Session 6
Resistance Risk Evaluation

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PESTICIDE RESISTANCE AND THE EC PLANT PROTECTION PRODUCTS DIRECTIVES

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ABSTRACT

Data relating to the development of pesticide resistance required by both the UK Regulatory Authority and the EC's Authorization Directive (91/414/EEC as specified in Annex III) are discussed. The rationale for these requirements and their role in the regulatory process are outlined in this paper.

INTRODUCTION

The European Commission (EC) has harmonised the requirements for authorization/approval of plant protection products across the Community by the introduction of a series of Directives. Included in these Directives are requirements for resistance risk assessment and management strategies to prevent the selection of pesticide-resistant organisms. The two relevant Directives that refer to resistance are Council Directive 91/414/EEC - the Authorization Directive (Anon, 1991a) and Commission Directive 93/71/EEC - the Efficacy Section of Annex III (Anon, 1993). The latter specifies requirements under the heading of "Information on the occurrence or possible occurrence of the development of resistance". These requirements do not differ very much from the UK's own Data Requirements Handbook which has been in use since 1986 (Anon, 1986).

THE E.C. DIRECTIVES

The E.C. Directives state:

"Laboratory data and where it exists, field information relating to the occurrence and development of resistance or cross resistance in populations of harmful organisms to the active substance(s), or to related active substances, must be provided. Where such information is not directly relevant to the uses for which authorization is sought or to be renewed (different species of harmful organism or different crops), it must, if available, nevertheless be provided, as it may provide an indication of the likelihood of resistance developing in the target population.

Where there is evidence or information to suggest that, in commercial use, the development of resistance is likely, evidence must be generated and submitted as to the sensitivity of the population of the harmful organism concerned to the plant protection product. In such cases a management strategy designed to minimise the likelihood of resistance or cross-resistance developing in target species must be provided."

DEFINITION OF RESISTANCE

The UK Government's Regulatory Authority considers a working definition of pesticide resistance to be :

"Pesticide resistance by an organism is an inheritable change of a population in its sensitivity to an active ingredient which will be reflected in reductions in levels of field control during commercial use according to the product label. Such a change in sensitivity can be confirmed by bioassay using a validated or recognised technique".

To explain further, it is accepted that while resistance of an organism to an active ingredient can often be demonstrated in the laboratory it need not necessarily mean that a grower will notice any lowering in the level of control in the field. It is the record of the occurrence of both factors which is necessary before it can be accepted that resistance has become significant to the use of the active ingredient. Dekker (1985) stated that this situation must be achieved by explaining clearly by what criteria resistance is being claimed.

INTERPRETATION OF DATA REQUIREMENTS

The issue of pesticide resistance must be addressed in all applications for approval/authorization of a new plant protection active ingredient. The registration authorities require information in order to decide if approval should be given and to guide them to a conclusion as to the need for further data and the need for development of strategies to minimise the possible occurrence of resistance. It is expected that applicants take a comprehensive world-wide view of the situation. The requirement for "information not necessarily relevant" is to discover the potential for the chemical group to select for resistance or for the selection of resistance in the target organism. It is not certain that cross-resistance will occur to a new active ingredient simply because there has already been selection in a target organism to a non-related chemical group, but the possibility cannot be dismissed. The amount of information required will depend on the perceived risk of resistance based on the evidence available at the time approval/authorization is sought.

The perceived risk of resistance will be 'low' if there is no evidence of resistance both to the pesticide/pesticide group and in the target pest to other active ingredients. If, however, the target pest has shown the propensity to develop resistance to other pesticides or if resistance has already been recorded to the pesticide/pesticide group either in the target pest or in other pests, then the perceived resistance risk may be 'high'. While accepting that evidence of resistance in the laboratory does not necessarily mean that control problems will be encountered in the field, such evidence will nonetheless be taken into account when determining the risk of resistance.

In both instances, the applicant must provide the information on which the resistance risk can be determined. Test protocols and the 'baseline' sensitivity of the target organism should also be made available when providing results of monitoring studies. Furthermore, in cases where the perceived risk of resistance is 'high', the applicant will be required to propose a management strategy designed to minimise the development of resistance in commercial situations.

Protocols for testing pest sensitivity

There are various protocols already published describing methods for the testing of susceptibilities of organisms to pesticides. Most of them allow for the production of baseline data that can be used for monitoring resistance. The Food and Agricultural Organisation (FAO) has published a series of recommended methods for the detection and measurement of fungicide and insecticide resistance (e.g. Anon 1980, 1982a, b) and the European and Mediterranean Plant Protection Organisation (EPPO) has also published methods for monitoring fungicide resistance (Anon, 1991b, 1992). However, these bioassays are not appropriate for all situations and new tests may need to be devised. These tests must be validated to show that they are capable of detecting changes in the susceptibility of the target organism.

Baseline response data

In cases where prior evidence is available that resistance has occurred to the pesticide/pesticide group or in the target organism, the data requirements state that the applicant should produce baseline data as to the sensitivity of the target organism to the proposed active ingredient. The production of such data are invaluable in resistance studies as once resistance is suspected it can never be unequivocally established that data produced subsequently are from truly susceptible organisms. Details of laboratory bioassays (or field tests if more appropriate) and results submitted to the registration authorities should be made publicly available. Anybody wishing to monitor for the development of resistance at a later date will therefore have access to the methods and the baseline data against which to judge their results.

Management strategies

The applicant has to provide a management strategy to minimise the likelihood of the development of resistance. An applicant should be able to construct a suitable strategy using knowledge of the mode of action of the new active ingredient to the target organism and the likely resistance mechanisms of the organism. The strategy should be publicised and brought to the attention of farmers and advisers either via instructions on the product labels or by technical literature. Management strategies to minimise the development of resistance could include measures such as marketing of the active ingredient only in formulated mixtures with other non-related active ingredients, or by restricting the number or timing of applications in order to reduce selection pressure. It is advisable that the development of strategies for resistance management are borne in mind when monitoring for resistance.

REVIEW OF EXISTING PLANT PROTECTION PRODUCTS

The EC is to undertake a major review of all approved pesticides with extant approvals within the Community. Agrochemical companies see the exercise as a formidable re-registration task which will include the need to produce data to answer the Directive's requirements on resistance. Although the data requirements as laid down by the Directives suggest that product re-registration should include a consideration of resistance, it is the opinion of the UK Regulatory Authorities that action will not be required by the agrochemical

companies for the initial review of the active ingredient for Annex I listing but may be required when the authorities highlight a problem during the authorisation process of products which follows in individual Member States.

CONCLUSION

We are not able to specify exactly what evidence is required to satisfy the data requirements relating to resistance mainly because of the varied and complex nature of resistance. The issue is very complicated in that interpretation of data is often difficult and new pesticide/target organism interactions may be unique. Therefore, testing techniques, sample sizes etc. could be very different from existing ones. Furthermore, strategies designed to prevent or delay the development of resistance are largely untested, with scientific opinion split on their validity.

We would therefore expect a dossier provided in support of an application for the approval/authorization of a new pesticide to contain the following:

i) Details of a protocol for testing pest sensitivity;
ii) Baseline response data (sensitivity/susceptibility); and
iii) in the event of evidence of a problem, a management strategy designed to minimise the likelihood of resistance.

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EARLY EVALUATION OF FUNGICIDE RESISTANCE RISK

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ABSTRACT

Early estimates of the initial variation for sensitivity, and attempts to induce resistance mutations in the laboratory, are essential for predicting the probability of evolution of resistance to a new fungicide. If the appropriate variability is present, the seriousness of the problem that should be anticipated is a function of the type and rate of population response to exposure to the new chemical. Whether the response will be qualitative or quantitative can be determined by studying the genetics of resistance in the laboratory before field testing. Measurements of the effects of resistance alleles on fungicide sensitivity and on fitness in the laboratory and the greenhouse may provide indications of the rate of response.

INTRODUCTION

One of the aspects needing investigation during fungicide development is the likelihood of loss of effectiveness because of the evolution of resistance to new products by target fungi. Early assessment of this risk is desirable, so that decisions can be made, if not to stop further development of compounds perceived to have too high a risk, to at least proceed cautiously. Work on appropriate marketing and use strategies to ensure the longest possible useful life of each new product could then be carried out within a meaningful context. Practical resistance problems arise when field populations of target organisms can respond to exposure to a chemical by a substantial change in their sensitivity. This contribution will try to indicate what laboratory and greenhouse tests can be made early, before field testing of a new fungicide, in order to answer two important questions :

a. Whether the use of the compound might cause a population response, and of what type. This depends on the availability of appropriate variation and the genetic control of such variation.
b. What will be the rate of evolution of resistance to the new compound. Main determinants of this rate are selection pressure and fitness.

Recent treatments of the subject of resistance risk assessment include that of Keiding (1986) for pesticides in general and those of Gisi and Staehle-Csech (1988a, b) and Brent *et al.* (1990) specifically for fungicides. It is to be understood, of course, that the results of resistance risk

assessments may help to set priorities in the development work, but can not be taken as conclusive forecasts on the resistance behaviour of new chemicals in all cases.

BACKGROUND KNOWLEDGE ON THE EVOLUTION OF FUNGICIDE RESISTANCE

It cannot be excluded that the genetic variability required for resistance to a particular type of toxicant may not be available to the intended target organism(s). Unavailability of appropriate genes may be the reason for which some protectant fungicides give apparently the same level of protection today as they did when first introduced (Georgopoulos & Skylakakis, 1986). Resistance to copper for example, has evolved in plant pathogenic bacteria which can no longer be controlled with the amount of copper available from fixed copper fungicides (Cooksey, 1990), but apparently not in the many fungal pathogens against which copper is used.

Genetic variation can pre-exist in a population, or arise *de novo* by mutation (or recombination) after environmental change. The change will then generate a selection pressure and the population response will be either qualitative or quantitative. A qualitative response to a fungicide is expected with major-gene control of resistance, causing discontinuous variation with at least two distinct, non overlapping subpopulations. Alternatively, if selection acts on continuous (polygenic) variation, the population response will be quantitative : there will be a decrease in mean sensitivity, but distinct subpopulations will be impossible to recognise even after long exposures. With major-gene control, the organism can achieve the highest possible resistance in one step, by mutation of one gene. In the polygenic system, the effects of individual genes, even if recognisable, are generally small. Highly resistant strains cannot be obtained in a single step, but only through recombination or sequential selection.

Of the fungicides already in use, the benzimidazoles and the phenylamides cause typical qualitative population responses because high resistance to these compounds is obtained by major-gene mutations (Shabi *et al.*, 1983 ; Crute & Harrison, 1988). Examples of a quantitative response are those to dodine, (Mckay & MacNeill, 1979) to ethirimol (Brent, 1982), and to the C-14 demethylation inhibitors (Heaney, 1988). Resistance to members of the latter group is generally polygenic, but major-gene resistance to triadimenol has been recognised in *Nectria haematococca* (Kalamarakis *et al.*, 1989) and discontinuous distribution for sensitivity to the same compound has been observed in populations of *Rhynchosporium secalis* (Kendall *et al.*, 1993).

Prediction of a qualitative or quantitative response is of considerable practical importance. Generally speaking, resistance controlled by major genes may be expected to lead to disease control failures faster than polygenic resistance. Thus far only fungicides causing qualitative population responses have been classified by practical experience as high-

risk fungicides (Georgopoulos, 1987). With major gene resistance, the increase in the frequency of the mutant forms is exponential and, unless impractically large numbers of samples are tested, monitoring is unlikely to detect field resistance until it is too late. Monitoring, however, may provide early warning in cases of polygenic control of resistance where the decrease in mean sensitivity is linear with time. This has adequately been explained by Brent *et al*. (1990).

The distinction is also important in order to decide on the initial use rate of a new fungicide. With major gene resistance, lowering of the selection pressure by reducing the dosage may slow down the change to a predominantly resistant population. Individuals which will not be killed will be wild types and will contribute sensitive progeny. In contrast, reduction of selection pressure in cases of polygenic control may encourage resistance evolution. In polygenic resistance, each gene contributes in a minor way and survival of individuals with a few such genes at a lower dose will give them the opportunity to acquire additional genes by mutation and/or recombination. If a higher rate is then applied, this will intensify the response to selection. Using a high dosage initially, will eliminate individuals with low resistance and the frequency of recombinations of polygenes will greatly be reduced.

OBTAINING DATA ON THE INITIAL VARIATION IN SENSITIVITY

Though only a few isolates of each target organism are used in the initial screening work, a larger number must be tested for sensitivity to those compounds which are considered for development. The sample cannot be large enough to detect rare mutants. Novel, DNA-based detection methods which might permit testing large numbers of isolates, require knowledge of the molecular basis of resistance. Estimates of the initial variation in sensitivity currently rely on bioassays which are time consuming, but it is advisable to include, e.g. a few dozens of isolates in these assays. The methods may differ for each fungus-fungicide combination, but it is important that some general principles be observed (Georgopoulos, 1982).

In the case of non-obligate parasites, sensitivity can be measured on artificial media, and it is usually not difficult to determine the response of each isolate to a number of concentrations, e.g. by replica plating. The range of concentrations is decided on the basis of preliminary tests, but it is important not to ignore the solubility limitations of the compound studied (Georgopoulos, 1982). Volatility may make it necessary to separate treatments. In tests with artificial media it is best to use technically pure material, so that interference of other components of the formulation can be avoided. However, when sensitivity needs to be tested on treated plant material, some formulation is usually needed for such treatments.

If isolates from different areas and from fields with different fungicide use histories are included, indications on the

type of problems to be anticipated may be obtained during this study of the initial variation in sensitivity to each fungicide candidate. If, for example, in spite of a small sample size, discontinuous variation is found, evidence not only of major-gene control of sensitivity differences, but also of cross-resistance to some of the previously used fungicides has been obtained. Testing laboratory or field strains with specific resistance to some of the known fungicides for sensitivity to the new compound can clarify the latter point. In contrast, when continuous, even if quite considerable, variation is found, this does not indicate cross resistance to previously used chemicals. Experience has shown that such variation may exist even without any previous selection. For example, populations of *V. inaequalis* never exposed to dodine were found to contain individuals with up to 100-fold differences in the concentration required to inhibit spore germination (Mckay & MacNeill, 1979). It is, therefore, important in baseline sensitivity testing to study the type as well as the extent of preexisting variation.

ISOLATION AND CHARACTERISATION OF RESISTANT MUTANTS

If little initial variation for sensitivity to a new compound is recognised, isolation and characterisation of resistant mutants in the laboratory will be needed for the additional tests of risk assessment. Where the potential fungicide target involves pathogens which are difficult to manipulate genetically, suitable species may be substituted. *Neurospora crassa* and *Aspergillus nidulans* are often preferred, but pathogenic fungi, such as *N. haematococca* and *Ustilago maydis*, have the advantage of allowing the effects of fungicide resistance genes on pathogenicity to be examined. To have a better chance of recognising substantial changes in sensitivity it is best to use an organism highly sensitive to the compound studied.

Mutations occur spontaneously at very low frequency which can substantially be increased by the use of physical or chemical mutagens. Since treatments increasing the mutation rate also result in the death of many of the treated cells, one must avoid the extremes: having too few survivors at a high dose or having rather few mutants among many survivors at a low dose of the mutagen. A treatment giving 90-95% lethality is usually appropriate. It is advisable to aim at the isolation of a number of resistant strains, so that a thorough study of the variability available to the organism can be attempted when desirable. This will require exposure of several millions spores, or other propagules, to the mutagen and plating of the survivors on medium containing the fungicide.

It is suggested that two fungicide concentrations are used, the lower one being at least twice the minimal concentration which completely prevents growth from spores of the original strain when plated at high density. Failure to obtain strains with stable resistance to the new compound in this way is strong evidence against at least a high risk. If resistant strains are obtained but are only slightly less sensitive than the original strain, they can be used in attempts to achieve higher resistance

by sequential selection (De Waard, 1988). In case of an organism which does not lend itself for genetic analysis, success in such a stepwise increase may be taken as an indication of polygenic control of resistance. On the other hand, high resistance obtained in one step points towards major-gene control.

The type of population response to the use of a new fungicide can safely be predicted, if the genes responsible for changes in sensitivity are identified and their interactions studied. The pathogenic vegetative phase of most fungi is haploid, hence dominance or recessiveness of a mutant gene is irrelevant and examination of the phenotypes of the F_1 from a resistant (R) x sensitive (S) cross is sufficient. In diploids, such as *Phytophthora* sp., recognition of a Mendelian ratio requires selfing of the F_1 and examination of the phenotypes of the F_2 generation. In such fungi, it is very important to know the type of intra-allelic interaction. A recessive resistance gene will not affect the phenotype of the heterozygote and, therefore, resistance should be expected to evolve more slowly. If resistance is semi-dominant, one should be careful in recommending reduced application rates with the aim to lower the intensity of selection. A rate high enough to eliminate the heterozygotes, if possible, is highly advisable.

Analyses of RxS crosses usually show that each strain obtained by one-step selection carries one mutant gene for resistance: in case of a haploid organism a 1R : 1S ratio is found in the F_1. It cannot be excluded that one RxS cross may yield a higher ratio, up to 3:1. In such a case the resistant parent in the cross must carry two mutant genes. This finding indicates that resistance to the new compound is probably polygenic because the probability of inducing two resistance mutations in the same nucleus is very low, unless the genes involved are numerous. If no evidence of polygenic control is obtained in this way, analyses of RxR crosses will be required.

Even if resistant mutants differ considerably in the degree of resistance, they do not necessarily carry resistance genes at different loci. In *V. inaequalis*, for example, very high, high and moderate resistance to benzimidazoles is controlled by different alleles of the same major gene (Shabi *et al.*, 1983). Involvement of different loci can be accepted only if some sensitive recombinants are obtained from some RxR crosses. Recognition of several non-allelic genes for resistance to the same fungicide, however, does not show that resistance is polygenic. An early study of 100 mutants of *N. haematococca* resistant to aromatic hydrocarbon fungicides identified five chromosomal loci involved in resistance (Georgopoulos & Panopoulos, 1966), but recombinants carrying mutant genes at two or more of these loci were not less sensitive than single-gene mutants because of epistasis. Consequntly, variation was discontinuous. On the other hand, because it is not practically feasible to analyse a large number of RxR crosses, the fact that only a few loci involved in resistance to a new fungicide have been recognised does not exclude polygenic control. The characteristic of quantitative inheritance, and the cause of continuous variation is the positive interaction between non-allelic genes in the

haploid nucleus and the additivity of their effects. An increase in the degree of resistance correlated with the number of mutant genes present has been conclusively shown, for example, in the case of resistance of *N. haematococca* to dodine (Kappas & Georgopoulos, 1970) and to fenarimol (Kalamarakis *et al.*, 1991) and of *A. nidulans* to imazalil (van Tuyl, 1977).

EVALUATION OF THE RATE OF RESISTANCE EVOLUTION

Having shown that the potential target organism(s) possess genes which can mutate to give resistance to a fungicide considered for development, and that the population response will be qualitative or quantitative, additional information is needed to predict the speed of response. The rate of resistance evolution may be affected by factors related to the environment (greenhouse or open field, conditions favorable for high disease pressure etc.) and disease management practices (sanitation, host resistance, etc.). Other factors being equal, the determinants of the rate at which the proportion of strains carrying given resistance alleles will increase are :

a. *intensity of selection* which is a function of the decrease in fungicide sensitivity caused by each gene and
b. *relative fitness*, i.e. the survival and subsequent reproductive success of the resistant strains in the absence of a discriminating fungicide concentration, as compared to the wild type.

Although selection pressures are usually referred to, they are difficult to measure. The degree of resistance of the various types of mutants is important, but in laboratory measurements, the degree of resistance is often overestimated because of the low water solubility of most fungicides (Georgopoulos, 1982). Even if an accurate numerical value is obtained, its significance may differ, depending on the properties of the chemical studied. Often, increasing the amount that can be made available for biological activity beyond a certain level is not possible in the field, so that even strains with low resistance are very difficult to control.

Relative fitness estimates are also difficult. In the laboratory, strains carrying particular alleles for resistance to a candidate fungicide may be compared to the wild type with respect to sporulation, time required for spore germination, germ tube elongation, linear growth and ability to survive under extreme conditions of temperature, humidity, osmotic pressure etc. Pathogenicity is, of course, a very important fitness determining characteristic. Not only the ability to infect a susceptible host, but also the rate of tissue colonisation, latent period, sporulation capacity and intensity must be considered. Information has been obtained by various workers by the method of mixed inoculations in laboratory or greenhouse tests. Reduced fitness of a laboratory selected strain may or may not be due to the resistance mutation itself. The problem can be resolved by examining the behavior of progeny from R x S crosses. Even if it is shown that recognised resistance genes affect important fitness determining characteristics, it

can not be excluded that in nature rarer alleles, not affecting fitness, may be selected for by exposure to the new fungicide. Of interest in this regard, are recent observations of Faretra & Pollastro (1993) regarding high resistance to dicarboximides in *Botryotinia fuckeliana*. The high resistance allele is responsible for increased sensitivity to media of high osmolarity and this is considered the cause of low pathogenicity and fitness. In heterokaryons, however, in which nuclei carrying the allele for high resistance coexist with wild type nuclei, the high dicarboximide resistance phenotype is dominant, while that of osmotic hypersensitivity is partially recessive.

CONCLUSION

Tests needed for resistance risk assessment at the time a new compound is considered for development as an agricultural fungicide are rather simple. No particularly great effort and expenditure is required compared to the benefit of acquiring knowledge so important for decision making. Depending on the information that becomes available from the risk assessment tests, some more or less safe predictions may be possible. If major-gene resistance is obtained in the laboratory and the experiments do not show significantly lower fitness of the mutant strains, there can be little doubt that we have a high-risk fungicide. A low resistance risk is quite likely if repeated attempts fail to isolate strains with considerable resistance to the new compound. Intermediate situations of moderate risk may be anticipated if polygenes are shown to be involved, or major-gene mutations increase resistance at a more or less high cost in terms of fitness. The seriousness of problems likely to arise in such moderate-risk situations may vary, depending, amongst other factors, on the magnitude of this cost.

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