* in the location and not prevalence of fungal propagules or the extent of the damage caused by them.

Sampling procedures

For the establishment of a true baseline, samples must be obtained from areas and crops that have not been treated with the fungicide, or a fungicide showing cross resistance to it, either in the season of test or in previous seasons. Exceptions can occur when it is desired to produce a baseline in retrospect and are valid when there has been no instance of resistance provided it is accepted that some selection against the most sensitive individuals in the populations may have happened, albeit with no effect on field performance of the fungicide. In cases where the baseline is being constructed for a new molecule from established chemistry for which resistance is already known it may be advisable to construct the baseline using reference samples from established culture collections providing such samples can be regarded as not having been exposed to the area of chemistry before.

Where fresh samples are being gathered from a field, crop or part of a crop, decisions must be taken regarding how to sample the test area. Sampling should be done at random, or by taking samples on a field diagonal or 'W' shape. It is quite permissible to discard samples showing no disease at the point of sampling and select another, diseased, plant. This is acceptable because the sampling is not being carried out in order to produce a distribution map of the pathogen across the field and there is no point in despatching non-diseased plants to the test laboratory. A sample unit could comprise an individual plant part e.g. a wheat stem for *Tapesia* spp. or an individual leaf e.g. for *Venturia inaequalis* on apple. The objective is to obtain samples which cover the full range of population variability. For this reason some knowledge of the epidemiology of the target pathogen is desirable so as to avoid sampling procedures which would select sub-samples of the same population rather than from different populations. Thus, if taking multiple samples of a relatively 'less mobile pathogen' (for example *Tapesia* spp, *Septoria nodorum*) from an individual field, the samples should be taken from the full area available and not restricted to one select part of the field.

For typically airborne spores e.g. *Erysiphe graminis* on cereals, samples of equal sensitivity are quite likely to be more widely dispersed especially if sampling is conducted during the active epidemic phase. In very early season this may be less so. In such circumstances the air flora will generally contain more variability than that found in a restricted area on the ground and sampling may be done using specialised apparatus designed to sample large volumes of air.

Sample number and theoretical baselines
It is accepted that unlimited resource will not be available for baseline establishment and that resource limitations may determine the number of samples processed and hence the number of data points generated.

The baseline must represent the variability of the fungal population to the fungicide. A baseline generated from a limited number of isolates from a limited number of plants from a single location has little chance of being representative of the whole population, although it may be valid for those particular circumstances. Decisions on the scope of the baseline thus have to be made with clear objectives in mind and knowledge of the biology and population structure of the pathogen. Reference to data published from baselines for the fungus to other fungicides may give some guidance.

When considering how many data points will produce a representative baseline there are two factors of variation to be considered:

- the inherent variability of individual fungal isolates to the fungicide, and:

- variability introduced by experimental error in the testing procedure.

We wish to measure the first of these and minimise the second. A method that has a high variability due to experimental procedures will be less reliable than a low variability method. The use of replicated assessments for the same isolate and dose rate can illustrate this variability and to some extent using a mean value of the replicates will help control the variation. However, excess variation between replicates (Coefficient of variation >10%) could indicate a significant flaw in the testing procedure and a need for a more robust method.

The objective is to obtain a realistic picture of the sensitivity distribution with the minimum number of data points such that deviations away from this distribution can be identified in subsequent monitoring exercises. Because of the differences between fungal pathogens and fungicides it is impossible to be prescriptive as to how many data points are required for any given situation, but general guidance can be given:

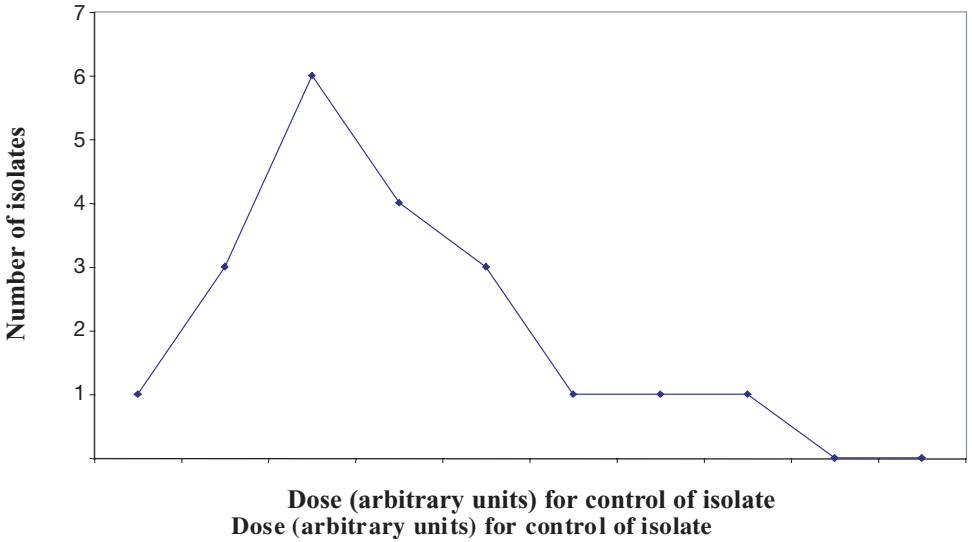
Clearly a baseline constructed on 5 points is unlikely to be of much use unless the individuals in the sample represent the full population variation. Consider the fact that this would not be realised until more than 5 isolates had been tested! Similarly, a baseline constructed from 500 points may be excellent but not always necessary. For the majority of cases it is likely that a baseline will not be adequately defined with less than 20 points and 50 points are more likely to give a reasonable picture. Baselines covering broad geographical areas are likely to require more data points than those directed at specific locations. The accuracy with which deviations from the baseline can be detected in future will depend on the accuracy of its original production.

Should the number of data points selected show a predominantly flat response with no cut off point for lower or upper sensitivity values, it will be very difficult to detect future shifts in sensitivity. In such circumstances the dose rates used should be revised so as to encompass the population variability limits.

Caution must be used if the determined baseline ends abruptly with a large proportion of the population in a particular (high) dose category as it is quite possible that the sampling procedure has also not encompassed the population limits or the gaps between the highest dose rates are too high. Dose rates selected for evaluation must cover the normal ‘sensitive’ range of isolate response and include a rate that gives total control, but must also allow an accurate assessment of the shape

of the sensitivity response. The dose rates used *in-vitro* will in all probability have little relationship to those used in the field because fungal sensitivity *in-vitro* is invariably higher than that in the field. For some *in-vivo* methods field dose rates may be applicable.

Examples of theoretical baselines are given below to illustrate the influence of number of data points. The examples are shown as line graphs but could equally be produced using histograms.



Dose (arbitrary units) for control of isolate

Figure 1. A theoretical baseline created from 20 isolates using 10 dose rates. The control values could be based on Minimum Inhibitory Concentrations (MIC), or EC_{50}.

In Figure 1, the response shows a typical skew distribution with the majority of isolates being controlled at a dose rate of 3. The response then falls off with some isolates not being controlled until the dose reaches a value of 9. No isolates in this survey survived a dose rate of 10. Note the tail from dose rates of 6 to 8. If resistance developed, the reduced sensitivity of isolates may cause a shift in the distribution peak to the right and should be quite obvious. Alternatively the peak at dose 3 could be reduced and the number of isolates showing a response at rates 6 – 10 increase. In either situation, the shape of the curve would change as more isolates fall into the right hand area. This could be considered as a suitable baseline even though the sample number is fairly small.

In contrast, Figure 2 shows a very irregular baseline derived from the same sample size and test criteria.

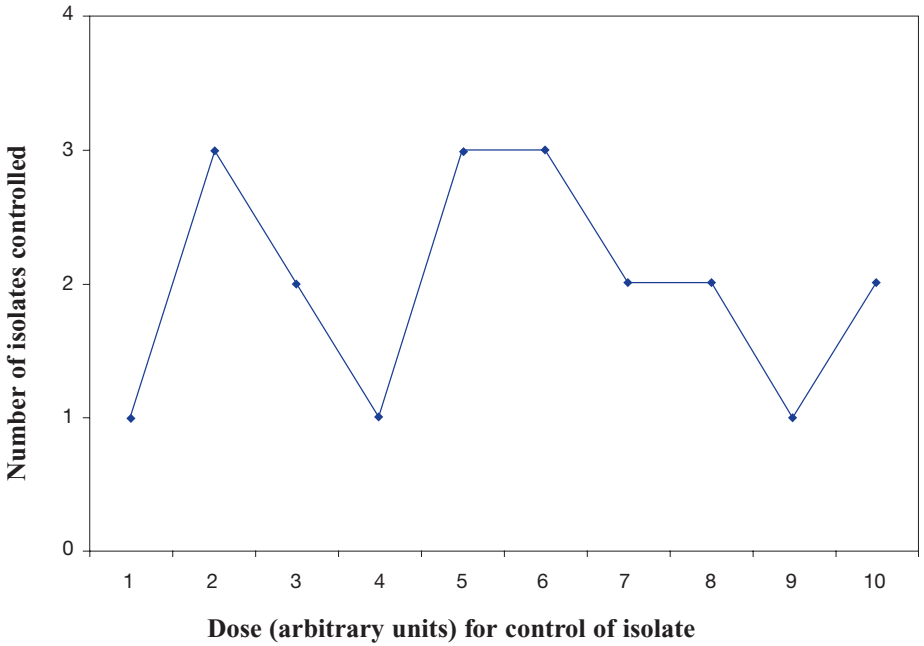


Figure 2. An irregular baseline derived from 20 isolates.
The control values could be based on Minimum Inhibitory Concentrations (MIC), or EC_{50}.

In Figure 2 it is clear that the sensitivity profile is open to question. The maximum number of isolates in any dose category is 3 and a complete control point has not been reached. Note also that due to the low number of isolates in any dose category how the response produces an apparent irregular line but which in reality is varying between a minimum of 1 and a maximum of 3 isolates in any category. The apparent two peaks at dose rates 2 and 5/6 are most likely artefacts caused by low sample numbers. They could, of course, be real points and indicate a sensitive peak at dose 2 and a 'resistant' peak at dose 5, but this is unlikely for a new compound unless resistance is already known or there have been irregularities in the field performance of the product during development. If there is concern, this possibility should be investigated, but a more reasonable task would be to review the procedures for establishment of the baseline because it would be very difficult to identify shifts

in the sensitivity pattern with any certainty using these parameters unless selection caused a massive shift to the right. In a situation like this the recommendation would be to increase the number of isolates tested, increase the dose at the extreme of the distribution so as to obtain a clear end point and also revise the dose rate intervals within the assay.

Figure 3 illustrates what could happen if isolate number is increased to 50 and the top dose rates modified to give a clear end point.

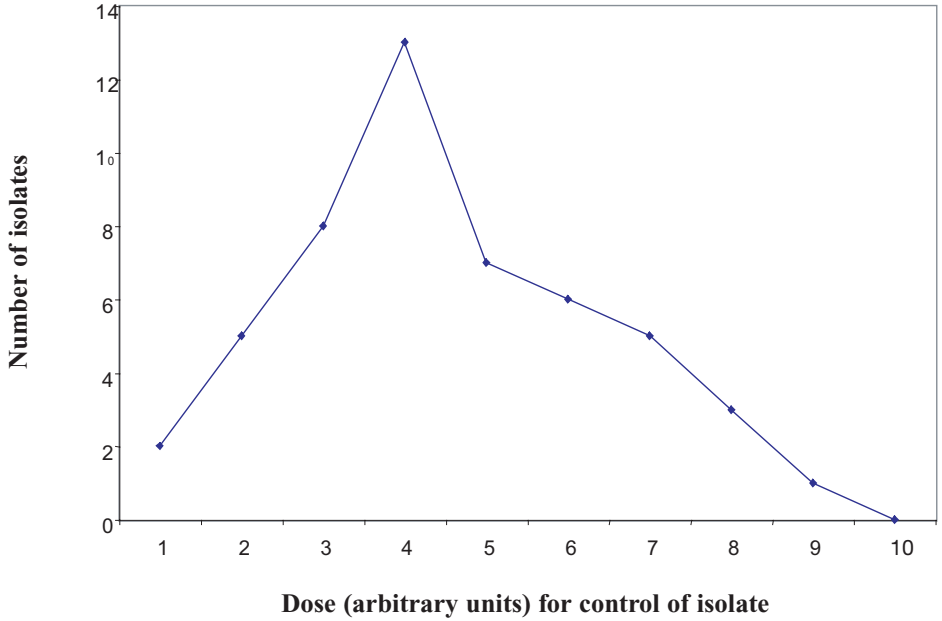


Figure 3. Baseline derived from 50 data points.

Note how the form of the baseline has become smoother with a clear end point. The distribution of points is more reliable and shifts in the pattern would be easier to detect.

Dose rates and intervals.
There is no set procedure for determining the dose rates to be used. Doses may increase in a logarithmic fashion i.e. increasing in a sequence of: 1, 10, 100, 1000, 10000 etc or be based on a sequence such as: 1, 2, 4, 8, 16, 32, 64 etc. The choice has to be made following prior experimentation before baseline research is begun. The sequence does not have to be regular, and conditions may decide that doses are more closely spaced at the lower end of the range than the upper. In general, however, any eventual statistical analysis may be made easier if the dose rate intervals are regularly spaced.

Figures 1 – 3 have illustrated the use of 10 dose rates. Figure 4 illustrates what can happen if the number of dose rates is reduced, using the data from Figure 2.

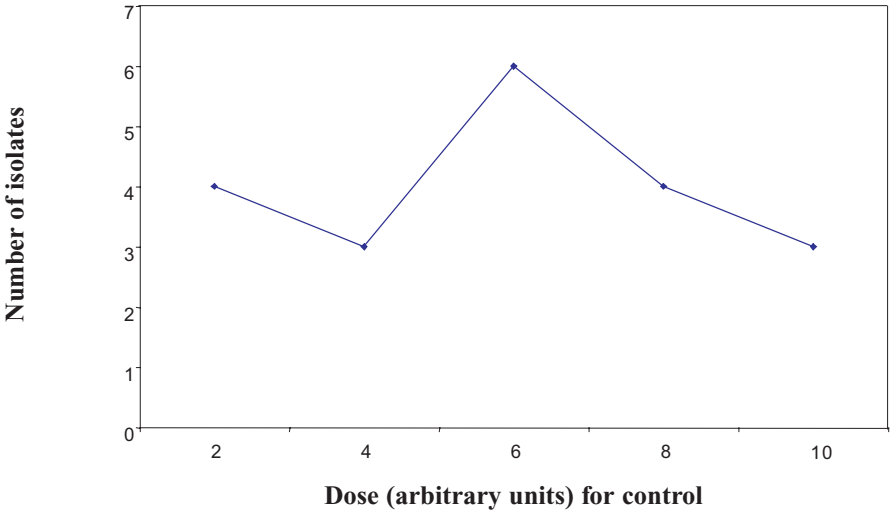


Figure 4: Baseline data from Figure 2 reformatted for 5 dose rates.

In Figure 4 note how the general shape of the curve is smoother but a comparison with Figure 2 illustrates that this is because variability in response to dose rate is being hidden. The baseline so produced is not satisfactory because there is no clear end point and the response is showing too little variation across the dose rates. Only if subsequent monitoring showed a large move in sensitivity towards the higher dose rates and a subsequent loss of the most sensitive isolates would the distribution be of any value.

The test procedure should be revised to include higher and lower dose rates.

This illustrates an obvious, but frequently ignored, fact. As the number of dose rates increases, particularly if the intervals between them are small, the shape of the baseline will be related to the number of isolates tested, with flatter distributions generally being produced where the ratio of sample number/dose rates is low.

Baselines for herbicides and insecticides will frequently be constructed from fewer data points than for a fungicide. The reasons for this are both biological and practical. Weeds are far less mobile than fungi and populations within a given area likely to be less variable due to this restricted mobility. Fewer samples are thus needed to cover the population variability. Insects are more mobile and able to disperse over much wider areas than weeds. But compared to fungi, the number of individuals produced per generation is much lower and differences between individual populations likely to show less variability. Testing multiple weed or insect samples also raises practical problems of sample transport, multiplication and maintenance in the test laboratory.

Expressing the baseline.
Figures 1 – 4 have been expressed as simple frequency curves but they could have easily been expressed as histograms and shown the same information. The dose rates expressed on the x axis are shown equally spaced but in reality would most likely be transformed to such a scale from tests run on a logarithmic or other non linear scale. More recently, much interest has been shown in the use of the lognormal distribution (Limpert 1999).

The frequency curves could have been expressed as cumulative frequency curves as shown in Figure 5 for the same data as from Figure 3.

An advantage of the cumulative frequency curve is that it is progressive and can easily be used in subsequent monitoring campaigns to look for visual shifts in position and differences between populations. Remember, however, that the smallest recorded rise in the Y axis (cumulative frequency %) will be given by '100 ÷ Number of isolates.' So if the isolate number is 20, the smallest % rise will be 5%, and 2% for 50 isolates. Cumulative frequency could also be expressed as a histogram. Pie charts could also be used but are considered of little value for illustrating changes in distribution pattern.

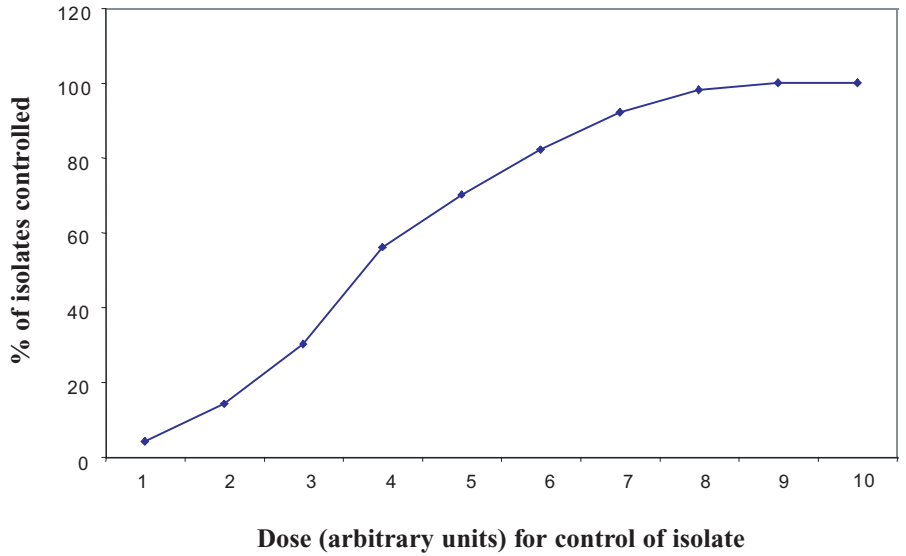


Figure 5. A cumulative frequency curve derived from data shown in Figure 3.

Geographical spread of the baseline.

Is the baseline for a restricted area of use such as a local district, or a country, a continent (e.g. Europe) or for global use? If commercial plans limit product development to a particular country it is clear that the baseline should concentrate on that country in the first instance but that plans are made to expand the knowledge should product introduction plans change. How do we deploy the resources used in baseline construction? In considering an individual country, is it reasonable to assume that sensitivity responses are equal over all the country? Can we assume that a baseline produced for one country e.g. England, will apply to another, e.g. France? The answer to this question can have serious consequences related to the sampling scope of the baseline. To obtain a true baseline for a country, the isolates sampled must be representative of all the population variability present in that country. Bias in sampling must be absent. It would thus be unwise to believe that samples derived from one field would represent a country and a better strategy would be to construct the baseline from field samples from different areas of the country. If the individual field sample size is adequate it will be possible to determine whether or not regional differences exist in the country, and if none are present, to pool the data into an overall country baseline. Experience to date indicates that for a new molecule from new chemistry the sensitivity profiles of populations in an individual country

are likely to be similar and that the main concern is to ensure that the samples tested illustrate the extremes of variability in the population. This concept can be expanded to consider a single baseline for different countries, but with the following proviso: *It may only be reasonable to expect that a baseline from one area will apply to another area if all the conditions of crop growth and pathogen development are as near identical as possible*. It may thus be quite reasonable to assume that a baseline established for a cereal pathogen in England may also be applicable for Northern France or Germany where crop growing conditions are similar. It may not be so reasonable to use a baseline from Europe as a reference point for e.g. Australia or New Zealand.

There are, however, ways to establish whether such common data patterns can be used and so avoid unnecessary duplication of effort in constructing baselines (see 'Bridging data from other crops and locations').

BASELINE ILLUSTRATED EXAMPLES

The following examples have been chosen to illustrate different approaches by crop protection companies to baseline production in practice during the development phases of individual molecules.

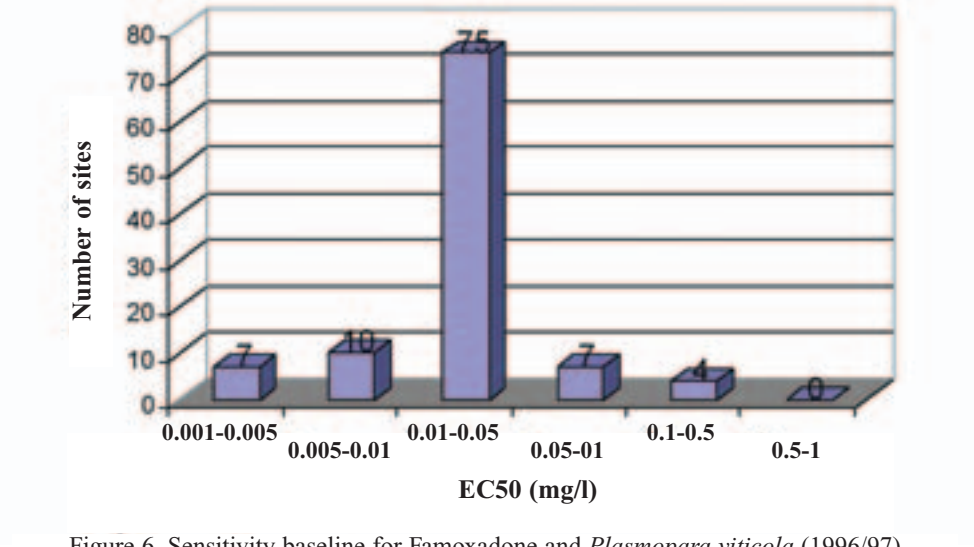


Figure 6. Sensitivity baseline for Famoxadone and *Plasmopara viticola* (1996/97)

New chemistry

Example 1. Frequency distribution: famoxadone and Plasmopara viticola.

Figure 6 illustrates the sensitivity baseline distribution for the QoI compound Famoxadone and *Plasmopara viticola*. At the time of production of this baseline, the mode of action of the QoI compounds was known but they had not been introduced for widescale use in the vine downy mildew market. It is reasonable to assume, therefore, that the sensitivity distribution shown is a truly 'natural' unselected distribution.

The baseline was constructed using an *in-vitro* assay based on the inhibitory effect of famoxadone on the release of *P. viticola* zoospores from sporangia using nine fungicide concentrations ranging from 0.001 to 3mg/l. Samples were obtained from France (47), Italy (31), Portugal (19), Spain (3) and Germany (3). After testing, an EC_{50} value was calculated for each isolate by probit analysis and the values grouped into the categories shown. Statistical analysis showed there was no difference in sensitivity profiles between countries and the data were thus combined to give an overall baseline. Reliability of the test procedure was checked and confirmed by including a reference isolate in 28 consecutive tests.

This is an example of a baseline that would allow departures from the expected sensitive response pattern to be easily identified. Full details are given by Genet and Vincent (1999).

Example 2: Cumulative frequency distribution: Anilinopyrimidine and Botrytis cinerea.

Figure 7 shows a baseline constructed as a cumulative frequency curve for the sensitivity of *Botrytis cinerea* to the anilinopyrimidine fungicide pyrimethanil. At the time of construction the fungal populations had not been exposed to anilinopyrimidine fungicides and it was reasonable to expect that the sensitivity profile was representative of natural variation. The samples came from all the major vine growing areas of France. Due to the large sample size (>600) it was possible to establish that there were no regional differences so the data were combined to give an 'all France' baseline. Data were generated using a microtitre plate method that measured the germination and subsequent growth of fungal spores in a defined medium over a range of several concentrations. From these data, the IG_{50} values for individual isolates were calculated (Birchmore *et al*. 1996).

Note the dose rates are plotted on a log scale. The distribution shows that there is an initial low proportion of the population that is controlled at very low concentrations of pyrimethanil, with the proportion controlled then rising rapidly as the

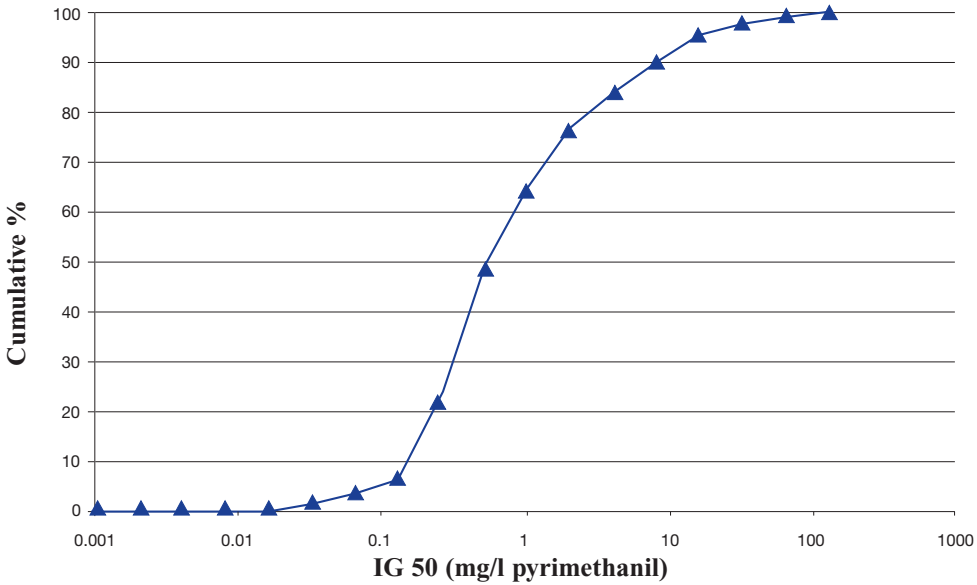


Figure 7. Cumulative frequency baseline distribution for pyrimethanil and *Botrytis cinerea*. Derived from French vineyards, 1995

concentration of fungicide rises. There is then a small proportion of the population that is not controlled until the fungicide concentration reaches higher values, but no isolate survives a concentration of 100ppm.

Old chemistry, new use, no prior knowledge

Example 3: Propamocarb and Phytophthora infestans

Propamocarb had been used for many years in the horticultural market for control of soil-borne *Pythium* and *Phytophthora* spp and some foliar downy mildews with no problems of resistance before being introduced for control of *Phytophthora infestans* on potato. Because of this history, it was concluded that the risk of resistance developing was low, especially as it was introduced in mixtures with established multisite, low risk molecules. However, P. *infestans* is a high risk pathogen and the possibility of resistance developing could not be ignored. A baseline was established (Figure 8) along with a sensitivity monitoring campaign as part of the product stewardship programme. The method used (Bardsley *et al*. 1996,

1998) was virtually identical to the standard floating leaf disc method developed for phenylamides (FRAC 1992). The criterion assessed was the development of sporulating lesions on potato leaf discs, and the median sporulation score was calculated for each set of leaf discs using the following score system:

Score	Criteria
0	Sporangiophores absent
1	1-4 Sporangiophores per disk
2	5-12 Sporangiophores per disk
3	Moderate sporulation; only visible under binocular microscope
4	Profuse sporulation visible with naked eye

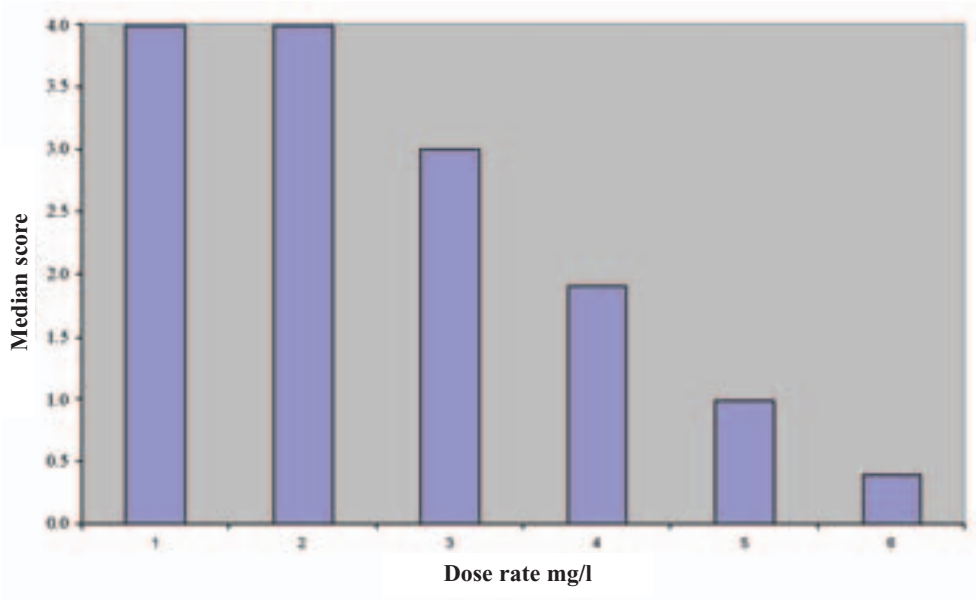


Figure 8. Sensitivity distribution for *P. infestans* and propamocarb (1994)

The data, presented here in histogram form, illustrate the sensitivity profile and can easily be converted to a regression line if a mathematical analysis is required.

New chemistry, established mode of action, some prior selection.

Example 4: Spiroxamine and cereal powdery mildews

Spiroxamine is an SBI fungicide with an identical mode of action to the morpholines (tridemorph, fenpropimorph) and fenpropidin. These latter fungicides had been used on cereals for control of *Erysiphe graminis* for many years and although no resistance had been found leading to field failures there were signs of some small shifts in population sensitivities (Felsenstein 1994; Felsenstein *et al*., 1994). Spiroxamine was thus being introduced into a situation where some prior selection had most likely occurred. In this situation it was necessary to construct a baseline for spiroxamine in order to monitor its future performance within the overall chemical group. A full account of the work and methods used is presented in Felsenstein & Kuck (1998).

The analytical method chosen was to establish population EC_{50} values for various European populations and, by using a standard reference isolate, calculate the 'Resistance Factor, RF' as the ratio of the tested population EC_{50} to the standard reference isolate EC_{50}. By using this relatively simple calculation it was possible to detect possible shifts in the population sensitivity over time.

The results obtained are shown in Figure 9.

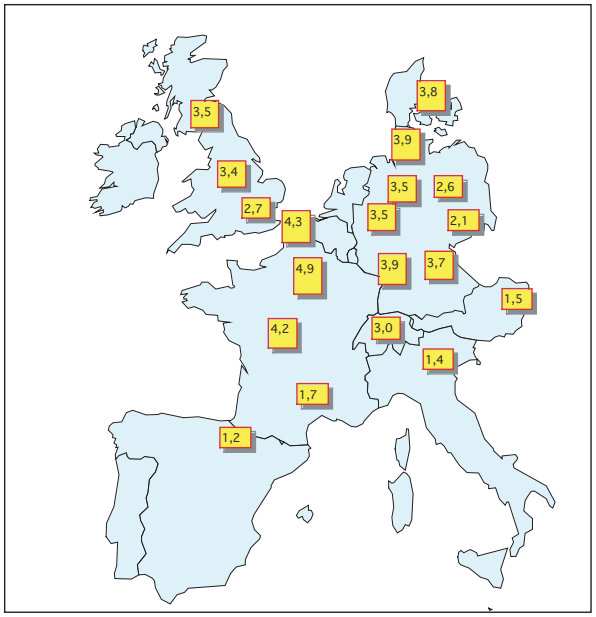


Figure 9. A baseline for spiroxamine and *E. graminis* on wheat. Values shown are Resistance Factors (RF) calculated as: EC_{50} test population/EC_{50} value for standard isolate.

The data in Figure 9 show that there was a clear difference in sensitivity of mildew populations throughout Europe, with the most sensitive populations being in the south.

BRIDGING DATA FROM OTHER CROPS AND LOCATIONS

In some instances it may not be economically viable to construct a baseline for a specific use. The use of efficacy data as a substitute could be considered an option but there are cases where this is not desirable, particularly with new chemistry and very important pathogens. In such circumstances it is possible to use 'bridging data' to allow a previously established baseline to be used in a new situation. The following examples will illustrate the principles:

Extending a baseline to a new geographical area
For a major pathogen it is logical for the baseline to be constructed for areas where product use is likely to be intensive. As an example, for cereal fungicides the major markets could be centred on France, Germany and the UK and it is reasonable for a baseline to be established either jointly or individually for these countries. But cereal diseases also appear in other European countries and it may be desirable to have a baseline applicable for that extra country. Is it possible to use the established baseline for the extra country? The answer is most likely 'yes', but needs to be checked by gathering *bridging data*.

The process is straightforward. To confirm that the established distribution is applicable to the new country it is necessary to test a number of isolates from the new country and show that they fall within the bounds of the established baseline. Once this is established it is possible to use the established baseline with confidence for the new country.

In practice, other items of information can be used to support the conclusions. If the established baseline has been constructed for a number of countries, and if it is shown that individual country distributions do not differ from each other, then it is reasonable to conclude that there is a common population sensitivity within the broad geographical area covered. Such a situation is shown in Figure 10 for sensitivity distributions of *Gaeumannomyces graminis* f.sp. *tritici*, the cause of take-all of wheat, to fluquinconazole. The data clearly show that the sensitivity

distributions are common for the three countries. This gives added confidence to the use of a combined *G. graminis* f.sp. *tritici* / fluquinconazole baseline for Europe without the need for extensive testing in other countries.

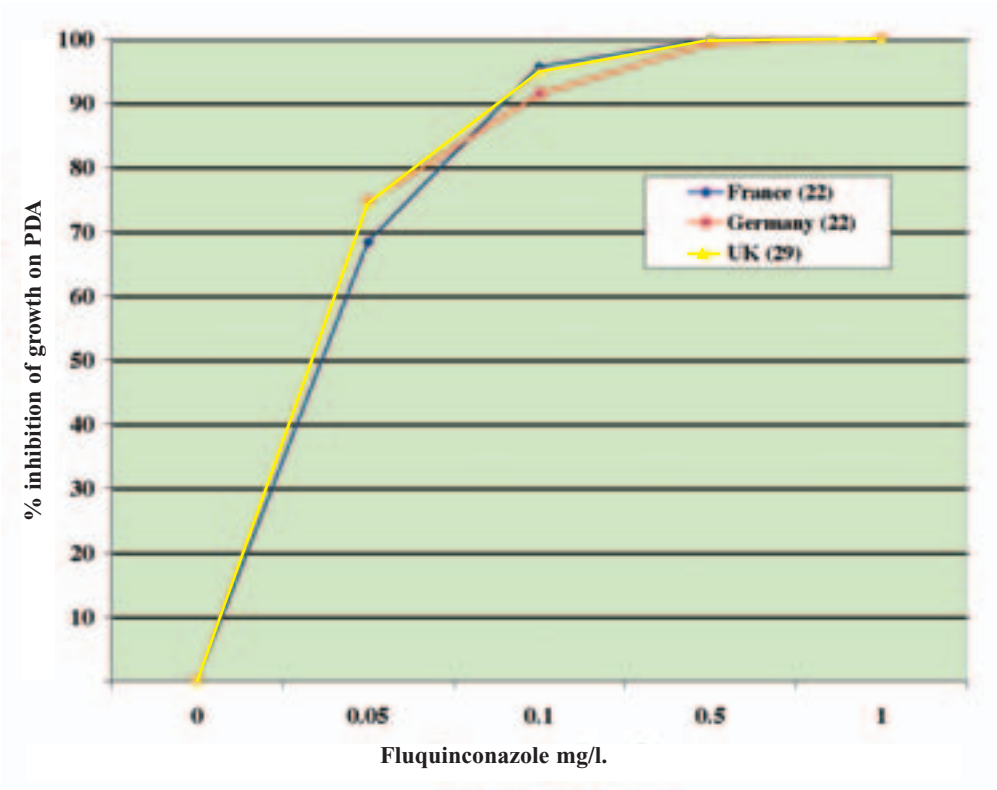


Figure 10. Sensitivity distributions for *Gaeumannomyces graminis tritici* to fluquinconazole in Germany, France and the UK. Numbers in parenthesis are the numbers of isolates sampled. (Data from Russell *et al.* 2002)

Care must, however, be taken in using this approach. In the example above there were no major differences between isolates within the individual countries. Had such differences been found this could have indicated local population differences, which, if large enough would create problems in data interpretation during subsequent monitoring operations.

Extending a baseline to a different crop
This is only possible when the same pathogen species is responsible for causing disease on all the crops concerned.

Such a situation can be illustrated by reference to *B. cinerea*. The major market for control of this pathogen is on vines for control of grey mould, particularly in France. It is thus reasonable to expect that a sensitivity baseline be established for this use in France, possibly extending the baseline by bridging data to include other countries.

But *B. cinerea* also causes grey mould on a wide variety of other crops e.g. strawberries, peppers, tomatoes, cane fruit. The economic justification for producing crop specific baselines for such crops is questionable but it is possible to use the same 'bridging data' approach as used for new geographical areas. Samples should be taken from the new crop and the sensitivity profile of a range of isolates compared with the established baseline from the major crop. Providing the isolates fit into the established baseline it should be possible to use the established baseline for the new crop with confidence.

Such an approach was taken when establishing baselines for the sensitivity of *B. cinerea* to pyrimethanil for vines and tomatoes. (Birchmore *et al*. 1996). An extensive baseline was established for vines from France and Switzerland, with no country differences being found. Isolates from Spanish tomatoes fitted well into the vine distribution, although as a group they showed less variation than the vine isolates and presented a different median response. Overall, however, and since the data from tomatoes were contained completely within the vine distribution data, it was concluded that the range of sensitivity responses found from vines could be taken as a good indication of the population variation likely to be present on tomatoes. Similar (unpublished) approaches were taken to establish that the vine baseline was also applicable to strawberries.

MOLECULAR TESTING PROCEDURES

Great advances are being made in the development of molecular analysis procedures to identify and monitor the development of resistance to plant pathogens. In theory, once a molecular marker is available to identify resistance in the pathogen it should be a simple matter to use the technology to search for resistance in target populations and to assess the effect of various management strategies on its development. Issues of sample size, sample distribution and other aspects covered earlier, remain unchanged.

The main problem with the use of such technology is that, by definition, resistance must be identified before suitable test procedures can be developed. In situations where the molecular basis of resistance is not known it would thus be impossible to use molecular methods to establish a baseline.

Maybe the most widely publicised example of the use of molecular techniques in establishing a baseline and subsequently monitoring the development of resistance is given by the QoI group of fungicides, including the strobilurin derivatives azoxystrobin, kresoxim-methyl, trifloxystrobin, pyraclostrobin, picoxystrobin, fluoxastrobin, the oxazolidinedione famoxadone and the imidazolinone, fenamidone.

These fungicides act by inhibiting fungal respiration in complex III (cytochrome bc1 complex) by binding at the ubiquinone oxidising (outer side) of the mitochondrial membrane, (Lyr 1995; Sauter *et al*. 1999).The molecular basis for resistance to this mode of action was first identified by Di Rago *et al*. in 1989 and eleven possible point mutations that can cause resistance have been described. For most plant pathogens it is now accepted that resistance can be due to a change in the cytochrome b gene, in particular a mutation at position 143 confers resistance. The mutation changes glycine to alanine, hence the nomenclature G143A, (Sierotzki *et al*. 2000 a,b; Heaney *et al*. 2000; Gisi *et al*. 2000). This marker has been used by most companies and public sector research laboratories to monitor resistance of several fungal populations.

When using such technology, the concept of a 'sensitivity baseline' largely disappears. It is replaced by a frequency distribution of the mutant gene in the fungal populations. In itself this can act as a reference point and providing future monitoring is conducted using identical techniques, it should be possible to identify increases in mutant (resistant) gene frequencies as a result of fungicide use. Unfortunately, the use of such techniques is not as simple as may first appear. Research conducted by the member companies of the Fungicide Resistance Action Committee (FRAC) has shown that it is possible to detect G143A mutations in fungal populations never exposed to QoI fungicides. It would thus appear that the mutation is naturally occurring in fungal populations, albeit at low frequencies. It can also be found, again at low, but variable, frequencies, in situations where the use of QoI based products is giving perfectly acceptable disease control.

The current problem is that it is not always possible to correlate the frequency of detection of the mutation with the likely decrease in field performance of the fungicide. Two aspects are of particular concern. The G143A mutation occurs in a mitochondrial gene. At the level of the individual fungal isolate, it is not yet certain

what frequency of mutant genes in a particular fungal cell are required to render that cell resistant to the fungicide. It is then also unclear what proportion of 'resistant' isolates are needed in a field population to lead to a failure of disease control. In such situations monitoring by molecular techniques must be supported by careful observation of field performance and standard *in-vivo/in-vitro* resistance assessments in order that correct conclusions can be reached. Neither must it be assumed that the G143A mutation is the only one operating. Resistance of *V. inaequalis* to QoI fungicides is not always dependent upon this mutation (Steinfeld *et al*. 2001) and more recently a second mutation, F129L, has been identified in *Magnaporthe grisea* and *Pythium aphanidermatum* (Gisi *et al*. 2002) as well as in *P. viticola* on grapevine and *Alternaria solani* on potatoes (FRAC). More research is urgently needed into these phenomena.

DETECTING SHIFTS FROM THE BASELINE

The baseline is to be used as a reference point to assess whether or not resistance is developing during commercial use of the at risk molecule. This is achieved by sampling from areas of product use and conducting sensitivity assays using the same protocols as used to establish the baseline. Concern that resistance could be developing will arise when the sensitivity distribution pattern observed in commercial practice deviates from that shown in the baseline.

Assuming that resistance is not detected as part of the baseline distribution, the emphasis will be on looking for changes to the shape, positioning or segmentation of the sensitivity profiles obtained from populations of fungal isolates exposed to the fungicide compared to the baseline. In conducting such studies, several factors must be taken into account:

- Sample sizes must be large enough to allow reasonable conclusions to be reached. The probability of detecting resistance is related to the sample size (Appendix 2)
- The sample area must be clearly defined; it could be a field, orchard, vineyard or a geographical region or country.

Whatever the origin of the test samples, several rules must be observed when conducting such studies:

- The baseline being used must be appropriate in terms of crop, pathogen and geographical region.
- The methods used must be identical to those used to establish the baseline.
- Collection, transport, storage, purification and inoculum preparation procedures must match those used for the baseline.
- Full details of the sample history such as treatment regime this season and previous seasons, and cropping history of the site must be obtained.
- Data on the performance of the fungicide in controlling the pathogen are required so that efficacy can be compared with sensitivity.

Once these criteria have been met it is possible to proceed with the monitoring assay and check to see if there has been a shift in sensitivity.

Care in data interpretation
Extreme care must be taken in reaching any conclusions as it is too easy to conclude that shifts have occurred when they have not. The following two examples illustrate this:

Example data from a monitoring programme for quinoxyfen and *E. graminis* are shown in Figure 11. Original baseline data were published by Hollomon *et al.* (1997). Spores were collected using a car mounted jet spore trap from various regions in Europe in each year and tested for sensitivity using wheat leaves sprayed with a range of concentrations of quinoxyfen. The EC_{50} of each isolate was calculated using probit analysis. To validate comparisons between years, standard reference isolates representing an untreated population from the early 1970's were included. The data show that differences did occur between years, with 1998 and 1999 showing an apparent increase in sensitivity as EC_{50} values decreased. However, the differences also occurred in the reference isolates, and so can be attributed to experimental variation. It is clear that there was no departure from sensitivity patterns seen in comparison to the reference isolates. Without a careful consideration of all data and inclusion of reference isolates, it would have been difficult to understand what was happening (Bernhard *et al.* 2002).

A similar situation is illustrated in Figure 12 for pyrimethanil and *B. cinerea* from French vineyards.

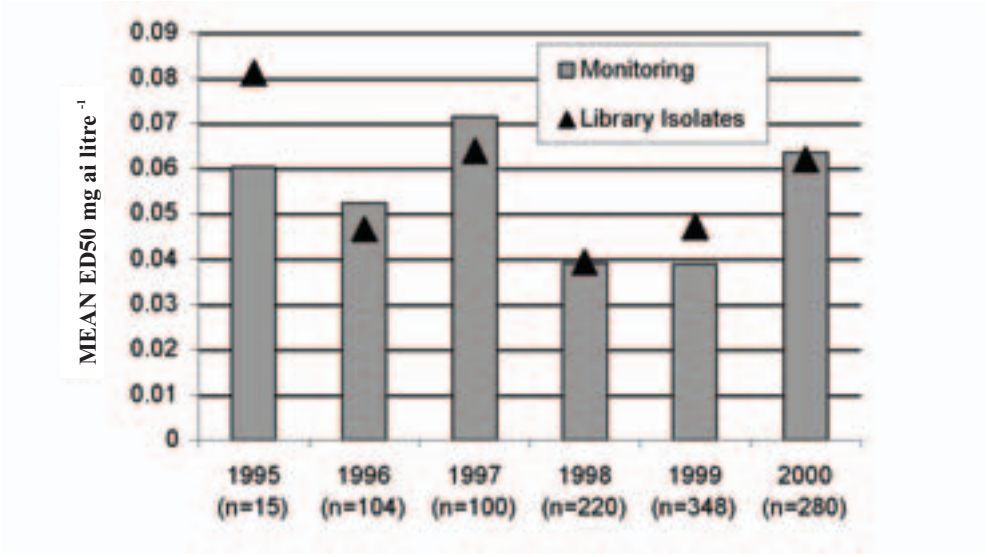


Figure 11. Monitoring data for quinoxyfen and *E. graminis* 1995 - 2000.

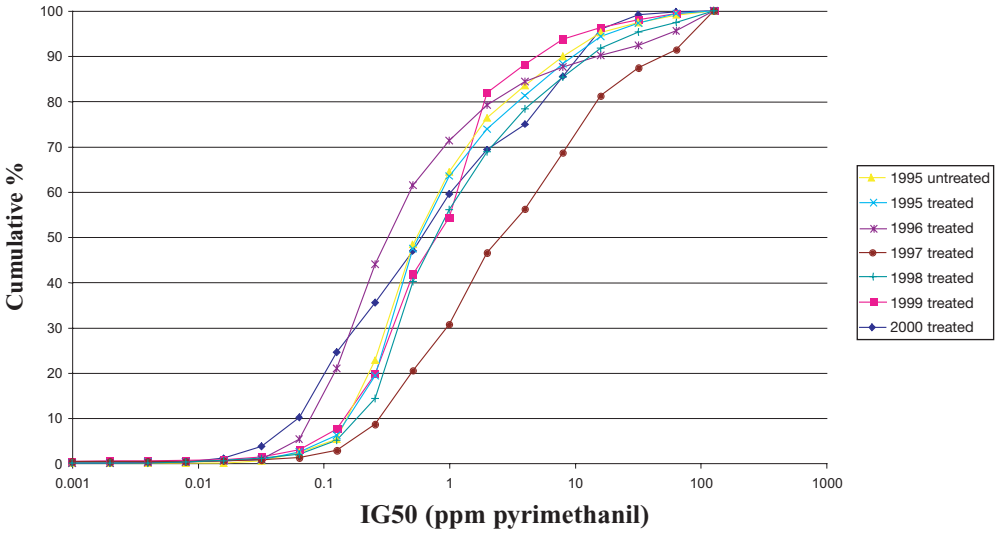


Figure 12. Monitoring data for pyrimethanil and *B. cinerea* in French vineyards 1995 - 2000.

The data presented in Figure 12 show several very important points:

- In 1995, the year when the baseline was established, a set of plots were treated using the agreed resistance management strategy. The data show clearly that the sensitivity profile was identical between the two data sets i.e. no resistance developed as a result of the management strategy.
- In 1996 the sensitivity profile appeared to move to the left, a possible indication of a move to greater sensitivity. However, while such a phenomenon is not impossible, its association with fungicide treatment is illogical. It could, therefore, either be an illustration of variability in testing procedures from one year to the next or an illustration of true sensitivity changes in the population from year to year with no influence of chemical treatment.
- In 1997 the sensitivity profile showed an apparent decrease in sensitivity as the line moved to the right. Note, however, that the maximum IG_{50} value had not changed. This could have been interpreted as a move towards decreased sensitivity, but bearing in mind the variability seen in 1996 it was not considered likely and was thought due to natural variability in the *B. cinerea* population. This was supported by observations on the efficacy of the product in the field; no problems having been seen.
- The conclusions from 1997 were supported by subsequent monitoring data for 1998, 1999 and 2000. All these distributions showed responses similar to the 1995 baseline although there was some variation around it. There was thus no reason to suspect a shift in sensitivity.

The important conclusion from these investigations is that it can be dangerous to jump to conclusions from a single year's comparison.

An example of a shift from the baseline.
A confirmed example of a shift from baseline sensitivity is shown in Figure 13 for *Venturia inaequalis* and the QoI kresoxim-methyl. The original baseline from 1996 was a typical log normal distribution, but the move to a much reduced sensitivity for some isolates from Germany by 1999 is clear.

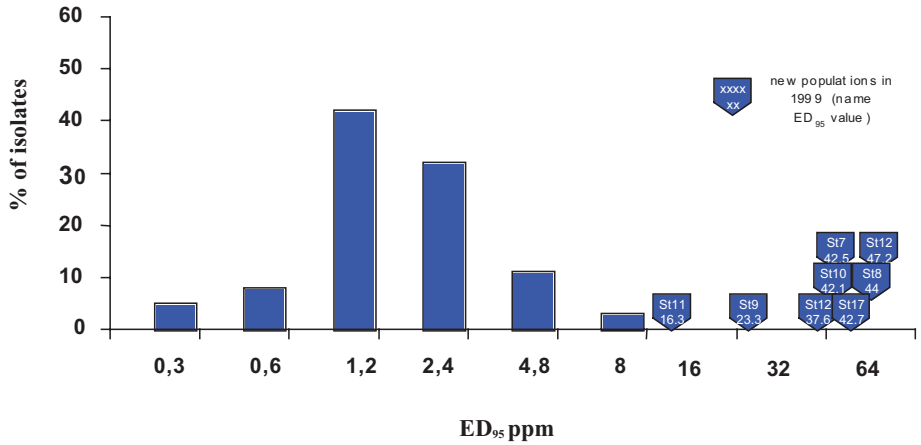


Figure 13. Data illustrating a shift from the 1996 baseline for kresoxim methyl and *Venturia inaequalis* in 1999 (*in vivo* test, n = 38).

USING DISCRIMINATORY DOSES

The establishment of baselines by the use of bioassay procedures described in this monograph has involved the use of a range of doses and the assessment of the fungal isolates to each dose. The sensitivity of the isolates can thus be illustrated in a continuous distribution. Although not impossible, it is unlikely that the baseline will encompass isolates that are 'resistant' and capable of causing a control failure in the field. However, when after sales monitoring is conducted after intensive use of the product and when shifts away from the baseline can be detected such that some isolates are declared resistant, it may be possible to simplify the monitoring procedure and use a *discriminatory dose*.

A discriminatory dose is a single dose rate at which, depending upon the reaction of the fungal isolate, it is possible to declare the isolate sensitive or resistant, although it is possible in some circumstances to split the 'sensitive' category down into further sub-divisions e.g. sensitive, less sensitive. The dose rate used and the reaction criterion for the test must be carefully defined and proven by extensive

research to accurately predict the field situation. The test procedure used may be identical to that used to establish the baseline or maybe modified. It must, however, remain unchanged throughout the monitoring exercise unless adequate safeguards are put in place to validate a change of procedure.

A common procedure is to select a single dose rate based on the responses of isolates from known field resistant situations and to measure the response of test isolates at this dose rate. *In-vitro* agar plate tests are popular for this procedure, with the growth of the test isolate being recorded as a % of its growth in the absence of the test fungicide. A resistant isolate is often defined as one with growth of 50% or more in the presence of fungicide.

Responses obtained can depend upon the nature of the resistance being investigated. In situations where the resistance is disruptive (Figure 14) and the fungal population can be split into two discrete distributions, one sensitive and the other resistant the discriminatory dose can be placed so as to obtain a clear 'plus' or 'minus' response. Biasing the dose rate towards the truly resistant part of the distribution will eliminate questionable responses of sensitivity.

Such situations are mostly associated with resistance mechanisms under single gene control and, in the absence of other information, could be a useful pointer to resistance being due to a single site mutation.

What could be worrying is if a disruptive pattern picture emerges during the production of a baseline before the product has reached the market. In these circumstances various questions must be asked, the most important of which is whether or not the apparently resistant segment of the population is truly resistant. Suitable assays must be devised to answer this question. It could also happen that the apparently resistant segment is of 'intermediate resistance' or could be classed as 'less sensitive'. Again, this would need to be established.

If a truly resistant portion of the population is found in the baseline setting process this does not necessarily mean that resistance will develop rapidly in practice, but it does indicate a risk that must be taken into account when establishing resistance management strategies.

If the resistance pattern is not disruptive but continuous (Figure 15), a clear demarcation between sensitive and resistant categories may be more difficult to show because individuals in the population are likely to show a much wider range of

responses. This shows as a difficulty in interpreting and classifying the responses of isolates that show growth in the 40 – 49% range because such isolates are not fully sensitive but, according to the set criteria, are not resistant. To ignore their response and record all as 'sensitive' could be seen as ignoring valuable information because they could represent an indication of a movement in the whole population towards a more resistant state. For this reason it can be valuable to split the 'sensitive' range down into further subdivisions. The subdivisions then become a further pseudo- baseline and valuable information can be obtained by monitoring the evolution of this distribution over time.

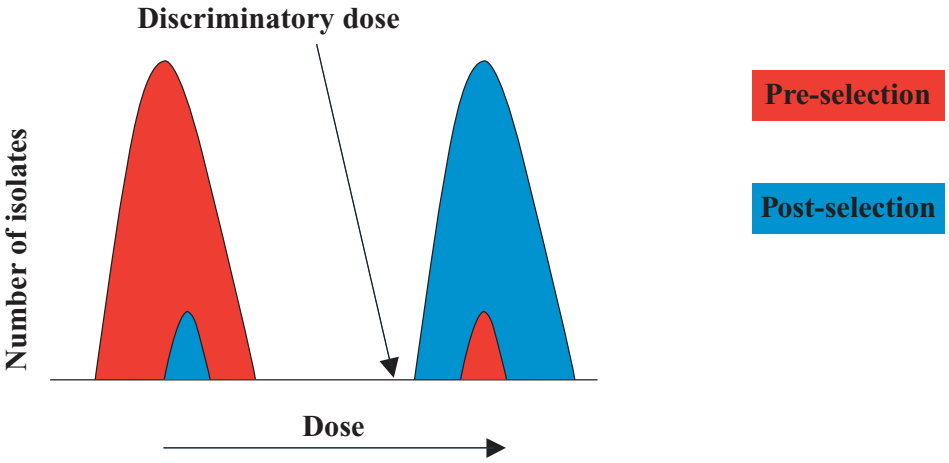


Figure 14. Illustration of disruptive selection. Pre-selection the majority of the fungal population is sensitive with a minority resistant. Post-selection the majority is resistant. There is a clear demarcation between sensitive and resistant populations.

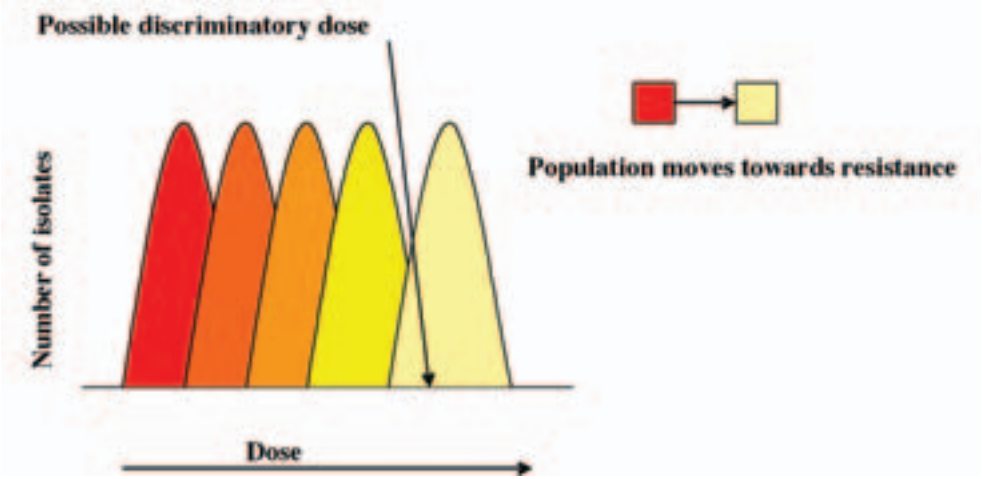


Figure 15. With non disruptive resistance the population sensitivity tends to move gradually with no single isolate being completely resistant in the initial stages of selection. Such patterns are often associated with resistance mechanisms controlled by several genes.

Such a procedure was established for prochloraz and eyespot (caused by *Tapesia* spp.) on wheat. The discriminatory dose selected was 0.5ppm prochloraz and the criterion for resistance established as growth of 50% or more at this rate compared to growth on unamended agar (PDA). This discriminatory dose was used since resistance was recorded in the north of France in 1991. Data obtained using this dose over a number of years are shown in Figure 16

The data show that in 1991 resistance was not extensive but increased in frequency over the years until by 1998 a large proportion of the population was considered resistant and the truly sensitive isolates (those showing minimal growth at 0.5ppm prochloraz) had become very low. The technique has been used subsequently to show that overall resistance in the eyespot population in France has decreased (Gaujard & Russell 2002).

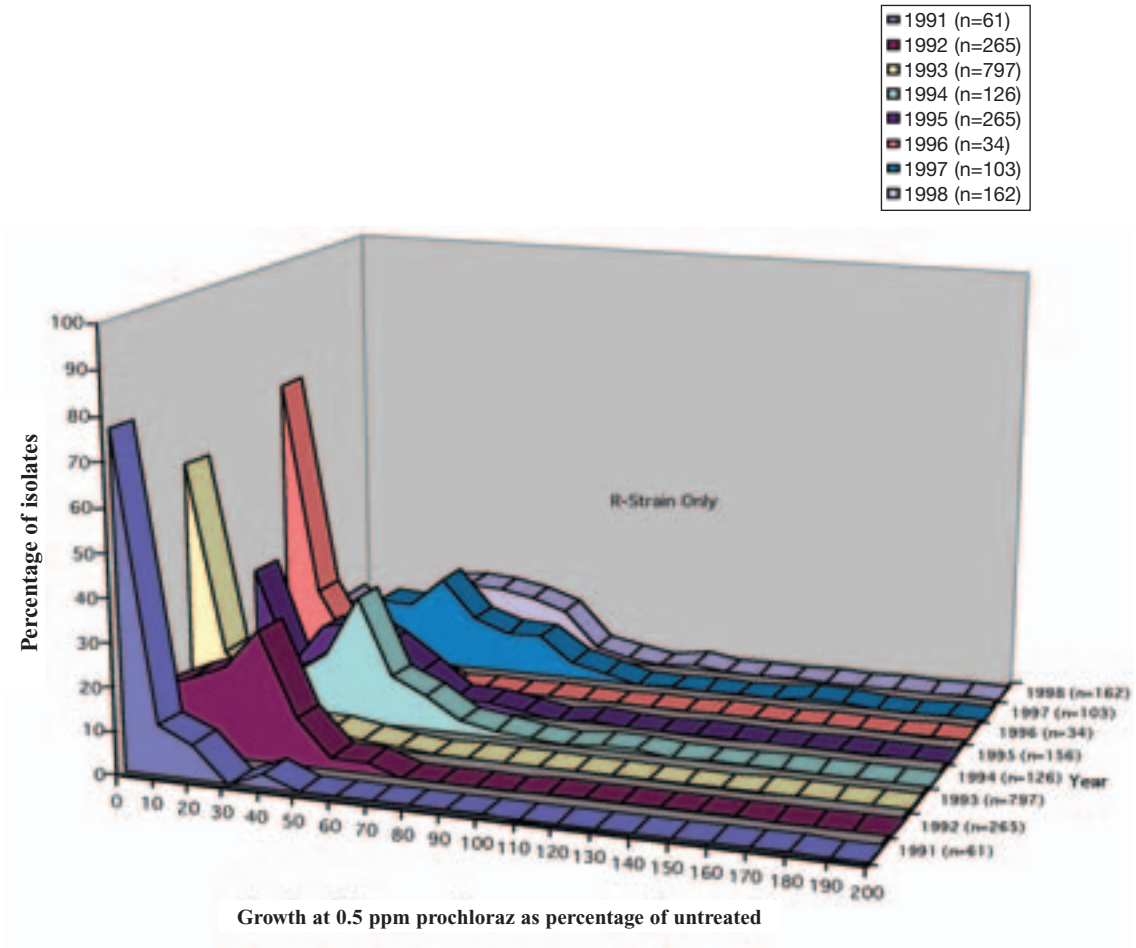


Figure 16. Data obtained using the discriminatory dose rate of 0.5ppm prochloraz for sensitivity of wheat eyespot *(Tapesia acuformis)*. Isolates showing growth of 50% or above are considered resistant (Schering/AgrEvo/Aventis data).

BASELINES AND REGULATORY PROCEDURES IN EUROPE

Up until the early 1990's there was no requirement for a product to be marketed and used in accordance with an approved resistance management strategy. Resistance management and the strategies employed to maintain product efficacy were voluntary, greatly aided by the work of the Resistance Action Committees (RACs) of the Global Crop Protection Federation (now Crop Life International). It was pleasing to see that because of the problems that resistance could cause, the vast majority of producers marketed their products within the resistance management guidelines issued by the RACs and that most users abided by the advice given and so reaped the benefits.

This position changed with the introduction and implementation of EC Directive 91/414/EEC (Anon 1991) and subsequently Commission Directive 93/717/EEC (Anon 1994). There was now a requirement, as part of the registration process for new active ingredients and the products containing them, and the re-registration of established products, for information on the actual or possible occurrence of resistance to be provided together with details of a management strategy to avoid resistance or to manage it if it was already present.

This was welcomed by the industry but presented some problems. The published directives gave no guidance to the registration applicant on what information was required to support the application and similarly gave no guidance to the regulators on what information to expect, nor on how to interpret it when provided. This situation led to the European and Mediterranean Plant Protection Organisation (EPPO) being asked to produce a Guideline for use by applicants and regulators in order to help them construct the dossier and interpret it respectively. A review of the process by which this Guideline (OEPP/EPPO 1999) was produced is given by McNamara and Smith (2000). The Guideline has now been revised and extended as a result of feedback on the original (OEPP/EPPO, 2002). As the provision of base-line sensitivity data is a significant part of the process, a summary of the regulatory requirements are presented here.

The data requirements and risk analysis process

The Guideline sets out a suggested way of approaching the problem of assessing the risk of resistance developing and of then developing strategies to prevent, limit or manage its development. The Guideline is not a 'statutory requirement' but it is

realistically expected that most applicants will follow the suggested formats and that regulators will follow the advice contained within it.

Maybe the most important factor to realise is that the onus is on the applicant to produce the analysis and to justify the conclusions and proposed resistance management strategies. The process requires the applicant to consider several aspects that affect the risk of using the product on a particular crop to control a particular pest under particular conditions. If the risk factors without any resistance management are considered too high, then a management strategy has to be proposed and justified. Full details of the steps involved are contained in the Guideline but entail an evaluation of:

Inherent risk
This is a risk factor that is generally beyond our control and applies to both the pest to be controlled and to the at risk active ingredient. Many pathogens e.g. cereal powdery mildews, potato late blight, grey mould *(B. cinerea)* are well known to be able to develop resistance quickly. Introduction of a new active for their control would automatically generate concern. For new actives in all disciplines, but especially plant disease control, history has shown us that we should expect a moderate to high risk of development of resistance in the absence of management strategies. As very little actual risk data may be available at the time of registration of a new active from new chemistry, it would be wise to regard all new chemistry as showing a potential risk of resistance development. These risk factors need to be considered and explained in the registration dossier, drawing on historical experience where applicable.

Agronomic risk
The risk produced by the combination of the pest and product risks analysed as the 'inherent risk' can be increased by certain conditions of use and leads to a consideration of the agronomic risk when no resistance management strategies are employed. Such factors include monocropping, short rotations, use of susceptible cultivars etc. The full list is given in the revised Risk Analysis guideline, OEPP/EPPO (2002).

Modifiers
Modifiers can be considered to be any means by which the unacceptable risk of unrestricted use of the product is reduced to an acceptable level. They include:

- Reducing the number of applications of the product
- Selling the product as a coformulated mixture with a non cross resistant partner, or recommending application in tank mix with a non cross resistant partner

- Recommending specific application programmes including non cross resistant partners. These take several forms:
  - recommending alternations with other non cross resistant products
  - recommending restrictions on the number of sequential applications (blocks) with our new product
- Recommending specific spray application timings to avoid excessive selection pressure for resistance
- Any combination of the above

Of course, different modifiers could be used in different environments for the same pest-crop system, and it is not expected that the same modifier strategy will apply to all crops that the product is to be used on.

The applicant is expected to produce a management strategy based on stated modifiers and explain why it will work.

Supportive data
Baseline studies: The applicant is expected to provide evidence of having the baseline reference data together with a method of assaying possible resistance should it be suspected.

Biochemical mode of action, Mode of resistance and Cross resistance. These parameters can be very useful in assessing the risk of resistance developing. Unfortunately they are rarely known for a new active from new chemistry at the time of a registration application. For this reason, the Guideline requests that evidence on these parameters should be provided where known, but that if not known, information should be provided on the tests completed. Similarly, evidence of cross resistance, or lack of it, to known molecules with resistance problems should be provided.

Evidence to support the strategy. Wherever possible, the applicant should provide data to illustrate that the proposed management strategy including the various modifiers will not select for resistance and allow it to dominate the population to be controlled. Of course, this can be difficult to do given the timescales of a new product development programme before a registration submission, but it is considered that in most cases some data can be gathered. This will include application of the proposed strategy, monitoring of the response of the pest population to the application, and comparison with the baseline.

Strategy implementation and monitoring

The applicant is expected to provide an example product label which should explain to the user how to use the product in order to manage resistance. The applicant should also inform the regulator how the proposed strategy will be promoted in the marketplace, and what steps will be introduced to allow the success of the strategy to be monitored.

APPENDIX 1

DETERMINING AN ASSAY METHOD, ASSESSMENT PROCEDURES AND PARAMETERS

Once a decision has been made to establish a baseline using bioassay procedures, a reliable technique must be developed for creating the baseline. The technique must also be able to be used in subsequent monitoring operations investigating possible development of resistance. If and when resistance develops, the same method may be used to identify resistant isolates but it is possible that more direct methods will have been developed at that time.

If the molecule is from known chemistry it is quite possible that research has already established suitable methods. If the molecule is covered by a FRAC Working Group then suitable techniques may have been published by FRAC. If, however, none of these situations apply then new techniques, either *in-vitro* or *in-vivo* must be developed.

The techniques must:
- be robust, reliable and repeatable
- be as simple as possible to operate in terms of technology and user skills
- be as cheap to operate as possible and capable of a high throughput in a short time
- be able to be related to sensitivity responses in the field.

In vitro testing

In vitro testing can be performed for both non obligate and obligate pathogens, although for the latter the methods are generally restricted to forms of assessment involving spore germination. Exceptions could happen where normally accepted obligate pathogens are able to be cultured on a special growth medium e.g. *Phytophthora infestans*.

Non obligate pathogens
If the pathogen can be cultured easily on artificial agar or other media it is reasonable for research to begin based on establishing dose response procedures on this medium. Most techniques will involve use of a ‘solid’ agar medium as it is easy

to observe fungal growth parameters on the surface, but techniques using liquid media are possible. However, not all non obligate fungi or fungicides are suitable for these techniques. Fungi such as *V. inaequalis* can give problems because of their slow growth while care must also be taken to ensure that the physicochemical properties of the fungicide are suitable to obtain the required fungicide concentrations in the test medium. Where fungicides are incorporated into agar or other media and the preparation has to be sterilised, care must be taken to ensure that the sterilisation procedure does not affect the fungicide. The fungicide may be added before autoclaving if this is possible, otherwise it must be added just before test initiation using appropriate sterile techniques. Agar media containing fungicide can be prepared in bulk batches and stored before use to pour test Petri dishes providing checks have been carried out to ensure that the fungicide does not degrade in storage. When using a batch ex-store it must be mixed efficiently to ensure dispersion of the fungicide through the medium. Producing a batch of Petri dishes containing test media and storing them for more than seven days before use is not recommended because it is possible for the fungicide to migrate through the agar and so lead to false concentrations on the surface layers. Such a phenomenon was found for prochloraz and potato dextrose agar (not published).

Various techniques are possible including mycelial growth inhibition, spore germination assays, and germ tube elongation assays. The artificial medium may be a complete medium, for example potato dextrose agar or malt agar, or be a nutrient deficient medium such as water agar. The choice will depend on the biological and physico-chemical properties of the fungicide coupled with available information on its physiological and/or biochemical mode of action. For instance, a spore germination assay will not be applicable for a fungicide that does not act on this part of the fungal life cycle, a nutrient rich medium may not be the best choice for a pathogen that acts by enzyme inhibition if the nutrients provided in the medium circumvent the mode of action of the fungicide (for example pyrimethanil, Birchmore *et al*., 1995). Specific conditions used in the test procedures can only be determined by experimentation, with a prime concern being repeatability. At this point it may also be necessary to decide whether the baseline response is to be established using single spore isolates or with a population of spores or mass mycelial isolates. Each technique has its supporters and sound scientific arguments can be made for each method. It is possible that practical considerations will lead to technique selection.

Single spore isolates: The important factor to consider when beginning single spore work is that the fungus will need to be isolated from plant tissue and techniques

established to obtain single spores for testing. Consideration must also be given to the genetic variability known to be present in the fungus and its population structure in the field. Testing many individual spores from the same lesion may not be representative of the field population while testing a single spore from single lesions each of which comes from a different location could potentially miss low level variability in different populations.

Populations of spores and mass mycelial isolates: These procedures arise when individual lesions are regarded as the sampling unit. They benefit from being simple to operate and generally require a minimum of sub-culturing after isolation from the plant before the fungus is ready for testing. However, the test sample will most likely contain much variability as it may comprise more than one true isolate. This could produce a biased response to the fungicide depending on the individual responses of the isolates present, but use of an adequate number of samples for the baseline is likely to minimise this problem. The use of such techniques in later monitoring exercises will mean that care must be taken in interpreting data from the surveys because the response of the sample will be determined by the response of the least sensitive individual present. When survey data are compared over seasons this is not of any consequence but using such data to estimate the proportion of resistant individuals in a population in a single season will undoubtedly provide a biased (higher) estimate.
Note that the sensitivity values obtained in agar plate tests can be influenced by the test medium. An EC_{50} value determined on one agar medium may not be repeated if the same test is run on another medium.

Assessment procedures: Assessment procedures need to be established. For assays based on spores, decisions will need to be made on what will constitute a germinated spore and how to assess germ tube growth inhibition. An often used criterion is that a spore is considered to have germinated when the germ tube has grown to twice the diameter of the spore, or twice the width whichever is applicable. Where germ tube growth is being assessed, the tester must decide on the parameters; assessing at a set time interval will be important and measurements could be true linear measurements of germ tubes or estimations of growth percentage compared to untreated spores.

If spores are allowed to develop the assay could be based on the resultant mycelium, in which case data could be based on radial growth assays. For mass mycelial isolates, the normal method will involve assessing the % inhibition of mycelial growth compared to the fungus growing in the absence of the test

fungicide. Growth measurements are normally taken on two colony diameters at right angles to each other and the mean colony radius calculated. Care must be taken to ensure that appropriate measurements are taken. If the inoculum is a mycelial plug from an untreated plate, it should be placed mycelium surface down to ensure contact with the test medium. Examination of the plug before taking any measurements must be done to ensure that the fungal growth being recorded is actually growing on the test medium and not on the top of the 'inverted' plug:

Growth on test agar gives accurate measurement

Growth on top of agar plug gives false measurement

The conditions for testing and the duration of tests must be standardised in order to ensure repeatability. Care must be taken to ensure that techniques are robust. As an example, during the early development work on prochloraz and the subsequent production of baselines for cereal eyespot it was realised that the use of prochloraz as the active ingredient was not appropriate. If glass vessels were used for chemical preparation, the prochloraz was very prone to sticking preferentially to the sides of the glass vessel, leading to considerable and variable errors in concentrations of prochloraz solutions. Using the commercially formulated product rather than active ingredient solved these problems but care then had to be taken to ensure that all subsequent monitoring work was conducted using the same commercial formulation because different formulations were found to produce different agar plate mycelial growth test results. The rank order of isolates in terms of sensitivity IG_{50} values was not changed, but the actual IG_{50} values were.

When beginning to develop a test protocol based on the use of a formulated product it is also necessary to ensure that none of the formulants has an effect on the fungus. Many commercial products contain various wetters, spreaders and preservatives which could affect fungal growth *in-vitro* even if they are regarded as non fungicidal *in-vivo*.

Obligate pathogens

For obligate pathogens *in-vitro* testing is restricted to observations on spores, except in the case of *Phytophthora infestans* where mycelial growth tests are possible.

The majority of such tests are carried out on downy mildew fungi, especially *P. viticola* where the tests can assess fungicide effects on differentiation of sporangia (Genet and Vincent, 1999) or determine the concentration of fungicide needed to restrict zoospore motility. Using such techniques demands a great deal of organisation as procedures must be in place to maintain fungal isolates upon receipt from the field. In the case of *P. viticola* this is normally achieved by transferring the isolates onto young vine seedlings and using the subsequent fresh sporangia for testing, usually conducted in the wells of a microtitre plate. This process has the advantage of 'purifying' the cultures from extraneous contamination that may develop during transport of samples to the testing location as well as providing a little more flexibility in test organisation. At the same time, when the procedures are used during subsequent monitoring operations, the process has been criticised as possibly leading to a loss of any less sensitive isolates during the purification process. Where this is a concern it should be investigated, but it is doubted if the fear is justified.

Decisions need to be made regarding what constitutes a sample; whether it is a single lesion or whether it consists of pooled subsamples from the same leaf, plant or location. Some degree of replication in test procedures should be established and criteria agreed upon for acceptance of a valid test result. Decisions would also have to be taken on whether or not to store individual isolates for future reference, bearing in mind that such practice could become very costly in terms of physical space and resource.

In vivo testing

In vivo procedures would be necessary for those obligate pathogens for which it is impossible to devise a suitable *in-vitro* test procedure. It may also be desirable to use *in-vivo* procedures for non obligate pathogens if the use of *in-vitro* techniques is considered inappropriate.

In general, the use of *in-vivo* techniques is easier for non obligate than for obligate pathogens because the transport, storage and subsequent preparation of samples prior to test may not involve the use of live plants. Samples may be able to be transported and stored as lesions on dried plant material or, following isolation onto artificial media, stored as cultures until required for testing.

For obligate pathogens the situation is more complex as conditions must be available for inoculating plants or plant parts as part of the sample isolation/purification and

storage procedure as well as the test technique. Other considerations include a method for storing samples and isolates if there is a time delay between sampling and testing. It is most unlikely that isolates will be able to be stored on dried plant material so consideration could be given to freezing isolates for storage. If this option is taken, research must be carried out to confirm that the freezing/thawing cycle does not affect the sensitivity profile of the isolate, particularly the possibility of increasing the sensitivity of a previously 'less sensitive isolate'. A continued supply of plant material will also be needed during the test campaign period. This is very relevant if a baseline and subsequent monitoring is to be conducted out of season or established for countries in different hemispheres. It is reasonable to assume that for reasons of cost, resource and physical space it will not be possible to construct a baseline using the same number of samples as used for *in vitro* testing.

Various test methods are available and the choice will be made depending upon the pathogen and the properties of the fungicide. They range from use of detached plant parts, possibly leaf discs floating on a fungicide solution as used for phenylamides and propamocarb for *P. viticola* and *P. infestans* and *P. infestans* respectively (FRAC 1992, Bardsley *et al*. 1996) or leaf segments placed on agar containing a fungicide (FRAC 1991) to tests in which the whole, young, plant may be used, as for strobilurins and *V. inaequalis*. Similar considerations relating to single spore work or mass mycelial isolates apply, although obviously the use of single spore work will involve a greater degree of sample isolation and multiplication than mass mycelial work. Assessments will normally be based on disease control expressed by lesion development with extra observations being made on effects on spore production from the lesions. Test techniques must be standardised, including the use of a particular plant variety and the age of plants used for testing.

Assessment parameters

Baselines can be constructed using parameters such as;

- Dose giving total control of the isolate (Minimum Inhibitory Concentration, MIC). This could apply to both *in vitro* techniques on artificial media or to *in vivo* techniques involving assessment of disease control on test plants.
- The EC_{50} or EC_{90} values, i.e. the dose that reduces the growth or other parameter (of mycelium or spores) to a value of 50% or 90% of that of growth in the absence of fungicide. These values are sometimes referred to as IG_{50} or IG_{90} values (Inhibition of Growth)

MIC values are obtained directly from the dose rate evaluations with due care being taken to conduct tests with adequate replication to reduce experimental error. The EC_{50} or EC_{90} values can be determined from the dose rate response data by statistical approximation (recommended) or by using a graphical plot and estimating the 50% or 90% points from the graph. Whatever method is used care must be taken to ensure that the estimate is reasonable; use of an estimated EC_{50} or EC_{90} value that lies outside the dose range used is certainly not recommended, particularly when dose rates are on a logarithmic scale.

APPENDIX 2

A NOTE ON THE DETECTION OF RESISTANCE AND SAMPLE SIZE

The detection of resistance during a monitoring campaign depends upon:

- The true frequency of resistant isolates in the population
- The sample size evaluated

How these relate can be illustrated by the following simple examples:

Case 1: The *TRUE* frequency of resistance in a population is 1%. Thus the sensitive population is 99%

If we take 10 random individual samples from this population and test them separately, the chance of them all being declared sensitive is given by: $0.99^{10} = 90.43\%$

The probability of declaring 1 of the 10 resistant is: $\frac{10! \times 0.99^{9} \times 0.01}{1!\,(9!)} = 9.1\%$

The probability of declaring 2 of the 10 resistant is: $\frac{10! \times 0.99^{8} \times 0.01^{2}}{2!\,(8!)} = 0.42\%$

Obviously with a sample size of 10 the highest probability is to detect just one resistant isolate, but the chances of doing this are low. If we succeeded, we would also be overestimating the true probability by a factor of 10 (1 in 10 rather than 1 in 100).

Case 2: The *TRUE* frequency of resistance is 1% but we take a random sample of 50 single isolates and test them individually.

In this situation the probability of declaring all 50 isolates sensitive, i.e. not finding any resistance, is: $0.99^{50} = 60.5\%$

The probability of detecting 1 resistant isolate is: $\frac{50! \times 0.99^{49} \times 0.01}{1!\,(49!)} = 30.5\%$

Clearly the chance of detecting resistance increases as the sample size increases, but note that even if 1 isolate out of 50 were declared resistant, the observed frequency would be double the true frequency (i.e. 1 out of 50 = 2% rather than the true 1%).

This process is summarised below:

% PROBABILITY OF DETECTING AT LEAST 1 RESISTANT ISOLATE IN THE SAMPLE SIZE GIVEN

	True Frequency Of Resistance In The Population				
	0.001	**0.01**	**0.05**	**0.1**	**0.2**
Sample Size					
5			23	41	67
10	1	10	40	65	89
15			54	79	97
20	2	18	64	88	99
25	3	22	72	93	
50	5	39	92	99.5	
75	7	53	98		
100	10	63	99.5		
150	14	78			
200	18	87			
500	40	99.4			
750	53				
1000	64				
1500	78				
2000	87				

Some probabilities for detecting resistance when taking various sample sizes and for different true frequencies of resistance in the population. Values have been rounded up in some cases.

The Table shows that when resistance is at a low level it is very difficult to detect without large sample sizes. However, such situations can be helped by using pooled samples. In these cases, isolates are not tested singly but tested as populations containing many spores. The rational behind this is that in a sample of 100 spores, if they are tested together as a single sample, if only one of them is resistant the test could give a positive result. Testing several bulk samples thus increases greatly the chances of detecting the resistance. Such a process will not give an absolute measure of the frequency of resistance in the population at a single point in time but can be used to measure its development over time.

APPENDIX 3

DANGERS AND PITFALLS

The following comments are from the author's own experience and are given as a guide to any individual or laboratory intending to establish a baseline and subsequent sensitivity monitoring campaign.

Departure from a protocol
The simpler the method, the better, providing it generates reliable data. But take care. Remember that methods can be copied by other laboratories or maybe you are copying a method yourself. Consider that any deviation from an established method can produce changes in a data profile and lead to false conclusions, e.g. the declaration of a shift in sensitivity when none has occurred or even a declaration of no shift when it has actually happened. As an example, many *in-vitro* methods use potato dextrose agar (PDA) for a growth medium. There are many sources of PDA, varying from commercially available powders to recipes designed to allow the scientist to prepare media from potatoes and agar. The sensitivity profile of a fungus tested on one form of PDA is likely to differ from that derived from tests on another form. This can happen very often when PDA is made *in situ* from raw ingredients. Such practice will produce media of different nutritional status which will be translated into variability in sensitivity response levels between batches of media. It is far better to define a particular pre-prepared medium e.g. Oxoid or Difco and use it for all tests.

Different people can produce different results
Unlikely as it may seem, this can happen. Experience shows that it is best to have all work conducted by the same technician or scientist, or groups of the same, for as long as possible. Differences in results may not be as large as those created by a deviation from established protocols, but can be enough to cause concern. Why these differences occur is difficult to explain. They are most likely due to slight differences in interpretation of a protocol and experimental procedures. They can be controlled by the use of standard reference isolates and, where possible, a period of joint working such that differences are identified and corrected.

Baselines are specific to your laboratory
This is an extension of the points given above. There is a common misconception that a laboratory can embark on a resistance monitoring exercise and use another laboratory's published baseline as the reference point. Such a procedure is highly dangerous and almost certain to lead to false conclusions. Due to differences in the interpretation of test procedures, differences in laboratory conditions and assessment procedures, maybe even differences in the test fungicide (active ingredient or formulated product) the data obtained may not be part of the population of data that have been published. The only way to avoid problems is to generate a baseline for a particular laboratory or to exchange reference isolates such that individual laboratories can use 'bridging data' to enable valid comparisons.

An example of problems that can occur is given by a study undertaken for the assessment of sensitivity of *Tapesia* spp to prochloraz by the then Schering Agrochemicals. Four research centres took part in the study: Schering Agrochemicals at Chesterford Park, the Plant Breeding Institute (before commercialisation), IACR-Rothamsted and ADAS. Each organisation provided six isolates of *Tapesia* spp for testing at all laboratories. A standard protocol was agreed upon and all test materials were supplied from a common batch. In theory all laboratories should have produced identical results, making due allowance for experimental variation. This was not the case. When the data were compiled and analysed it was clear that there were significant numerical differences in the sensitivity values found between laboratories. However, the rank order of isolates was the same irrespective of the laboratory. The test also used three different test media: PDA, malt agar and Czapek Dox. Great differences were found in sensitivity values generated on these media. The fungal isolates were (numerically) most sensitive when tested on Czapek Dox and least sensitive when tested on malt agar. Data for PDA were in-between these extremes.

The lesson was clear: it was unreliable to use sensitivity data generated from one laboratory to compare with baseline data generated by another laboratory, but decisions on relative sensitivity of isolates tested within an individual laboratory were valid and consistent between laboratories.

Wherever possible, investigations should be carried out to ensure that the *in-vitro* methods used correlate well with tests made on plants.

The shape of the curve and its placement on a dosage axis can vary according to the method used for determination.

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Phil Russell obtained his BSc and PhD at UCW Aberystwyth before joining the Applied Biology Department of The University of Cambridge as a Demonstrator in Plant Pathology and Plant Breeding. At the end of his 5 year tenure he joined Fison's Agrochemicals as a plant pathologist in charge of initial field testing of new fungicides. The Company merger with Boots Agrochemical Division to form FBC Ltd, followed by the merger with Schering saw Phil move through various roles including spells in Field Development and Central Marketing before he became Head of Fungicides Dept for Schering Agrochemicals. It was at this time that his interest in fungicide resistance and its management developed and he maintained the interest as Head of Development Support for AgrEvo (Schering plus Hoechst) before becoming Fungicide Resistance manager for Aventis Crop Science (AgrEvo plus Rhone-Poulenc) based in Lyon. He was Chairman of the FRAC Steering Committee for 8 years, is a Special Professor in Plant Pathology at the University of Nottingham and was awarded a BCPC medal in 2002 for his contributions to Crop Protection.

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