Fungicide resistance in crop protection

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Preface

In recent years awareness of the importance of fungicide resistance in crop protection has been growing. In response, an international post-graduate course on this topic was held in Wageningen, The Netherlands, from 13 to 20 August 1980.

Because of the great interest and large number of applications to attend the course, it was repeated in 1981 (22 July - 5 August). The aim of the course was to inform phytopathologists who may be confronted with fungicide resistance in their work about the origins and nature of the phenomenon and to discuss practical measures that might prove helpful in combatting the problem.

The course was organized by the Foundation of Post-graduate Studies of the Agricultural University at Wageningen on the initiative of the Chemical Control Committee of the International Society of Plant Pathology, and with the support of the Food and Agricultural Organization of the United Nations and the chemical industry. The course programme was chosen by a scientific course committee, whose members were R.J.W. Byrde (U.K.), L. Chiarappa (FAO), J. Dekker (The Netherlands), J.W. Eckert (U.S.A.), S.G. Georgopoulos (Greece), F.J. Schwinn (Switzerland) and H.D. Sisler (U.S.A.).

There have been requests from many sides to make the information provided in the course available to a broader public. This has led to the publication of this book, which is based on the course lectures.

We should like to take this opportunity to thank Mr I. Cressie of Pudoc for his assistance in editorial matters, Mr W.C.T. Middelplaats for drawing the illustration on the cover and the figures, and Mr F.W. de Vries for the compilation of the index.

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Contents

Introduction

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General

Agricultural crops are under constant attack by noxious organisms. The total crop losses that result are estimated at 25-45 % (Cramer, 1967) of production. To safeguard world food production, crop protection measures are indispensable. When the use of resistant varieties, crop rotation and other cultural practices, or alteration of the environment are inadequate to suppress pathogens sufficiently, the use of chemicals becomes essential. Nowadays chemicals play a predominant role in plant protection. Without pesticides active against insects, nematodes, fungi, bacteria and weeds, modern agriculture would not be possible and hunger and misery in the world would increase dramatically.

But control of diseases and pests with chemicals may also encounter problems, for example when the causal organism becomes resistant to the toxicant. This resistance involves one of the most fundamental properties of living matter, namely, the ability of organisms to adapt to changing environmental conditions and to survive under new, often adverse, circumstances. The evolution of organisms would have been impossible without this property, as illustrated by the natural history of life on earth. The application of a pesticide also constitutes an adverse change in the environment for an organism that is sensitive to such a compound: it may adapt to the new situation by becoming resistant.

There are various examples of development of resistance by organisms to biocidal chemicals used by man to control those organisms, such as the development of resistance by bacteria to antibiotics and insects to insecticides. Until a short time ago there were, surprisingly, hardly any problems of resistance to fungicides, even though some of them had been used on a large scale for control of fungal diseases for almost a century. However after the introduction of systemic fungicides - about a decade ago - several problems with fungicide resistance occurred in practice.

The concept resistance

In a fungal population that is originally sensitive to a fungicide, forms may arise or already exist that are less sensitive to the fungicide. Such a decrease in sensitivity may be caused by genetic or non-genetic changes in the fungal cell. The latter type of change is not stable and usually disappears rapidly in absence of the toxicant. As it is therefore hardly of any importance in practice, it is not discussed in this book. Decrease in sensitivity due to genetic changes in the pathogen is more serious, however. Recently it was recommended by the FAO Panel of Experts on Pest Resistance to Pesticides (1979) that the term resistance should apply to these hereditable changes in sensitivity in fungi and bacteria, and that the word tolerance should not be used in this sense as it is ambiguous. That recommendation is followed in this book.

History

The beginning of large-scale application of fungicides to combat fungal plant diseases is marked by the discovery of Bordeaux mixture by Millardet in 1882 in Bordeaux. This preparation, a mixture of copper sulphate and lime, remained the most used fungicide for more than fifty years. Hardly any problems with resistance to this fungicide in practice have been reported. To a certain extent, this is also true for the organic mercury compounds, introduced around 1914, the dithiocarbamates, introduced in the 1930s, and various other organic fungicides developed later. All these compounds have in common that they provide only protection at the surface of the plant. In spite of the results obtained with these protectant, or conventional, fungicides, their shortcomings were obvious. Therefore after the Second World War, the search started for fungicides that could penetrate the plant, and thus eradicate pathogens even after infection, or protect plant parts not in direct contact with the fungicide. Such compounds, which are taken up by the plant and transported throughout the plant system are called systemic fungicides. Various research groups in the U.S.A., U.K., the Netherlands and other countries began research on these fungicides. The chemical industry also played an important role in the search for systemic fungicides, testing thousands of available and newly synthesized chemicals.

Various fungicides that have systemic action were discovered in this way. Most of them were not fit for practical use, however, since they gave phytotoxic side-effects. It was difficult to find compounds selective enough to kill the fungus (a lower plant) without harming the crop (a higher plant). It also became clear that there might be other problems. While working with 6-azauracil, an experimental systemic fungicide, Dekker (1967) found strains of *Cladosporium cucumerinum* that suddenly had

become resistant to the chemical. Moreover, in laboratory experiments with some fungicidal antibiotics, such as antimycin (Leben et al., 1955) and cycloheximide (Hsu, 1963) in the U.S.A., and kasugamycin in Japan (Ohmori, 1967), resistant fungal strains were found. These occurrences, observed before the era of systemic fungicides had begun were a warning that fungicides, too, might encounter resistance problems.

Indeed, this happened soon after the introduction of systemic fungicides into practical agriculture, a little more than a decade ago. Notorious cases of resistance have occurred with benzimidazole fungicides and thiophanates in most of the originally sensitive pathogenic fungi, dimethirimol in cucumber powdery mildew, kasugamycin in *Pyricularia oryzae*, polyoxin in *Alternaria kikuchiana* (Dekker, 1977), and recently metalaxyl in pathogens belonging to the Oomycetes (Georgopoulos & Grigoriu, 1981). However, resistance problems are not confined to systemic fungicides, as demonstrated by the penta- and tetrachloronitrobenzenes (Georgopoulos, 1962) and organic tin compounds (Giannopolitis, 1978). These compounds are considered to be specific-site inhibitors, in contrast to most conventional fungicides, which act at many sites in the fungal cell. There appears to be less risk of development of resistance with this latter type of fungicide.

Since the 1960s more than fifty fungicides with specific action have become available, and problems with resistance have appeared in many instances.

Importance

The sudden appearance of resistance in a pathogen population may, when it is not recognized at an early stage, result in failure of disease control and, consequently, serious crop losses. As examples, the resistance to carbendazim of *Venturia inaequalis* in the apple-growing area around Hannover in West Germany in 1974 and the sudden appearance of resistance to metalaxyl in *Phytophthora infestans* on potatoes in the Netherlands in 1980 (Davidse et al., 1981) may be mentioned.

Sometimes the use of an originally effective compound has be restricted or even abandoned. Such a situation may put growers in a difficult position, if no adequate substitutes are available, and it may further reduce the financial returns of the company that developed - invariably at high cost - the fungicide. Should this occur repeatedly, it might make the agrochemical industry hesitant to invest in the development of new systemic fungicides, especially when the companies run the risk of costly law suits; in the long run this could mean the lack of powerful new chemicals to the disadvantage of the grower. There may also be legal consequences for those who have sold the compound, and for farm advisers. Serious crop losses and management problems could also be disadvanta-

geous for world food production. Problems of resistance are expected to increase when selective fungicides will be used to a considerable extent in the developing countries.

The importance of the problem of resistance prompted the Food and Agriculture Organization of the United Nations, which already in 1965 had begun to study problems caused by insecticide resistance, to organize meetings in Rome in 1975 en 1978 for a panel of experts on fungicide resistance. Those meetings resulted in a report that the FAO Panel of Experts on Resistance to Pesticides (1979) presented to the organization's director-general. The Committee on Chemical Control of the International Society of Plant Pathology (1979), too, issued a special report on problems and prospects of chemical control. In that report close attention is paid to the problems of fungicide resistance. This report and that of the FAO's panel contain recommendations for further research needed to fill the gaps in our knowledge.

Research needed

There is still much to learn about the development of fungicide resistance, for not only is it a rather recent problem, but also a very complex one, full of apparent contradictions. In particular, genetic, biochemical and epidemiological studies are required to gain new understanding of the phenomenon of resistance and its development.

Genetic studies of fungicide resistance have been carried out by various research workers (Georgopoulos, 1977). For these studies fungi with a perfect stage are used, which allows genetic analysis for the recognition of genes conferring resistance. Studies have been carried out with non-pathogens, such as *Emericella nidulans* (imperf. *Aspergillus nidulans*), *Neurospora crassa* and *Saccharomyces cerevisiae*, and with pathogens, such as *Venturia inaequalis*, *Ustilago maydis* and *U. hordei*, and *Nectria haematococca*. Genetic aspects of fungicide resistance in imperfect fungi may be studied when the fungi have a para-sexual cycle and the techniques exist for experiments based on this cycle. Detailed genetic studies have been carried out with some members of the aromatic-hydrocarbon group, benzimidazole fungicides and carboxamides. This type of study is often difficult, especially for fungi having polykaryotic cells or with obligate parasites. In many cases, resistance to specific-site inhibitors emerges by single-gene mutation. Conventional fungicides, however, act at many sites in the fungal cell and are considered to be general plasmatoxicants. It seems plausible that the possibility of a pathogen adapting to the chemical at many sites is very limited. It is important that genetic and biochemical studies are continued, to determine the basis of resistance to new compounds and extend knowledge about compounds already used.

Progress in clarifying the biochemical mechanism of resistance has been made with some systemic fungicides, of which the so-called carbendazim generators (benomyl, thiophanates), the carboxamides (carboxin, oxycarboxin) and some organophosphorus fungicides can be mentioned (Georgopoulos, 1977). The occurrence of resistant strains has even promoted studies on the mechanism of action of new fungicides, as it allows comparison of sensitive and resistant individuals of one pathogen. The development of new fungicides by the chemical industry and the complexity of the problem of fungicide resistance mean that much research still has to be done on the mechanism of action of fungicides and the resistance mechanism in pathogens. Such studies have direct practical importance, since fungi may be cross-resistant to other fungicides with a similar mechanism of action.

In addition to research on genetic and biochemical aspects of resistance, the study of the ecological aspects concerned is indispensable for an insight into the development of fungicide resistance in the field. Resistance may develop not only in pathogens, but also in antagonistic fungi, which could bring about changes in the microbial balance in the soil. Little is known yet about the epidemiological side of fungicide resistance. Emergence of resistant individuals by mutation or other processes does not always mean that a fungicide-resistant pathogen population will build up in the field. This may be influenced by several factors, in particular the relative fitness of the resistant strains. Studies on the dynamics of the pathogen population in relation to fungicide resistance are urgently needed.

Information about all the genetic, biochemical and ecological aspects of fungicide resistance will be the basis for devising counter measures to avoid or delay the development of fungicide resistance in practical agriculture. There is still much confusion and uncertainty about such counter measures. It would be worthwhile to investigate what role computer modelling can play here.

Dissemination of information

Most of today's plant pathologists have little or no experience with the problem of fungicide resistance. As a result resistance is seldom recognized at an early stage, and when it is recognized it is often not described accurately. Further, lack of information on how resistance develops makes it difficult to devise effective counter measures to cope with the problems. It is important, therefore, that the knowledge obtained by research and from experience is made available to practical agriculture. This is one of the reasons behind the organization of two courses on fungicide resistance in crop protection held in 1980 and 1981, and for the publication of this book, which is based on lectures given at these courses.

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Chemical control of fungal diseases: importance and problems

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Abstract

In comparison to herbicides and insecticides, fungicides are the smallest group of pesticides, but they show a good growth rate and future potential. Their main markets are in Western Europe and the Far East. The main target crops are grape-vines, rice, vegetables, deciduous fruit, potatoes and small grains. The classical protective organic fungicides are the most commonly used, although use of the highly active, systemic compounds with curative action is increasing. Fungicides will remain the main pillar of disease control for the foreseeable future. They control the majority of diseases satisfactorily. However the majority of fungicides are protectants, which have to be applied repeatedly, a fact of increasing importance in view of the rising cost of energy. Moreover their utility in forecasting systems of IPM programmes is limited. The systemic fungicides are much better suited to such systems, but they may create resistance problems, a risk which shows the need for more appropriate use concepts, on the one hand, and a larger variety of chemically different products, on the other. The present application methods for fungicides are wasteful: research on better targetting of applications is needed.

Keywords: agrochemical market statistics, pesticide use, fungicide use, economics, role of fungicides, limitations of fungicides, recommendations.

Introduction

Chemicals are undoubtedly the main, in many cases, even the only useful and economically acceptable means for effective and reliable control of fungal plant diseases. This in contrast to the control of insects, for which, in some crops at least, alternatives are available - viral or bacterial preperations, biological control agents - and for which strong efforts for the development of integrated control strategies have been go-

Table 1. World pesticide users' market (sales in US $ 1 000 millions).

	Sales to users			Proportion pesticide types in total 1980 market (%)	Increase 1980 compared to 1978 (%)
	1978	1980	1984		
Herbicides	3.71	4.23	4.79	43.5	13.4
Insecticides	3.02	3.35	3.82	34.5	13.8
Fungicides	1.53	1.69	1.97	17.4	16.6
Soil Fumigants	0.19	0.20	0.23	2.1	14.5
Others	0.22	0.26	0.30	2.6	14.9
Total	8.67	9.73	11.11		14.2

Source: Anonymous (1979b).

ing on for several years. Even taking into account that similar efforts for fungal-disease control have also been begun recently, in all probability they will not play a major practical role in agriculture before the turn of this century. Thus, fungicides will be with us for quite a while, and it is therefore useful, as an overture to this book, to briefly review their role in disease control and the problems they pose. In particular, economic and practical aspects of chemical control of fungal plant diseases are discussed in this contribution.

Economic importance of fungicides

Insight into the economic importance of fungicides can be gained through the analysis of the world pesticide user market, for it is in such markets that user attitudes, preferences, priorities and practices are reflected. Indeed, applied science, which is oriented to practical application, may find much of interest from such market analysis.

Of the three major groups of pesticides, namely, herbicides, insecticides and fungicides, the latter is the smallest in terms of world-wide sales to users (Table 1).

Its growth rate is slightly higher than those of the other product groups, probably due to the recent introduction of fungicides in field crops that were not treated with them before (e.g. wheat, barley, peanuts and soybeans). The distribution of the agrochemical users' market by geographic region is shown in Figure 1.

The North American subcontinent is clearly the leading pesticide user, followed by Western Europe. These two account for almost 60 % of the total world users' market. This indicates the dominance of the developed countries over the developing countries in the consumption of agrochemicals. However, if one considers the product groups herbicides, insecticides and fungicides, the comparison of developed with developing countries looks different (Table 2).

Whereas both herbicides and fungicides are mostly used in developed

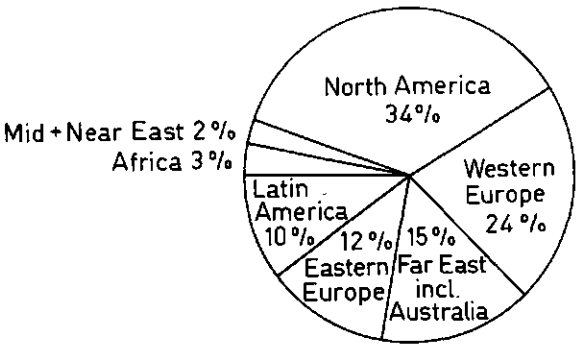

Figure 1. Distribution of the 1978 world pesticide users' market by geographic region; *total world sales of pesticides = US $ 9 700 million.* (Source: Ciba-Geigy, 1979).

countries, there is a major outlet for insecticides in developing countries, namely 40 % of the total insecticide consumption. The distribution of world agrochemical sales to users by crop (Figure 2) indicates why this is so. Figure 2 shows that the crop plant for which most agrochemicals are used is cotton, most of the producers of which are developing nations; maize, deciduous fruit, and vegetables follow in that order. Seventy-three percent of all pesticides are applied on these four crops. The importance of the herbicide, insecticide and fungicide groups for pest control varies widely from crop to crop (Table 3).

In cotton growing, for example, insecticides are the dominant pesticide used. In contrast, herbicides hold a leading position among the pesticides used in growing corn, soya and small-grain cereals. Only for growing fruit and vegetables are fungicides the most frequently used agrochemicals. In particular for fungicides, Western Europe is the dominant user followed by the Far East and Eastern Europe (Figure 3). Figure 3 shows clearly how small the importance of fungicides is in North America, in contrast to the large proportion (34 %) of the total agrochemical users' market accounted for by this subcontinent; it is the largest user of herbicides and the second largest user of insecticides (Table 4). In

Table 2. Use of pesticides in developed and developing countries in 1979.

	Proportion of pesticide used in developed countries	Proportion of pesticide used in developing countries (%)
Herbicides	80	20
Insecticides	60	40
Fungicides	85	15
Total	75	25

Source: Anonymous (1980b).

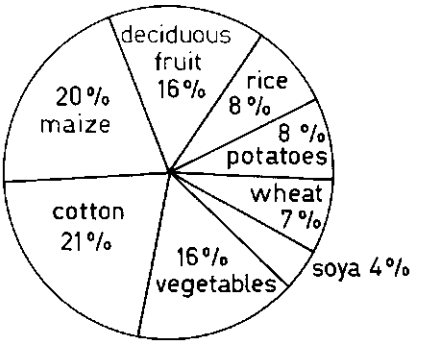

Figure 2. Distribution (%) of 1979 world agrochemical sales to users by crop. (Source: Anonymous, 1977).

Western Europe, Japan and Eastern Europe the fungicide market is larger than the herbicide or insecticide markets.

Analysis of the fungicide market by crop (Table 5) shows vines ranked first, followed by rice, vegetables, deciduous fruit, potatoes and small-grain cereals. Around 70 % of the total amount of fungicides sold is used on these six crops, almost exclusively against foliar diseases. The high ranking of Western Europe in the geographical distribution of fungicide sales reflects the importance of viticulture, wheat and deciduous fruit, vegetable and potato growing on this subcontinent. The high ranking of the Far East is largely due to the importance of the Japanese rice-fungicide market. Note also the evenly distributed use of fungicides in vegetable production around the world, and the strongly varying importance of the small-grain-cereal fungicide market. Western Europe is the leading user of fungicides on small-grain cereals, as about ten years ago the economic importance of foliar diseases of wheat and barley became evident. In contrast, the US, the largest wheat producer in the world, uses very little fungicides on this crop. On a world-wide basis, rice and small-grain cereals may be considered the two crops with the highest potential for increase in the use of fungicides.

Table 3. Proportion (%) of herbicides, insecticides and fungicides used in selected crops in 1979.

	Cotton	Corn	Rice	Soya	Wheat	Fruit & Vegetables	Total
Herbicides	26	78	35	92	75	10	43
Insecticides	71	20	47	6	10	38	37
Fungicides	3	2	18	2	15	52	20
Total	100	100	100	100	100	100	100

Source: Anonymous (1980a).

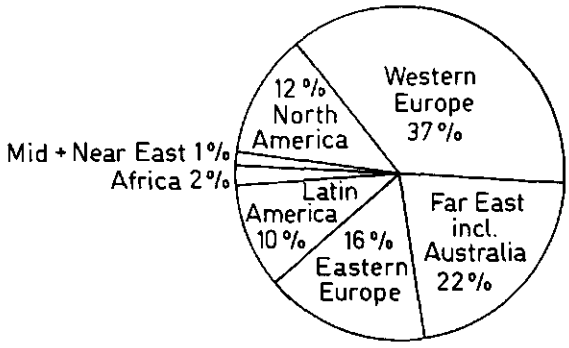

Figure 3. Distribution (%) of 1978 world sales of fungicides by geographic region; total sales of fungicides to users = US $ 1 700 million, or 18 % of total pesticide users' market. (Source: Ciba-Geigy, 1979).

The distribution of the fungicide market by chemical group is shown in Table 6. Unfortunately, these figures are estimates for 1980 made in 1975. I have been unable to find more recent figures. From my knowledge of the new types of fungicides introduced in the past five years, I am certain that since 1975 the proportions have changed in favor of the systemic fungicides, although the dithiocarbamates still hold their leading position.

Practical importance and problems of fungicides

To discuss technical aspects of fungicides clearly, one must distinguish the terms used to describe them. The terms commonly used are presented in Table 7.

As stated in the introduction, chemicals are the only effective and reliable means of directly controlling plant diseases. Thus to the person faced in his daily work with the immediate problems of disease - be he farmer, adviser or researcher - chemicals will remain his main weapon

Table 4. Distribution (%) of 1979 world sales to users of herbicides, insecticides, fungicides and other pesticides by geographic region.

	Herbi-cides	Insecti-cides	Fungi-cides	Others	Total
North America	52	23	15	44	34
Western Europe	21	13	37	38	22
Japan	8	13	20	7	12
Eastern Europe	12	12	19	6	13
Rest of world	7	39	9	5	19
Word total	100	100	100	100	100

Source: Anonymous (1980a).

Table 5. Fungicide sales (US $ millions) to users by crop in geographic regions in 1978.

	North America	Western Europe	Japan & Far East	Rest of World	Total	Proportion of total market(%)
Corn	5.0	4.4	4.0	8.4	21.8	1.6
Cotton	12.7	2.1	4.5	14.0	33.3	2.6
Wheat	5.8	62.7	5.4	28.2	102.1	8.0
Rice	2.7	1.5	144.6	33.2	182.0	14.1
Soya	13.1	1.8	5.2	7.4	27.5	2.2
Tobacco	2.3	3.6	10.5	9.4	25.8	2.0
Peanuts (Groundnuts)	26.7	-	6.9	4.7	38.3	3.0
Sugar-beets	4.3	8.1	9.9	7.8	30.1	2.3
Coffee, Cocoa, Tea, Rubber (Plantation Crops)	-	-	33.8	68.0	101.8	7.9
Citrus	13.9	8.1	16.2	9.5	47.7	3.7
Apples	19.6	56.6	22.2	25.4	123.8	9.6
Vines	11.6	200.5	11.3	63.3	286.7	22.4
Potatoes	13.6	45.0	10.8	38.9	108.3	8.5
Vegetables	18.1	47.3	44.0	45.2	154.6	12.1
Total	149.4	441.7	329.3	363.4	1283.8	100.0

Source: Anonymous (1979b).

against plant diseases. Therefore they are for him of great practical importance (Anonymous, 1979a). In spite of the continuous efforts by the chemical industry to develop highly active and safe products, the chemicals in the arsenal available to the farmer in his struggle with disease vary in their effectiveness. Table 8 gives a general picture of the present state of fungal-disease control. For several types of disease, chemical control is still weak or not possible at all. Even for those diseases that can be controlled, one has to realize that the major fungicides used to do that job are technically old-fashioned: most of them (Table 6) are *residual* products suited for a prophylactic use only. In other words, they are neither *systemic* nor *curative*. The progress during the past 15 years in the development and introduction of systemic and curative fungicides

Table 6. Projected 1980 world fungicide market (sales in US $ millions) by fungicide group.

	Sales
Copper compounds	180
Dithiocarbamates	600
Phthalimides	515
Mercurials	40
Systemics	46

Source: Anonymous (1975).

Table 7. Terminology used to describe technical attributes of fungicides.

Fungicidal attribute	Complementary terminology for attribute
Mobility in the plant	residual systemic (acro-, basipetal, two-way translocation)
Role in protection	protective curative
Time of application during disease cycle	prophylactic eradicative

is certainly considerable - and encouraging. However, it has led to the appearance of a new problem too, namely, resistance. Resistance is caused by the exclusive and continuous use of a fungicide. Therefore, a larger variety of technically high-grade fungicides needs to be made available to farmers.

Chemicals are the foundation of the most manageable of the disease control tactics at our disposal. However, the present range of fungicides available have a number of limitations:

- Most of the fungicides available are *protectants*. This means that the farmer has to apply them following a prophylactic schedule, i.e. whenever the probability of infection has reached a certain threshold value. Other issues aside, the increasing energy costs of this method are causing a major problem. But even these chemicals could be used in a more sophisticated way if the forecasting methods were better. Currently, efforts are being made to improve these, as part of research on integrated disease management. Even so, because of their inflexibility in use, the value of

Table 8. State of chemical control of major fungal diseases.

State of control	Pathogens attacking leaves, twigs and fruits	Pathogens attacking roots and stems
good	downy mildews, late blight, powdery mildews, rusts, smuts, leaf-spot pathogens (Venturia, Pyricularia, Septoria, Helminthosporium), fruit rots.	Pythium, Phytophthora
fair	Botrytis, leaf-spot pathogens (Alternaria, Cercospora, Stemphylium)	Rhizoctonia, Pseudocercosporella
weak	vascular wilts, (Phoma tracheiphila, Ceratocystis), Nectria, Geotrichum	Fusarium, Verticillium
none	Chondrostereum	Phymatotrichum, Armillaria, Gaeumannomyces, Plasmodiophora.

protectants in integrated control programmes is limited.
- The new *systemic* compounds, even though they have a significant *curative* action, are widely used according to the standard pattern, i.e. as if they were protectants. In other words, farmers have not yet learnt to make full use of their particular features: their systemic mode of action and their 'kick-back' (curative) activity. No doubt, they need to be educated to make a more intelligent use of these fungicides. More research is needed that will demonstrate that they can change the way they use fungicides without running the risk of falling profits. Of course, the degree of change possible varies from crop to crop. Here, too, more applied research needs to be done. Systemic fungicides do seem to be much better suited for integrated control programmes - another area in which more research is needed.
- All plant-protection chemicals, and thus fungicides too, are applied wastefully. It is well known that irrespective of the application method used, the major portion of the chemical does not reach its target, i.e. the plant pathogen. Of the three most common application methods, namely foliar, soil and seed application, the latter gives the best ratio of utilization, although it is still far from being satisfactory (Graham-Bryce & Hartley, 1979). Thus there is ample space for improvement in application technology, which will eventually lead to a more precise, judicious and economic use of existing fungicides. Their biological potential is greatly underutilized at present.
- A limitation of increasing importance is the restrictive policies of many national registration authorities: it is becoming more and more difficult to register product mixtures and products for special crops.
- A further, but only minor, limitation of fungicides presently used is their toxicity to certain crops. Of course, none of them would be marketed unless they met the needs of at least one particular crop plant or crop group, but wider use would be possible if they were better tolerated by other crop plants. Examples of such limitations are the copper sensitivity of many row crops and fruit trees, the zineb sensitivity of pears, and the general phytotoxicity of dinocap and sulphur in hot weather.
- The physico-chemical incompatibility of certain fungicides limits their combination in mixtures that would be desirable for disease control, including the reduction of the risk of resistance, and the saving of energy through joint application.
- Human and environmental hazards limit the present use of fungicides, although one must admit that no major problems have so far arisen when the instructions for use have been followed. There have been some severe cases of acute poisoning, for example in Iraq in the 1970s following the consumption of mercury-treated seed, but, this and most other known cases can be put down clearly to misuse. There is little documented evidence of adverse environmental effects due to use of fungicides. But that situa-

tion has changed recently in so far as both the ethylenebisdithiocarbamates and some of the phthalimide fungicides have come under RPAR (rebuttable presumption against registration) by the Environmental Protection Agency in the USA because of reported chronic toxicity. Since these cases are still under investigation, it would be premature to comment further here.

Conclusions

From the information presented in this contribution, there can be little doubt about the leading role of chemicals in the continuous fight against plant diseases. Albeit the present range of fungicides looks fairly broad and satisfactory, it has great limitations in view of the needs of the integrated approaches to disease control that are attracting more and more attention in applied research. The dominating conventional fungicides do not have the flexibility of use needed for such approaches, and the range of systemic, curative products is still too small. In addition, little experience has been gained to date in coping with resistance to these products, a phenomenon that is seriously threatening their usefulness. Thus, more effort by academia and industry is needed to understand the various aspects of resistance and to develop viable use strategies. Beyond this, the search for new types of fungicidal molecules, to broaden our spectrum of weapons, is needed. A further area that calls for more attention in basic and applied research programmes is application techniques. The high percentage of the product quantities applied that never reaches its final target clearly indicates the need for improvement. Recent advances in seed treatment technology and electrostatic spraying systems should stimulate this avenue of research.

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Socio-economic impact of fungicide resistance

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Abstract

The impact of development of fungicide resistance on various groups in society, namely, farmers, consumers, manufacturers, extension officers and regulatory authorities, is discussed. Special attention is paid to the position of the agrochemical industry on the problem of fungicide resistance.

Keywords: fungicides, resistance, registration policy, industry-adviser co-operation, resistance strategies, agrochemical industry.

Introduction

When development of resistance to fungicides became a problem in practical agriculture - after the introduction of systemic organic molecules - discussion began about strategies that could be used to cope with this phenomenon. If the strategies that have been developed prove successful in practice, resistance may have no socio-economic impact. However, since they are still unproven and controversial, and since most of the strategies developed by man in his continuous fight against harmful organisms have turned out to have shortcomings and limitations, it is certainly worthwhile to look at the socio-economic impact of fungicide resistance in more detail. Yet, this is a fairly new topic, about which very little information is available. Thus I am forced to philosophize on the subject rather than present a great deal of facts.

The title of this contribution refers to two major aspects: social impact and economic impact. But since they are strongly linked, they will be treated as one. To begin, I will put a question. 'If resistance arises, who is going to suffer from it, or, in other words, upon which groups in society is it going to have an impact?' The groups that may be affected are:

- the user (farmer)

- the consumer
- the manufacturer
- the adviser (extension officer)
- the national regulatory authorities
- the agrochemical industry.

Before discussing the impact of fungicide resistance on these groups, attention will be paid to pest control, which has had to contend with the problem of resistance to synthetic insecticides.

Experience with insecticide resistance

Experience with insecticide resistance, which has been known for several decades (Brown, 1977), has some valuable lessons for us on the impact of resistance. Resistance is a common phenomenon in all the major chemical classes of insecticides introduced to date, primarily with insects that are either vectors of human microbial pathogens or affect public hygiene. In this area certainly, resistance has had a strong social impact in the past and it continues to do so today. It now seems that vector resistance to pesticides is the biggest single obstacle in the struggle against vector-borne diseases, and it is largely responsible for preventing the successful completion of some control programmes, mainly in developing countries (Pal, 1976). In the case of malaria, for example, resistance in mosquitoes has developed in 14 countries, and the total population of areas affected by it is estimated to be 250 million, which represents about one third of the total population of the malarious areas of the world. In this case resistance has a manifold impact:
- it exposes the population to a higher disease risk, thus affecting human health directly
- the change-over, due to resistance, from DDT to other insecticides has led to a substantial increase in the treatment costs, causing severe hardship in developing countries, which are often unable to defer funds from other urgent needs
- it has forced WHO and other international bodies to develop new strategies for the effective control of malaria vectors.

Clearly, it is in the control of human disease that insecticide resistance has had its strongest socio-economic impact to date. But also in insect control in agricultural crops, resistance has caused socio-economic problems. Cotton, the crop upon which more insecticides are applied world-wide than all other crops combined, may be used as an example. Even though resistance is a wide-spread phenomenon in this crop, as an economic problem it is still restricted to relatively small areas for most pest species, as a recent survey conducted by Ciba-Geigy has shown. Probably, the problem caused by the cotton bollworms (*Heliothis virescens, H. zeae*) on the American continent in the 1960s is one of the few examples

of heavy economic losses due to the development of resistance (Reynolds, 1976). Not only farmers suffered, by losing profit and by being forced to switch to other crops, but also the economy of some countries was badly damaged. In Nicaragua, a country that relies on cotton for 20 % of its total exchange earnings, production decreased annually by 16 %, which almost resulted in the country going bankrupt. However even in such severe cases problems were overcome by the use of integrated control strategies, which involved in particular the more intelligent use of new pesticides (Vaughan & Leon, 1976). In spite of all the experience gained in insecticide resistance, Brown (1976) was right in stating that 'we must agree that our pest management in face of the insecticide resistance factor over the past 30 years leaves considerable room for improvement'. I would add to this statement that since the question is no longer 'if' resistance against insecticides develops, but rather 'how soon' (Reynolds, 1976), such improvements are urgently needed.

Impact of fungicide resistance

On the farmer

Several cases of serious resistance have occurred in practice. First resistance to the pyrimidines, later to the benzimidazoles, and, most recently, to the acylalanine metalaxyl. Where resistance occurred, it certainly had an impact on the farmer, as he lost part of his profit because of the attack on his crop by resistant strains. This was the case in northern Germany when the apple scab (*Venturia inaequalis*) developed resistance to benzimidazole fungicides, as did *Cercospora beticola* on sugar-beets in northern Greece, and in Western Europe when late blight in potatoes (*Phytophthora infestans*) developed resistance to metalaxyl (Staub & Schwinn, 1980). However, before more can be said about the economic impact of resistance on the farmer, exact figures of losses are needed. To my knowledge, they do not exist for fungicides.

On the consumer

In sharp contrast to the farmer and the other groups mentioned in the introduction, the consumer is not immediately affected by fungicide resistance. He will only realize that resistance has become a problem in practice when, because of severe losses caused by resistant pathogens, either food costs increase or certain commodities, like flowers or fruits, are locally and temporarily unavailable. Therefore in the developed countries, at least, with their broad international base of food supply, the consumer will not suffer because of resistance. In developing countries however, if resistance occurred in a staple crop or a high-

value export crop, it might have immediate impact on their populations and national economies.

On the manufacturer

Nowadays manufacturers are confronted with a new situation of product liability as resistance to newly introduced fungicides has developed. It is known that farmers who have lost money through the occurrence of resistant strains in their crops have filed suits against chemical companies, who the farmers hold liable for product failures. This is a new development as the product liability of the manufacturer was previously limited to the technical quality of the product. A number of the law suits have been won by the companies, but since other suits are not yet settled, I will refrain from commenting on them further. However the possible consequences for the manufacturers of fungicides may be considerable, and they may influence them in their sales and research activities in the future. For example, if resistance occurs in a speciality crop grown on a small area (e.g. onions or cucumbers) the financial consequences of resistance for a company may be much higher than possible sales revenues over the years. Thus a company may decide not to register resistance-prone products for such crops. Legalities aside, the farmer is in principle entitled to technical guidance from industry - and his extension or advisory service, thus resistance has an immediate impact on these two groups, too.

On the adviser

The main duty of an extension officer is to advise the farmer which fungicides he should use at what time with what spraying schedule, to obtain an optimal yield. The adviser collects or generates his knowledge from technical information from the manufacturer and from his own experimental work. Further, he relies on the findings of workers active in fungicide research. Nobody can blame advisers for not having foreseen the risk of resistance to the first systemic fungicides appearing on the market, the benzimidazoles and pyrimidines, which were widely used by farmers because of their attractive qualities as fungicides. In the light of this practical experience, and now that there is evidence from research and practical experience, that other groups of modern fungicides are also subjected to resistance, advisers are, with good reason, extremely keen to learn as early as possible whether such a risk exists with any new type of fungicide. The farmer, too, now expects advisers to be aware of these risks and to know the strategies that must be used to minimize the potential damage to his crops. There is, therefore, a clear need for the evaluation of the risk of resistance to become a part of the routine product-development procedure - a direct impact on the chemical industry.

On regulatory authorities

So far, when filing petitions for the registration of a new pesticide, the chemical industry has had to demonstrate the pesticide's biological effectiveness, its usefulness for agriculture and its biochemical and toxicological harmlessness. With the increasing awareness of resistance as a potential threat to the efficacy of an agricultural chemical, the authorities may well ask the manufacturer for a statement about the potential risk of resistance developing to the chemical. But a knowledge of the potential risk should also encourage the regulatory authorities to be more cautious in restricting the use of older fungicides, like the dithiocarbamates or the phthalimides, that so far have not been subjected to the risk of resistance. Their availability is crucial to current strategies for coping with resistance, for it is these products that are the alternatives - alone or in mixtures - to chemicals that have met resistance. Thus the appearance of novel and potent products for disease control should by no means lead to cancellation of old but still useful protective products by the registration authorities. The broader our arsenal of weapons against the pathogens is, the better are our chances of success against resistance, especially using strategies of application that combine biochemically different fungicides. Actually, the loss of effective fungicides due to regulation would increase the possibility of resistance, by increasing the demand for new chemicals (most likely systemic). At the same time strategies for the prevention of resistance would be limited in their value or diversity. Therefore it is with mixed feelings that I await the outcome of the pending RPAR investigations by the Environment Protection Agency (EPA) of various major protective fungicides in the USA. It is in the interest of all groups involved in plant protection that these important tools for the control of pathogens remain at our disposal (Anonymous, 1979).

On the agrochemical industry

The agrochemical industry has been exposed to resistance to insecticides for several decades. During most of this time development of resistance may have meant a short commercial life for a new product in a certain market segment. However, to my knowledge it never led to the complete disappearance of an insecticide from the market. Indeed, some observers believe that resistance was one of the driving forces of the development of new insecticides. Besides this, increasing research efforts have been made over the past years to understand the various aspects of resistance as a biological phenomenon and to develop strategies to cope with it (Plapp, 1979; Dittrich, 1979; Georghiou, 1980). Thus industry could adopt a simple 'wait and see' attitude on fungicide resistance.

But, in the meantime, the situation has changed so radically that such a policy is no longer valid. First, the search for new chemicals for insect and disease control, and their development and introduction into the market, is getting more costly all the time, because the actual development costs are rising continually and because the chances of finding new products are decreasing. Nowadays, the development costs for a new fungicide are in the order of US $ 20 - 30 million, and there are not many market segments that can support such an investment. Therefore, the prospects for the size of the envisaged market and the life-time of a product must be promising. It is certainly not realistic to expect chemical companies to develop systemic fungicides with a potential risk of resistance for emergency use only, i.e. upon the outbreak of heavy epidemics or when multi-site-inhibiting fungicides are not effective. Thus, the loss of a newly introduced fungicide because of the development of resistance has such strong financial repercussions that no company can take a simple 'wait and see' position. Once a product has been introduced into the market, at the cost of much time, expense and effort, the development of resistance must be held at bay for as long as possible.

What can a company do? What are the chances of reasonably predicting the risk? Checking the risk of resistance must become a regular element of the development process of a new fungicide, a process which comprises several steps of biological testing under an increasing range of climatic conditions over a period of several years. That checking procedure could easily include model studies, in vitro and in vivo. One could even envisage special field trials to test for the build-up of resistant strains. However, the problem remains of how to judge the findings of such experiments, provided they yield meaningful results. If they are favourable, i.e. if no resistance emerges, does it mean that it will not occur after the products' introduction into the market, especially if it is highly attractive, and therefore will be widely, probably exclusively used? If they are unfavourable, i.e. if resistance emerges, does it mean that the compound should be dropped for further development?

It is the company researcher who is in the decisive position here, because it is he who advises his company's management, which in turn has to set priorities and allocate funds for product development. Even if he can convince his management to go ahead with development, and even if the compound surmounts the hurdle of registration, the question which still remains is whether to introduce a new fungicide aggressively and extensively with the risk of shortening its life or to promote it moderately and speculate on a possibly longer life-time.

Competition in the market place and the need to recoup investment and reach the zone of profit as early as possible during the limited life-span of a product usually dictate aggressiveness. However, even if the company decides to follow a careful policy, the farmer may overturn it by

simply prefering to use the new fungicide. Because of specific features, such as its systemic translocation and curative effectiveness, for example, it may be so attractive to him that he tends to use it exclusively and continually; in other words in a way which, if the risk inherently exists, almost inevitably leads to resistance. Marketing experts know how hard it is to convince the farmer to temporarily refrain from using an effective product unless he understands the reasons for such restrictions. Moreover, as long as his neighbour continues to use it, he is likely to do the same. Strategies for use must therefore be enforceable, otherwise they are inevitably unsuccessful. This, in turn, means that the best theoretical strategy may not necessarily be the most successful one in practice.

Chemical companies need the collaboration of agricultural advisers to encourage and enforce promising strategies in farming practices and to educate the farmer in new use concepts. Since the chemical industry and advisers are directly affected by failure due to resistance, they are bound to find ways and means of cooperation in this matter. Beyond this, the chemical industry may be forced to make some changes in its marketing philosophy, which to date aims for maximum sales in the shortest possible time. Further, the understanding of resistance needs to be improved in the marketing organization, and in my opinion it is now time for companies who sell fungicides of similar mode of action, to develop jointly strategies or rules to be followed in order to keep the risk of resistance low.

Conclusions

Resistance certainly has the potential to increase the farmers cost of production and decrease his profits, unless he can pass on the increased costs to the consumer or the manufacturer. Resistance also complicates the life of the agricultural adviser. But, above all, it adds to the already considerable uncertainties which the agrochemical industry faces when developing and marketing fungicides, or more generally, pesticides.

Nevertheless, the fact that resistance has developed has certainly had the positive and beneficial effect of forcing all groups to think more carefully about how to use pesticides in the best and most economical way. And it has opened up a new and broad discipline of both fundamental and applied research. It may well be that some of the integrated pest management (IPM) strategies, which are being developed in various countries and for certain crops (e.g. apples, potatoes, bananas and horticultural crops), for which disease control has reached a high level of technology, will turn out to be useful for preventing the build-up of resistance.

If all those involved can reach a general understanding as described in this paper and, especially, if industry, researchers and advisers co-

operate closer in defining the resistance risk, in developing adequate realistic strategies to prevent or delay it, and in educating the farmer to use pesticides in an intelligent way, there may still be a chance that the socio-economic impact of fungicide resistance will remain small. But given the many uncertainties, it is unrealistic to expect the immediate appearance of a magic formula for long-term success. It remains to be seen whether there is enough time left for us to develop and introduce new concepts of use that are urgently needed for the new fungicides, those we have and those to come.

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Detection and measurement of fungicide resistance

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Abstract

The general principles and methods useful for the detection and the measurement of fungicide resistance are described. Some of the possible errors in determining response to various concentrations and in sampling the fungal material are indicated.

Keywords: base-line sensitivity, extent of resistance, degree of resistance, fungicide solubility, dosage response, monitoring.

Introduction

Fungicide resistance may be detected and measured in various ways, depending on the fungus-fungicide combination; some examples of agricultural significance will be examined in the case studies presented later in this book. The general principles are the same, however. As with other organisms, the recognition of resistant strains of fungi must be made by comparison with data obtained with sensitive strains. Accordingly, it is essential that the 'base-line sensitivity' for the fungus-fungicide combination in question be established either by appropriate experiments with strains that are unquestionably wild type with respect to sensitivity to the fungicide involved or by the use of data from the literature. It must be remembered that toxicity may vary not only with fungicide concentration but also with inoculum size, composition of medium, temperature, pH, etc. For this reason the suspected resistant strains must be studied by the method used to establish the base-line sensitivity, under exactly the same conditions.

After resistance has been detected there are two parameters that should be measured: the extent of resistance, i.e. the proportion of the population that no longer exhibit the normal sensitivity of the species; and the degree of resistance, i.e. the magnitude of the difference in sensitivity. With some selective fungicides, e.g. the benzimidazoles, the

carboxamides, and the acylalanines, great differences in sensitivity between wild type and resistant strains exist. In such cases the detection and the measurement of resistance is fairly easy. With some other fungicides, e.g. dodine and members of the aromatic hydrocarbon group, the differences are small, although often very significant in practical agriculture, and detection and measurement require careful testing.

Methods in general use

Since the detection of resistance is essentially a matter of recognition of a difference in sensitivity, it is important that in each case the most appropriate method for an accurate determination of sensitivity is chosen. For some arthropod pests, standardized test methods have been prepared by the FAO Panel of Experts on Pest Resistance to Pesticides; these are accepted internationally. It is expected that in the near future such standardized test methods will be approved for some of the most important fungus-fungicide combinations.

Descriptions of several methods for the measurement of fungitoxicity can be found in the literature (American Phytopathological Society, 1947; Horsfall, 1956; Peletier, 1978; American Phytopathological Society and Society of Nematologists, 1978). Such methods have been adopted by several investigators for the recognition of resistant strains and the measurement of the degree of resistance.

The rate of increase of colony diameter on treated agar medium appears to be the most frequently used criterion for measuring fungitoxicity. Usually agar blocks of a few millimetres diameter are taken from the periphery of colonies grown on control medium to be used as inoculum. After incubation the diameters of colonies produced on fungicide-containing and control media are measured and from this the diameter of inoculum should be subtracted. This method has been used for the detection and the measurement of resistance to organomercurials in *Pyrenophora avenae* (Greenaway & Cowan, 1970), to diphenyl and sodium 2-phenylphenolate in *Nectria haematococca* (Vomvoyanni & Georgopoulos, 1966), to dyrene in *Sclerotinia homoeocarpa* (Nicholson et al., 1971) to dodine in *N. haematococca* (Kappas & Georgopoulos, 1968) to organotins in *Cercospora beticola* (Giannopolitis, 1978), to benzimidazoles in *Botrytis cinerea* (Bollen & Scholten, 1971; Miller & Fletcher, 1974), in *Fusarium oxysporum* (Sozzi & Gessler, 1980), and in *Sclerotinia fructicola* (Whan, 1976; Penrose et al., 1979), to dicarboximides in *Botrytis cinerea* (Gullino & Garibaldi, 1979), to polyoxin in *Alternaria kikuchiana* (Nishimura et al., 1973) and to kasugamycin in *Pyricularia oryzae* (Taga et al., 1978).

The rate of dry weight increase in liquid media containing the fungicide is used less frequently for measuring fungitoxicity because its measurement requires more time. It is, however, a more accurate criterion

than colony diameter. For organisms with yeast-like growth the increase in turbidity can be used as a criterion of growth. Liquid media have been used for the measurement of cycloheximide resistance in *Saccharomyces cerevisiae* (Wilkie & Lee, 1965), of resistance to benzimidazoles in *Aspergillus nidulans* (Hastie & Georgopoulos, 1971) and *C. beticola* (Georgopoulos & Dovas, 1973) and of resistance to carboxamides (Georgopoulos et al., 1972) and chloroneb (Tillman & Sisler, 1972) in *Ustilago maydis*.

Plating spores or other propagules on agar media containing a fungicide concentration that completely prevents the growth of the wild type is the usual method for first detecting resistance and its subsequent monitoring. Similar plating on varying concentrations of the fungicide and colony counts have been used less frequently to compare resistant and sensitive strains. Examples include resistance to dodine in *Venturia inaequalis* (Mac Neil & Schooley, 1973; Polach, 1973) to benzimidazoles in *A. nidulans* (Hastie & Georgopoulos, 1971), *Cercospora arachidicola* (Littrell, 1974), *Pseudocercosporella herpotrichoides* and *Septoria nodorum* (Horsten & Fehrmann, 1980), and to dicarboximides in *B. cinerea* (Maraite et al., 1980). A modification of this method was used by Jones & Walker, (1976), who, instead of incorporating dodine into the agar medium, used dodine solutions to impregnate assay discs, each of which was placed in the centre of a plate on which conidia of *V. inaequalis* had been plated. Sensitivity was then measured by the diameter of inhibition zones.

Germination of spores in fungicide solutions or on media containing fungicide has been used to detect and measure resistance to diphenyl, sodium 2-phenylphenolate (Vomvoyanni & Georgopoulos, 1966) and dodine (Kappas & Georgopoulos, 1968) in *N. haematococca*, to benzimidazoles in *V. inaequalis* (Wicks, 1974; Jones & Walker, 1976) and to fentin derivatives in *C. beticola* (Giannopolitis, 1978). It is often necessary in this type of test to consider not only germination but also germ-tube elongation and morphology. For fungi with yeast-like growth, bud formation rather than spore germination is the criterion used (Wilkie & Lee, 1965).

For fungicides with known pronounced effects on a cellular process, e.g. respiration or protein synthesis, the sensitivity of various strains is very appropriately measured by the effect of varying toxicant concentrations on the process. These experiments can be done either with whole cells (in vivo) or subcellular preparations (in vitro). Examples are the studies on carboxamide resistance in *U. maydis* (Georgopoulos et al., 1972, 1975; White et al., 1978) and *A. nidulans* (Gunatilleke et al., 1976; White et al., 1978) and cycloheximide resistance in *S. cerevisiae* (Cooper et al., 1967) and *Neurospora crassa* (Vomvoyanni, 1974).

Resistance can also be detected and measured on living, appropriately treated, plants of plant parts. This method is practically indispensable in the case of obligate parasites, e.g. the powdery mildews (Schroeder &

Provvidenti, 1969; Bent et al., 1971; Vargas, 1973; Shephard et al., 1975; Dekker & Gielink, 1979) and the downy mildews (Georgopoulos & Grigoriu, 1981; Katan & Bashi, 1981; this book, p.118-127). Details on experiments with these two groups of fungi are given on p.219-230 and p.118-127. The method is also applicable in the case of non-obligate parasites, e.g. the smuts (Kuiper, 1967; Georgopoulos et al., 1975).

Adjusting fungicide concentration

For measuring sensitivity in artificial media it is advisable to use a form of the fungicide that is as pure as possible. If only the commercial product is available there is always the possibility that other components of the formulation will affect the results, particularly in the case of resistant strains, for which higher amounts have to be used. Recrystalization from an appropriate solvent can be used to separate the active ingredients from some but not all other components. If blind formulations are available they should be used as controls.

The low water solubility of most fungicides is one of the commonest sources of error when measuring resistance. With some compounds, particularly the structurally non-specific, Ferguson-type toxicants, such as the chlorinated nitrobenzenes (Eckert, 1962), activity may increase beyond saturation. However, in general, material that is not in solution is inactive, so that increase of the amount of fungicide in a medium beyond solubility does not give an increase in effect. Insoluble reserves of compounds that tend to be accumulated by fungal cells may serve to replenish the amounts that are taken up, but what portion becomes active in this way is not easy to determine in all cases. Solubility increases if a mixture with an organic solvent is used, but most solvents are toxic and only small amounts, usually not exceeding 2.0 %, can be used. It is best to first prepare stock solutions of the fungicide in a suitable solvent and then add small amounts to the medium, making sure that no precipitate is formed; the same amount of solvent must be added to the controls.

After the response of the strains studied to the various concentrations of the fungicide has been determined, the degree of resistance is usually measured by a comparison of: the responses to the same concentration; the minimal growth inhibiting concentrations (MIC); or the concentrations that produce the same effect (e.g. ED_{50} or ED_{95}). Depending on the slope of the dosage-response curves, the first method may overestimate the degree of resistance. The second method is also not very dependable, particularly for fungicides of low water solubility. In work with sensitive strains, solubility (or volatility in the case of vapor-phase fungicides) limitations may be of little concern. Resistant strains, however, are often not completely inhibited at saturation and thus the MIC cannot be

determined in an acceptably accurate way. Since the method does not consider the effect of concentrations causing less than total inhibition, resistant strains are frequently considered to totally lack sensitivity. If accuracy is important, it is best to use the third method, i.e. to obtain the dosage-response curves by plotting response against only accurately determined concentrations. The lines are then compared on their position and slope, and the degree of resistance is given by the ratio of concentrations that give the same percentage of inhibition in the wild type and the mutant strain.

Fungal material

The objective of the test determines the amount and the type of fungal material used. During the development of a new fungicide, information may be required on its ability to select out resistant strains if it is applied commercially for plant-disease control. In this situation it is improbable that resistance may be readily detected in natural populations of the potential target organisms, unless these populations have previously been exposed to a related (in terms of cross-resistance) chemical. The usual test is to expose several millions of cells treated with a mutagenic agent to a concentration of the fungicide under development that completely inhibits the wild-type fungus. The mutagenic treatment should be sufficient to kill a large proportion of cells. The mutagen usually delays the onset of growth of survivors, which sometimes necessitates long incubation. If only a few of the survivors eventually grow in the presence of the fungicide, these probably represent resistant mutants. But if many colonies grow on each plate, it is likely that the long incubation caused a breakdown of the fungicide and the growth of non-mutant cells.

After commercial application begins, it is very desirable to be able to detect changes in sensitivity in the pathogen population at an early stage, before such changes result in failure of the fungicide and extensive crop losses. This requires testing of a very large number of isolates, each of which should represent an independent infection. Testing many spores from a few lesions (or from a few plants in the case of systemic infections) does not increase the sample size. In addition, it is important to use random samples that represent the whole of the population under consideration.

If the fungicide fails to control the disease, that failure cannot be ascribed to the development of resistance unless resistant strains of the pathogen represent a very considerable portion of the population in the area and those strains are able to cause disease on treated plants. Detection of resistance when disease control fails requires testing of a rather small number of isolates. However, it is important to collect sam-

ples from the well treated areas with the severest infection. If resistance is shown to be involved and the fungicide is withdrawn, monitoring is necessary to follow changes in the pathogen population as to the extent of resistance. This requires more-or-less random samples. If possible, each sampling should examine a new infection cycle of the pathogen. For this reason old infections should be avoided, e.g. by avoiding older leaves.

When transferring from the diseased material care must be exercised to avoid residues of the fungicides with which the plants have been treated in the field. In the case of non-systemic fungicides this is made easier by a thorough washing of the samples, which also removes old spores, which, perhaps, have low germinability. Then the material can be kept under conditions allowing renewed sporulation. An accurate description of resistance requires fungal material that is as genetically homogeneous as possible. Pure cultures of the strains to be compared must be established, preferably by transferring uninucleate, haploid spores.

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Mechanism of action of fungicides

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Abstract

A survey is given of the mechanism of action of a variety of protectant and systemic fungicides. Occurrence of resistance in the laboratory and in the field is indicated for most of the fungicides.

Keywords: protectant fungicides, systemic fungicides, mechanism of action, inhibition of biosynthesis, inhibition of respiration.

Introduction

For a proper understanding of the mechanisms of resistance to fungicides some general knowledge of their mechanism of action is essential. Therefore this contribution comprises a broad survey of the various mechanisms of action recognized so far for agricultural fungicides; close derivatives of several of these fungicides are used in human and veterinary medicine. Fungicides for which up till now no resistance is known have also been included.

Much detailed information on mechanisms of action of fungicides is available in various chapters of the monograph 'Antifungal Compounds' by Siegel & Sisler (1977); a comprehensive survey of the mode of action of systemic fungicides was published by Kaars Sijpesteijn in 1977. Therefore only references to publication since then are cited in this present review.

Mode-of-action studies require a great variety of experiments. The observed responses to the fungicide have to be critically analyzed to establish which effect is the primary cause of growth inhibition at the lowest effective dose of the toxicant. Many secondary effects may result from the primary effect. Moreover, for certain compounds additional effects become apparent at higher concentrations.

Many compounds primarily affect respiration or, more generally, energy production; many others affect biosynthetic processes; and a few com-

pounds destroy cells, by directly affecting membrane function. With the exception of 'Dexon', carboxin and carboxin-related compounds, fungicides affecting respiration are not systemic. In contrast, those affecting biosynthetic processes generally appear to have systemic properties.

In the survey most of the known mechanisms of action of agricultural fungicides are summarized. Fungicides whose modes of action are unknown or not known for certain are also included. Note that if a mutant is resistant to different fungicides it does not necessarily point to the same mode of action of those fungicides. However, it does indicate that this may be the case. Structural formulae of the toxicants discussed are given in Appendix A to this contribution.

Fungicides that interfere with energy production

Specific inhibitors

There is much evidence that the dimethyldithiocarbamates, e.g. thiram and its salts, at low concentration quite specifically inhibit the dehydrogenation of pyruvate. At such concentrations, a 1:1 complex of copper and dimethyldithiocarbamate is formed. Probably this combines with the dithiol compounds (lipoic acid or lipoic acid dehydrogenase) that are essential for dehydrogenation of pyruvate (Kaars Sijpesteijn & van der Kerk, 1965). At higher concentrations other mechanisms operate. Pyruvate dehydrogenation is also the target of seondary butylamine ($CH_3CH_2CHNH_2CH_3$), which is used to combat *Penicillium digitatum* (Yoshikawa & Eckert, 1976).

Carboxin, a systemic fungicide with a rather narrow antifungal spectrum, interferes with respiration, causing a block in succinate dehydrogenation. The site of action lies between succinate and coenzyme Q in the succinate-ubiquinone reductase complex of the electron transport system. The actual target of carboxin may be the nonheme-iron sulphur protein associated with this enzyme complex. Resistant mutants can easily be obtained in vitro. The related compounds mebenil and pyracarbolid act in the same way and this may also be the case with the later-developed carboxamides, e.g. methfuroxam.

'Dexon' acts rather selectively on Oomycetes; it seems to inhibit some flavin enzymes involved in the dehydrogenation of reduced nicotinamide adenine dinucleotide (NADH) in the respiratory chain (cf. Schewe & Müller, 1979).

There is much reason to believe that triphenyltin compounds, such as fentin acetate, interfere with fungal growth by inhibiting oxidative phosphorylation. Serious resistance in the field has been described by Georgopoulos in his discussion of fungicide resistance in *Cercospora beticola* (p.187-194). Dinocap and other dinitrophenols act by uncoupling oxidative phosphorylation from respiration.

Unspecific inhibitors

A group of fungicides that inhibit respiration quite unspecifically are the thiol reagents, among them zineb, maneb, chlorothalonil, captan, folpet, captafol and dichlone. These compounds, or their transformation products, react quite generally with -SH enzymes and other -SH compounds involved in respiration. Since they act at many sites they are often indicated as multisite fungicides.

Fungicides that interfere with biosynthetic processes

Inhibition of protein synthesis

The antibiotic cycloheximide inhibits growth by interference with protein biosynthesis. Low concentrations specifically inhibit this process at the stage where in a complex with the ribosomes, amino acids are transferred from tRNA into polypeptides. The precise mechanism and site of action is still not clearly known, however. For various fungal species, strains resistant to cycloheximide have been obtained in vitro.

The probable site of action of the antibiotic blasticidin-S is the final step of protein synthesis, which takes place on the ribosomes. The antibiotic kasugamycin, which like blasticidin-S is particularly active on *Pyricularia oryzae*, also affects some stage of protein synthesis. Considerable resistance to this compound has developed in the field.

Streptomycin is used against certain bacterial and fungal diseases. Its action on bacteria is ascribed to interference with protein synthesis, the primary target being the 30 S sub-unit of the ribosomes.

Inhibition of nucleic-acid synthesis

Ethirimol and demithirimol act exclusively on powdery mildews. Ethirimol has now been shown to inhibit specifically the enzyme adenosine deaminase in these fungi only. It is supposed that this enzyme, which converts adenosine to inosine, is essential for appressoria formation (Hollomon, 1979). In fungi insensitive to ethirimol, this enzyme was also insensitive. Although these data may suggest interference with nucleic acid synthesis, the picture is still far from clear. In practice, resistance to these compounds develops easily.

Metalaxyl and furalaxyl are particularly active against a variety of *Peronosporales*. There are very strong indications that metalaxyl interferes with nucleic-acid synthesis in *Pythium splendens*; presumably RNA synthesis is the primary site of inhibition (Kerkenaar & Kaars Sijpesteijn, 1981; Kerkenaar, 1981). Resistance in the field has been reported for certain pathogens.

Hydroxyisoxazole inhibits a variety of fungi. In *Fusarium oxysporum*, interference with DNA synthesis is its primary site of action (Kamimura et al., 1976).

Interference with nuclear processes

Benomyl and thiophanate-methyl are known to act after their conversion to the toxic agent carbendazim. The primary process inhibited by this broad-spectrum fungicide is mitosis. Carbendazim appears to form a complex with sub-units of microtubuli, thus preventing the normal assembly of microtubule sub-units into spindle fibres. In practice, resistance to the above compounds develops easily in certain fungi (see p.60-70). Recently thiabendazole was shown to have the same mode of action as carbendazim (Davidse & Flach, 1978).

The dicarboximides (vinclozolin, procymidone and iprodione) and the aromatic-hydrocarbon fungicides (chloroneb, hexachlorobenzene, quintozene, dicloran) are remarkably similar in their action. Moreover, many examples of cross-resistance have been observed for these compounds (Leroux, 1977). They all cause mitotic instability in *Aspergillus nidulans*, which points to interference with mitotic division (Georgopoulos et al., 1979). Nevertheless, the true nature of the primary effect is not yet clear. Interference with DNA biosynthesis has been observed for chloroneb (Tillman & Sisler, 1973) and vinclozolin, procymidone and dicloran (Fritz et al., 1977). No cross-resistance has been found with carbendazim.

Inhibition of cell-wall synthesis

The antibiotic polyoxin D inhibits chitin synthesis; the intermediate UDP-N-acetylglucosamine accumulates and protoplast-like cells are formed. The fungicide shows a structural resemblance with this intermediate substrate. It obviously acts as an anti-metabolite, resulting in inhibition of the enzyme chitin synthetase. Resistance to polyoxin D has been observed in practical agriculture.

Inhibition of lipid biosynthesis

The mode of action of IBP has recently been further clarified by Kodama et al. (1979). The compound is particularly active on *P. oryzae* and inhibits specifically the conversion of phosphatidylethanolamine to phosphatidylcholine by transmethylation of S-adenosylmethionine. Phosphatidylcholine is an important constituent of the membranes. Since IBP has some structural resemblance with the substrate phospatidylethanolamine it may well act as an antimetabolite of this substrate. Another phosphorothiolate fungicide, edinfenphos, has recently been shown to act in the

same way as IBP (Kodama et al., 1980).

As the incorporation of ^{14}C-methionine into phospholipids is also inhibited by isoprothiolane (Kakiki & Misato, 1979), the mode of action of isoprothiolane may well be the same; cross-resistance with IBP had already been observed by Katagiri & Uesugi (1977).

Many fungicides are now known to be sterol-biosynthesis inhibitors. Triarimol, a broad-spectrum fungicide that has been withdrawn from the market, was the first compound recognized to act in this way. In the years that followed several more-or-less related fungicides were developed for which a similar mode of action was observed: fenarimol (de Waard & Ragsdale, 1979), triadimefon (Buchenauer, 1976) and its reduced derivative triadimenol (Buchenauer, 1978), bitertanol (Kraus, 1979), imazalil (Leroux et al., 1976; Siegel & Ragsdale, 1978), and phenapronil (Girardet & Buchenauer, 1980). For prochloraz (cf. Leroux & Gredt, 1978), diclobutrazol (Bent & Skidmore, 1979) and CGA 64250 (Ciba-Geigy AG, Basel, Switzerland) a similar mode of action is very likely. It is remarkable that two unrelated fungicides triforine and buthiobate also act in a similar way.

All inhibitors of sterol biosynthesis cause a rapid alteration in the quantity and the nature of the lipid components in the cells. Especially the incorporation of ^{14}C-acetate into ergosterol was strongly inhibited at a very early moment, and several intermediates accumulated. Inhibition of demethylation at C-14 appears to be the principle site of inhibition in sterol biosynthesis. An effect on synthesis and function of cell membranes will certainly result from this; it may explain the morphological changes of the treated cells.

Tridemorph has many effects in common with these compounds (Kerkenaar et al., 1979); its exact target is not ^{14}C-demethylation, but a step following demethylation (Kato et al., 1980; Kerkenaar et al., 1981). Fenpropimorph may act similarly.

Although not always present, and even then not always to the same degree, in vitro cross-resistance has been observed between many inhibitors of sterol biosynthesis, (e.g. Barug & Kerkenaar, 1979). In the field, no resistance has so far developed.

Fungicides that interfere with cell structure

Dodine affects the permeability of the cell membrane, which results in leakage of the cell contents. Inhibition of respiration is a secondary effect (Brown & Sisler, 1960). The exact nature of the interference with the membranes is not yet known. Resistance in the field has been reported.

Fungicides whose mode of action is unknown or not known for certain

Presumably pyrazophos is itself not active. Its metabolite (PP), however, is highly fungitoxic to certain fungi in media of low pH. This metabolite affects respiration, but researchers are still uncertain whether this is its primary effect.

Prothiocarb and its derivative propamocarb are particularly effective against the *Peronosporales*, *Pythium* and *Phytophthora*. Prothiocarb does not inhibit respiration, which suggests it interferes with a biosynthetic process. Cholesterol, although not a cell constituent of *Pythium* somewhat antagonizes the fungitoxicity (Kerkenaar & Kaars Sijpesteijn, 1977). Similar effects were also described for propamocarb by Papavizas et al. (1978), who, moreover, observed leakage of cell constituents following growth in the presence of low concentrations of that fungicide. Papavizas et al. suggest that the mode of action might be related to cell-membrane function.

The action of prothiocarb on *Achlya radiosa* differs from that on *Pythium*. It can be ascribed to the effect of released ethylmercaptan (Kerkenaar & Kaars Sijpesteijn, 1977).

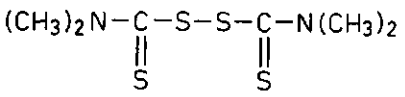
Etridiazole affects particularly *Phycomycetes*. Halos & Huisman (1976) reported that it caused inhibition of respiration of *Pythium* species. In contrast, Lyr et al. (1977) suggest that, in *Mucor mucedo*, the compound acts by liberation of phospholipases within the mitochondria.

'Curzate' (Dupont, Wilmington, USA) is effective in the combat of *Peronosporales* in the field. Little is known of its fungicidal activity in vitro. Unpublished results show inhibition of *Pythium* on agar media at relatively high concentrations.

Discussion

A short survey has been given of the mechanism of action of many of the fungicides used in agricultural practice or studied as experimental fungicides. In many cases resistant strains are known, especially for compounds that act on specific systems only. Compounds that protect the host plants by an indirect mechanism or are supposed to do so, e.g. tricyclazole, probenazole, CGA 49104 and 'Aliette', have not been dealt with because they are not fungicides.

Appendix A: Structural formulae of toxicants discussed in this contribution.



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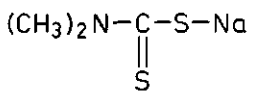

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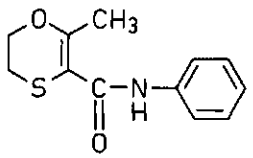

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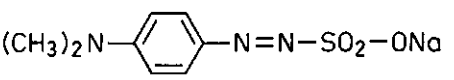
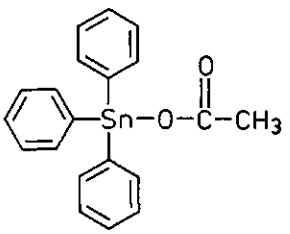


dexon



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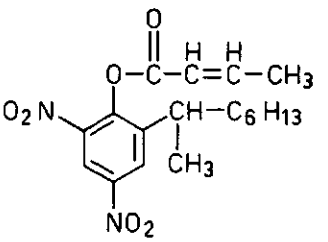

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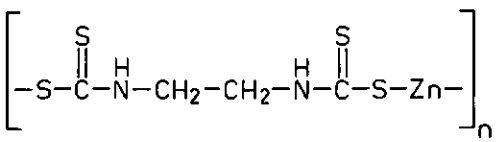

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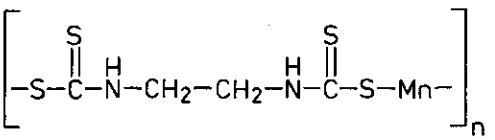

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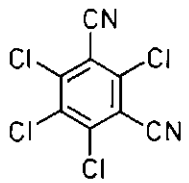

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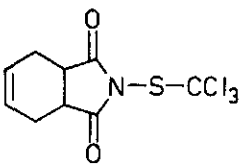

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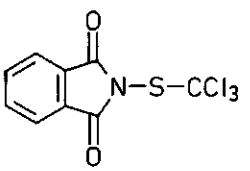

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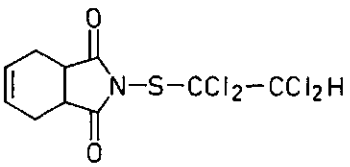

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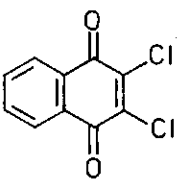

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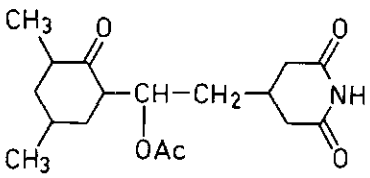

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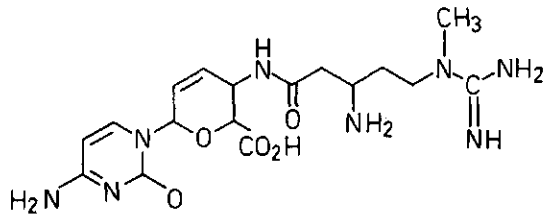

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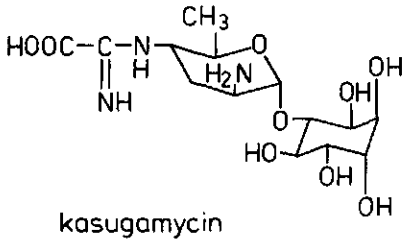
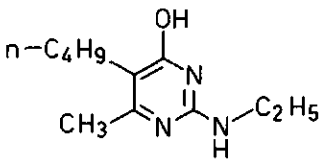


kasugamycin



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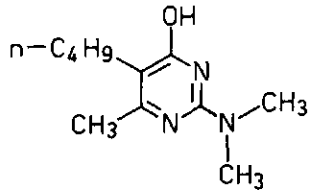

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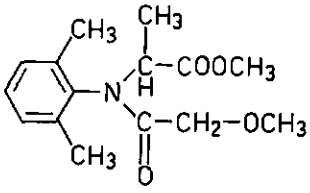

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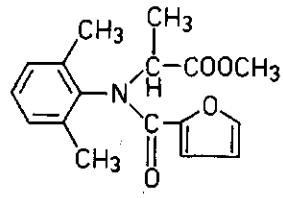

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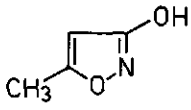

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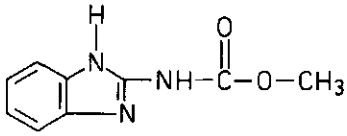

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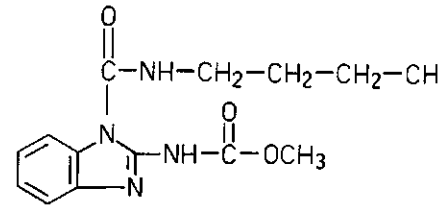

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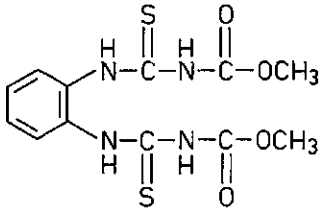

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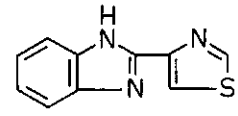

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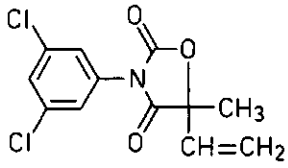

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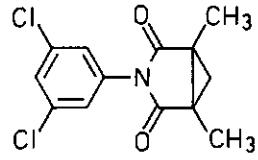

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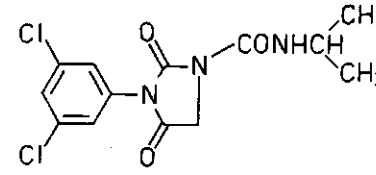

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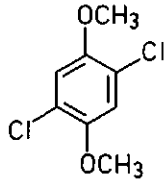

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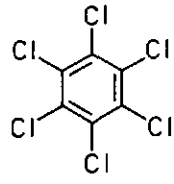

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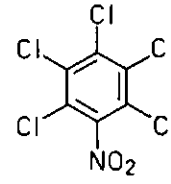

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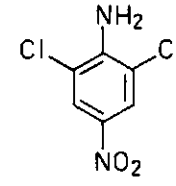

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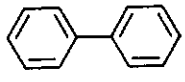

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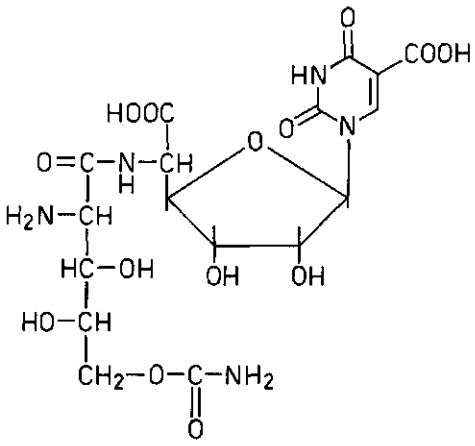

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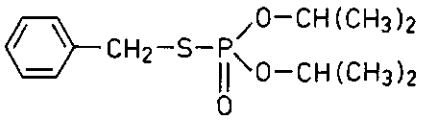

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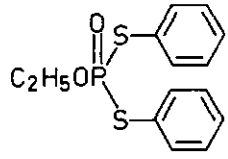

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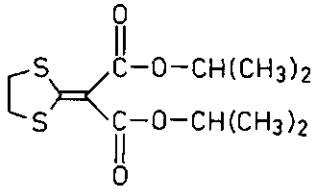

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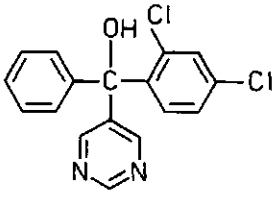

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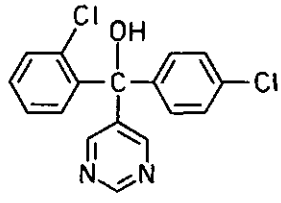

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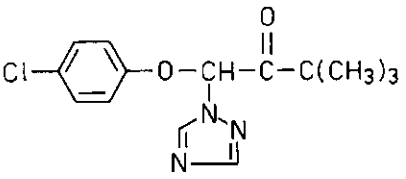

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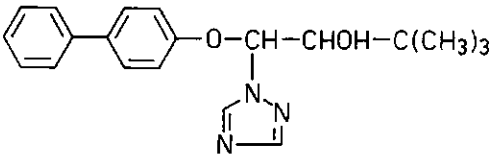

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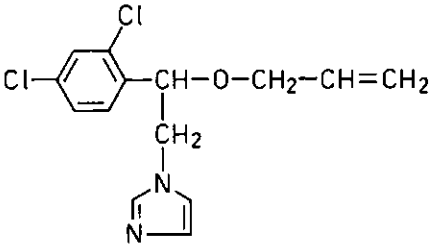

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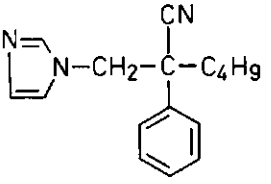

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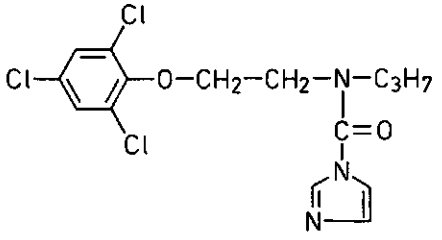

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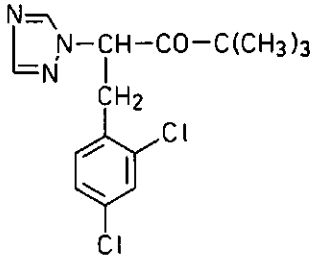

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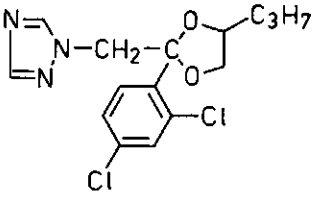

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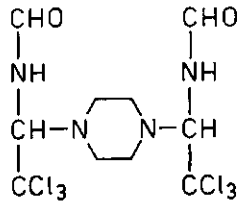

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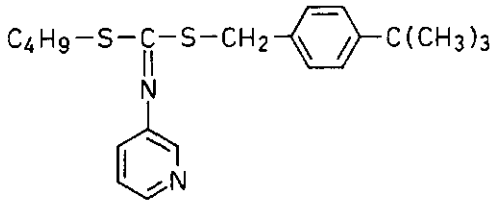

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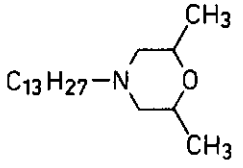

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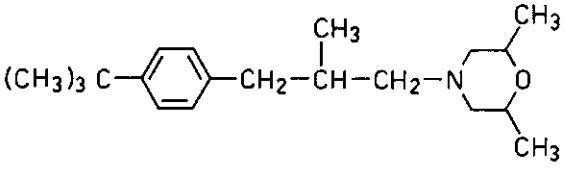

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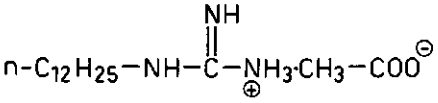

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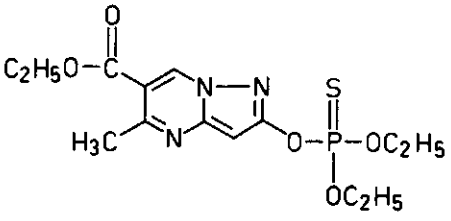

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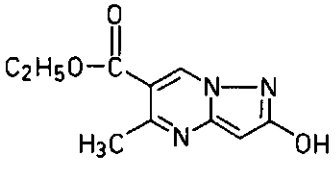
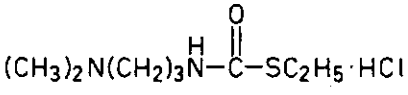


metabolite of pyrazophos



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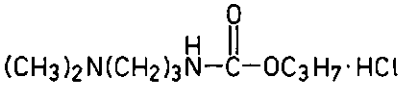

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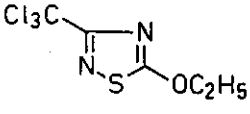

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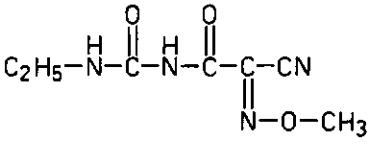

'Curzate'

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Genetical and biochemical background of fungicide resistance

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Abstract

It is explained how sensitivity to toxic compounds is controlled by genes that control a cellular process important for exertion of the toxic effect. Then attention is paid to the biochemical genetics of resistance to the main groups of agricultural fungicides. With most fungicides knowledge on either genetics or biochemistry is incomplete. In a few instances, however, resistance genes have been definitely identified and their action at the biochemical level is more or less understood.

Keywords: fungicide resistance, genetic control, gene action, benzimidazoles, carboxamides, sterol-biosynthesis inhibitors, aromatic hydrocarbons, polyoxins, pyrazophos, kasugamycin.

Introduction

Some chemicals are toxic because they interact with cell constituents in a way that adversely affects important cellular processes. The ability of an organism to develop resistance to such a chemical is due to its cellular components being able to exist in more than one form without preventing survival under normal conditions. Changes from one form to another that are disadvantageous for the organism are quite possible, but they are of little concern to those engaged in the chemical control of pathogens. The problem of resistance arises from changes that not only permit survival but also decrease sensitivity to the chemical used. In other branches of science, however, problems may arise from an increase in sensitivity. Ordinary anesthesia, for example, may prove fatal to some individuals with increased sensitivity. Such individuals are normal in every other respect, but due to an unfortunate hereditary enzyme constitution they cannot detoxify the anesthetic as most other individuals do, and hence they cannot recover from anesthesia (Elliott, 1973).

To understand a change of cellular components from one form to another,

it is important to recognise: whether there are genetic determinants (genes) involved and of what kind; and what cellular components (enzymes or others) are coded by these genes and in what way they have changed. This contribution summarizes the available information on the biochemical genetics of resistance to agricultural fungicides, with emphasis on recent findings; earlier work has been reviewed (Georgopoulos, 1976).

Genetics

If a change in sensitivity does not involve a change in chromosomal or extrachromosomal DNA, it is thought to be the result of phenotypic adaptation. Such adaptation may take place in culture after long exposure to the fungicide under conditions favourable for the organism (e.g. nutrition). Phenotypic resistance is usually easily lost upon transfer to a toxicant-free medium. Whether phenotypic adaptation to fungicides is possible under field conditions is not known. Instability of resistance of field isolates may indicate phenotypic adaptation, but it should not be confused with regression of resistance in the field, which may well be due to reduced fitness of genetic mutants.

Involvement of extrachromosomal genes in resistance to toxicants is very common in bacteria. Such genes are borne on plasmids, extrachromosomal, circular DNA molecules, and are transmitted more easily than chromosomal genes. Plasmid- or virus-like particles have been recognised recently in fungi and have been associated with heritable changes. The presence of a DNA plasmid in yeast is correlated with the determinant of one type of oligomycin resistance (Guerineau et al., 1974). Fungi also possess mitochondrial DNA with a definite role in heredity and in several instances resistance to toxicants has been found to be the result of mutations of mitochondrial genes (cf. Georgopoulos, 1977).

However most genetic studies on resistance to fungicides have revealed the involvement of chromosomal genes. As a rule several genes control sensitivity to the same fungicide or group of fungicides. The easiest way to recognise them is to use a heterothallic ascomycete whose perfect stage is produced readily and that gives easily analyzable progeny within a few days. These advantages are offered by the non-pathogen *Neurospora crassa* and, more or less, by some pathogens, e.g. *Nectria haematococca* or *Venturia inaequalis*. Some heterothallic Basidiomycetes, e.g. *Ustilago maydis*, are also used for the recognition of fungicide-resistance genes. The genetics of resistance can be studied by this meiotic analysis also in homothallic Ascomycetes when a method is available to distinguish between crossed and selfed perithecia. Mitotic analysis may be used also to recognise genes for fungicide resistance in the absence of the sexual stage. The methods of genetic analysis in the main groups of fungi can be found in a number of texts, e.g. Fincham et al. (1979).

Biochemistry

In the area of gene expression, i.e. information transfer from gene to protein, knowledge of resistance to agricultural fungicides is rather limited. Rationally, a resistance gene must first be transcribed to messenger RNA, which is translated in the ribosomes, and a protein molecule is formed. This protein may itself interact with the fungicide, i.e. be the sensitive site, or it may affect some other cellular property important in terms of toxicity.

The main mechanisms by which resistance to a toxic compound may develop are: modification of the sensitive site; bypass of the site due to operation of an alternative pathway; decreased uptake or increased secretion of the toxicant; detoxification; or decreased conversion of a non-toxic into a toxic compound. Of these mechanisms, the first two are important for the development of resistance to mainly site-specific fungicides, the remaining three to specific and multi-site fungicides. Note that with resistance to the same toxicant different mechanisms may operate in different organisms, or in different mutants of the same organism.

For the study of biochemical mechanisms it is usually necessary to examine the effect of the fungicide on the wild type and mutant at the cellular and the sub-cellular level. If resistance is expressed in experiments with whole cells but not with cell-free systems, then the mechanism does not involve modification of the sensitive site. Comparative experiments on the influx and efflux of the toxic molecule, on its metabolism and its binding to components of mutant and wild type cells are also required. These studies are time-consuming and require specially equipped laboratories.

Work with agricultural fungicides

The best available example in which the action of a known gene that controls sensitivity to agricultural fungicides has been completely elucidated is that of the *ben*-A gene for resistance to benzimidazoles in *Aspergillus nidulans*. This gene codes for β-tubulin, one of the sub-units of the tubulin molecule (Sheir-Neiss et al., 1978). Mutations of this gene affect the electrophoretic properties of β-tubulin and at the same time the ability of the protein to bind carbendazim, which is inversely correlated to carbendazim resistance. More details on the action of the *ben*-A gene are given on p.60-70.

Chromosomal genes for resistance to benzimidazoles have also been recognised in several other fungi (Borck & Braymer, 1974; Brasier & Gibbs, 1975; Shabi & Ben-Yephet, 1976), although their action has not been studied at the biochemical level. In *Mycosphaerella musicola* and *M. fijiensis*, in addition to chromosomal factors, one or more extrachromosomal

factors appear to be involved in resistance to benzimidazoles (Stover, 1977). This conclusion is based on the observation that one type of resistance could not be found in ascospore progeny, but no research on the nature of extranuclear determinants has been done. For other fungi, genes for benzimidazole resistance have not been identified, but biochemical mechanisms other than the one mentioned for *A. nidulans* have been suggested. Impairment of an active transport system for carbendazim has been considered responsible for resistance to this fungicide in *Sporobolomyces roseus* (Nachmias & Barash, 1976), while in *Verticillium malthousei* benomyl resistance appeared to be related to the ability to lower the pH of the medium by the production of an unidentified acid (Lambert & Wuest, 1976).

Another instance in which a gene for resistance has been identified and its action at the biochemical level has become known is the *oxr*-1 gene for resistance to oxathiin carboxanilides in *U. maydis*. These fungicides inhibit specifically the succinate-CoQ oxidoreductase system in fungi, bacteria and animal tissues. In *U. maydis*, two mutations at the *oxr*-1 locus give two levels of resistance of this mitochondrial system (Complex II) to carboxin. They can also recognize other carboxanilide structures, which are positively or negatively correlated to carboxin, depending on the mutation (White et al., 1978). Apparently, the gene codes for a component of the enzyme complex, and when it mutates the component's affinity for different carboxanilide structures may or may not be altered. However the complexity of the system has made it difficult to identify the component that interacts with the inhibitor and, therefore, may be controlled by the resistance gene. Recent research (Ramsay et al., 1981) indicates that the binding site in Complex II obtained from animal mitochondria is formed by two small peptides, C_{11-3} and C_{11-4}. If this is true also in *U. maydis*, gene action must involve peptide components. Chromosomal genes for resistance to carboxamides have also been recognised in *A. nidulans* and they have been found to affect the mitochondrial Complex II.

Lancashire & Griffiths (1971) isolated a number of *Saccharomyces cerevisiae* mutants resistant to trialkyl and triphenyltin derivatives, the latter of which are important in agriculture. In all of these mutants resistance was inherited in a non-Mendelian fashion, indicating extrachromosal control. The trisubstituted derivatives of tin bind to a specific site in mitochondria, inhibiting oxidative phosphorylation, and in one class of the *S. cerevisiae* resistant mutants a mitochondrial DNA mutation appears to be responsible for a change in the binding site. The chemical nature of the site was not determined.

Another *A. nidulans* gene whose action has been examined is the *ima* B gene, one of several that control sensitivity to the ergosterol-biosythesis-inhibiting fungicides. This gene appears to control the efficiency of

an energy-dependent system for efflux of fenarimol. Efficiency is higher in the mutant, and this apparently results in a lower fungicide concentration at the site. Note that the single *ima* B mutant and a recombinant carrying two genes for resistance (*ima* A and *ima* B) appeared to behave similarly for fenarimol efflux activity (de Waard & van Nistelrooy, 1980). The recombinant has been compared to the wild-type strain in a recent study with imazalil, another ergosterol-biosynthesis inhibitor (Siegel & Solel, 1981). This study supports, in part, the hypothesis that resistance to imazalil is also based on reduced uptake of the fungicide.

With resistance to many other agricultural fungicides, knowledge on either the biochemical mechanism or the genetic control is incomplete or totally lacking. Five genes for resistance to aromatic-hydrocarbon fungicides (quintozene, biphenyl, hexachlorobenzene, dicloran, chloroneb) have heen recognised in *N. haematococca* (Georgopoulos & Panopoulos, 1966); two have been recognised in *A. nidulans* (Threlfall, 1968). The same genes must control resistance to dicarboximides, which are classified with the aromatic hydrocarbon group on the basis of cross-resistance and the ability to increase the frequency of mitotic segregation (Georgopoulos et al., 1979). Practically no conclusive evidence on gene action is available. Different resistance mechanisms have been suggested (cf. Georgopoulos, 1977). Similarly, the mechanism of dodine resistance is not known, although the genetic control in both *N. haematococca* (Kappas & Georgopoulos, 1970) and *V. inaequalis* (Polach, 1973) has been clarified.

At the other extreme, we have a satisfactory knowledge of the biochemical mechanism of resistance to polyoxins in *Alternaria kikuchiana*, but without information on the genetic determinants involved. The uptake of polyoxin in the wild-type strain of this fungus is high and is antagonized by the dipeptide glycylglycine (Hori et al., 1977). In contrast, resistant strains can take up equally small amounts of the antibiotic in the presence and absence of dipeptides. In all cases resistance is associated with a very ineffective system for dipeptide uptake. It appears that a peptide permease must be coded for by a gene for polyoxin resistance, but such a gene has not been recognised. Similarly, genes for resistance to pyrazophos are not known, but a good understanding of a resistance mechanism in *Pyricularia oryzae* has been obtained (de Waard & van Nistelrooy, 1980). This mechanism involves a lack of ability to convert pyrazophos into the toxic metabolite PP (2-hydroxy-5-methyl-6-ethoxycarbonylpyrazolo(1,5-a)-pyrimidine). Apparently, the determinant regulating the biosynthesis of the converting enzyme is responsible for pyrazophos resistance.

With kasugamycin resistance in *P. oryzae*, involvement of three chromosomal loci has clearly been demonstrated (Taga et al., 1978). All kasugamycin-resistant ascospore progeny was without exception resistant to blasticidin-S. Both kasugamycin and blasticidin-S act by inhibition of

the protein-synthesizing system in *P. oryzae*. Although the action of the genes recently recognised has not been studied at the biochemical level, it is likely that they are involved in alterations of the protein-synthesizing system.

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Cross-resistance

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Abstract

The scientific and practical value of cross-resistance tests is outlined. Examples are given of toxicants that are uncorrelated, positively correlated and negatively correlated, with emphasis on groups of fungicidal compounds that are important in agriculture.

Keywords: positive correlation, negative correlation, collateral sensitivity, receptor site, synergistic action.

Introduction

When a fungicide fails because of the development of resistance by the target organism, in practice it is very important to know whether the effectiveness of other fungicides has been affected. The recognition of cross-resistance relationships may also be useful in other ways. In mode of action studies, for example, cross-resistance tests with inhibitors of known mechanism of activity may provide a very good indication of the cellular processes affected by a fungicide. For example, the recent observation that fentin-resistant strains of *Cercospora beticola* are also resistant to oligomycin (Chrysayi-Tokousbalides & Giannopolitis, 1981) strongly suggests that in this organism fentin derivatives act by inhibition of oxidative phosphorylation.

If a mutation affects sensitivity to one toxicant and not to another the two compounds are said to be uncorrelated in terms of cross-resistance. A positive correlation exists when the mutant is less sensitive or less resistant to both toxicants than the wild-type strain. If the mutant is more resistant than the wild type to the one chemical and more sensitive than the wild type to the other one speaks of negatively correlated cross-resistance. In most of the bacteriological and the medical literature the term collateral sensitivity is preferred to describe negative correlation. The term cross-resistance is then used only in cases of pos-

itive correlation. Whatever the preference, the terms should not be misused. A strain, for example, may be resistant to two different fungicides because of two independent mutations. Then the fungicides are not correlated.

Cross-resistance cannot be assumed without evidence that sensitivity to both chemicals is controlled by the same gene. The best way to prove this is to cross a resistant with a sensitive strain and show that all progeny resistant to the one fungicide are also resistant to the other. If this is not easy to do, one can test several independently isolated strains resistant to the one toxicant for sensitivity to the other.

Often a positive correlation can rather safely be predicted on the basis of chemical similarities between two compounds. There are, however, some very interesting exceptions to the rule. Not only may there be positive correlation between compounds that are chemically unrelated, but also compounds chemically quite similar may be found uncorrelated or even negatively correlated. This is not as peculiar as it may first sound. To remain functional, a receptor site cannot change very dramatically. A small modification of this site may reduce affinity for a toxicant, but, depending on the modification, it is conceivable that affinity for a structural analogue may not be affected, or may be even increased.

Aromatic-hydrocarbon group

This group includes some substituted aromatic hydrocarbons such as hexachlorobenzene, tecnazene, dicloran, biphenyl and chloroneb. In several fungi, mutants obtained by selection on one of these fungicides are resistant to all members of the group. (Georgopoulos & Zaracovitis, 1967; Kuiper, 1967; Tillman & Sisler, 1973). No exceptions have been reported. With most of these compounds the degree of resistance appears to be very high, because it is practically impossible to achieve a concentration inhibitory to the resistant mutants. But positively correlated fungicides act synergistically, and resistant mutants are easily inhibited by the concurrent use of two or more members of the group at low concentrations.

Recently, a positive correlation between aromatic-hydrocarbon fungicides and the dicarboximides (vinclozolin, procymidone, iprodione) has been recognised (Leroux et al., 1977; Sztejnberg & Jones, 1978). On the basis of their chemical structures (p.102), this positive correlation would be rather unexpected. However there must exist similarities in the mechanism of action between the dicarboximides and the aromatic hydrocarbons because, in addition to cross-resistance, the members of both groups have in common the ability to increase the frequency of mitotic segregation (sectoring) in diploid strains of *Aspergillus nidulans* (Georgopoulos et al., 1979).

The action of genes for resistance to the aromatic hydrocarbons has

not been adequately explained. It is not easy therefore, to suggest an explanation as to why sensitivity to both the aromatic hydrocarbons and the dicarboximides should be decreased by the same mutation. A highly sensitive site within the fungal nucleus appears to be common to both groups of fungicides (Georgopoulos et al., 1979). However, it is not known which component in the nucleus interacts with the toxicant and whether mutant and wild-type cells differ in that component. An alternative explanation has been suggested by Tillman & Sisler (1973), who indicated that the mutations may affect partitioning of every one of the fungicides involved at interfaces.

Benzimidazoles

In several fungi studied by van Tuyl (1977) a positive correlation between the carbendazim generators and thiabendazole appears to be the rule. However, a low percentage of mutants of *A. nidulans* and *A. niger*, obtained on benomyl medium, are not less sensitive to thiabendazole than the wild type, and somewhat higher percentages of those isolated on thiabendazole are more sensitive to benomyl (and to carbendazim) than the wild type. The mechanism responsible for the existing correlations is very well understood since sensitivity differences in vivo are reflected in differences in the affinity of tubulin for the fungicides (Davidse & Flach, 1977; 1978).

With some benzimidazole-resistant *A. nidulans* mutants that exhibit temperature sensitivity, there is a positive correlation between these fungicides and *p*-fluorophenylalanine (Morris & Oakley, 1979). This correlation appears to be the only piece of information available to us so far on the mechanism by which *p*-fluorophenylalanine cause non-disjunction of chromosomes. Griseofulvin, which also inhibits fungal mitosis, was not found correlated to the benzimidazoles for cross-resistance (Kappas & Georgopoulos, 1974). Some N-phenyl-carbamate herbicides are known antimitotic agents. A recent study of Leroux & Gredt (1979) has shown that with some mutants of *Botrytis cinerea* and *Penicillium expansum* these herbicides are negatively correlated to the benzimidazoles. In *Cercospora beticola*, however, no correlation has been observed (Georgopoulos, unpublished data).

Carboxamides

This group is probably unique among the fungicides because of the great variety of cross-resistance relationships existing among its members. In *Ustilago maydis* considerable resistance to carboxin results from mutations at the *oxr*-1 locus (Georgopoulos & Ziogas, 1977). Although strains carrying a mutation at this locus are more or less resistant to

many carboxin analogues, including oxycarboxin and pyracarbolid, some interesting exceptions can very easily be found (White et al., 1978).

The sensitivities of the succinic dehydrogenase complex, the enzyme system inhibited by the carboxamides, are given for selected carboxamides in Table 1. From Table 1, the moderately resistant strains, carrying the *oxr*-1A mutation, are 14-times less sensitive to carboxin than the wild type. The same mutation does not affect sensitivity to 4'-propyl-carboxin (lack of correlation) but increases sensitivity to 4'-phenyl-carboxin 25 times (negative correlation). For the highly resistant *oxr*-1B mutants, the resistance factor is 100 with carboxin, almost 300 with 3'-methylcarboxin, close to 3 with 3'-decyloxycarboxin, at least 16 with 4'-phenylcarboxin, and below 0.07 with a thiophene carboxamide, the 4-*n* butylanalogue of 3-methylthiophene-2-carboxanilide (White & Thorn, 1980).

Similar effects of modifications of the carboxin molecule on sensitivity were also observed for *A. nidulans* (White et al., 1978). With all the structural analogues available, it is conceivable to be able to overcome any type of mutation that decreases the sensitivity of a fungus to carboxin. The explanation that can be offered is that the mutations affect the carboxin-binding site, probably a peptide, in the succinic dehydrogenase complex. Any such modification of the site, which will decrease affinity for most carboxamide structures, may not affect or even decrease affinity for some others. It must be added that in *U. maydis* the *ants* mutants, which have low resistance to carboxin, are all highly sensitive to the inhibitors of the cytochrome system (Georgopoulos & Sisler, 1970). Thus while in the case of the benzimidazoles no compound is known to se-

Table 1. Sensitivity of succinic dehydrogenase from wild-type and carboxin-resistant mutant strains of Ustilago maydis to selected carboxamides.

Carboxamides	I_{50} (μM)		
	wild type	oxr-1A	oxr-1B
Carboxin	0.36	5.0	37.0
3'-methylcarboxin	0.075	1.7	20.0
3'-decyloxycarboxin	0.017	-	0.058
4'-propylcarboxin	0.16	0.24	15.0
4'-phenylcarboxin	4.8	0.22	> 75.0
3'-methylthiophene-2-carboxanilide	7.2	45.0	38.0
4-n butylanalogue of 3'-methylthiophene-2-carboxanilide	> 100.0	12.0	7.0

lectively inhibit the large percentage of mutants that are resistant to carbendazim and thiabendazole, the effect of all of the carboxamide-resistance mutations that have been studied can be reserved by the use of an appropriate inhibitor.

Sterol-biosynthesis inhibitors

Fungicides of this group, including triforine, fenarimol, nuarimol, triadimefon, imazalil, tridemorph, bitertanol, and prochloraz, are mostly positively correlated for cross-resistance (Sherald et al., 1973; Fuchs et al., 1977; Barug & Kerkenaar, 1979). Although the group comprises diverse chemical structures, the similarity in the mechanism of action is sufficient to explain why a mutation should affect sensitivity to all of these inhibitors, if resistance was the result of sensitive site modification. However, this does not appear to be the case. The sterol patterns of sensitive and resistant strains of *A. nidulans* do not differ significantly (Ragsdale & de Waard, 1977), indicating that the sterol synthesizing system is not affected by the resistance mutations. The data so far available indicate that the mechanism of resistance is related to membrane transport (de Waard & van Nistelrooy, 1980; Siegel & Solel, 1981). Whether such diverse chemical structures have common transport systems is not known.

It is peculiar to the ergosterol-biosynthesis inhibitors that although within the group cross-resistance is not always reciprocal (Barug & Kerkenaar, 1979), some of the mutants selected on such inhibitors show an altered sensitivity to quite unrelated toxicants. The *ima* B mutants of *A. nidulans*, selected on imazalil, are resistant to chloramphenicol and hypersensitive to cycloheximide, acriflavin and neomycin (van Tuyl, 1977). Some other mutants, selected on bitertanol, are more sensitive to carboxin than the wild type (Georgopoulos, unpublished data). Thus, some of the mutations recognised by the use of ergosterol-biosynthesis-inhibiting fungicides appear to be pleiotropic. But it is far from understood how those mutations cause such peculiar cross-resistance patterns.

Organophosphates

Resistance of *Pyricularia oryzae* to phosphorothiolates, e.g. 'Kitazin P', is always accompanied by an increased sensitivity to phosphoramidates, and vice versa. The two types of fungicides act synergistically on wild type but not on phosphorothiolate-resistant strains of the fungus (Uesugi et al., 1974). It has been shown that wild-type strains are capable of rapid metabolism of phosphoramidates, but this metabolism is inhibited by phosphorothiolates. Mutants selected for resistance to the phosphorothiolates are capable of much slower metabolism of the phos-

phoramidates (Uesugi & Sisler, 1978). This appears to be a satisfactory explanation for the negative correlation between phosphorothiolates and phosphoramidates.

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Benzimidazole compounds: selectivity and resistance

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Abstract

Benzimidazole compounds inhibit microtubule assembly, leading to interference with a great number of processes in which microtubules are involved, like nuclear and cellular division, cell migration and organelle movement. Since microtubules play a role in maintaining cell shape and structure, inhibition of their formation also affects the intracellular organization. Affinity of tubulin, the sub-unit of microtubules, to a particular benzimidazole compound is probably the most important factor that determines the activity in a certain organism. Modification of the basic structure required for activity creates compounds with a high degree of selectivity, which can be used in the therapy of a number of diseases of plants and animals. But the highly specific interaction between tubulin and benzimidazole compounds is vulnerable to any structural alteration of the binding site. A mutation that lowers the binding affinity of tubulin to a benzimidazole compound without any effect on normal tubulin functioning creates a resistant strain. This is what probably has happened already many times in the history of benomyl's use. In fundamental cell research, benzimidazole compounds are becoming increasingly important as valuable tools in the study of the structure and role of the microtubular system, whose origin was one of the basic molecular innovations in the evolution of prokaryotes to eukaryotes.

Keywords: benzimidazoles, thiophanates, benomyl, carbendazim, fenbendazole, fuberidazole, mebendazole, nocodazole, parbendazole, thiabendazole, *Aspergillus nidulans*, *Penicillium expansum*, tubulin, microtubules, anthelminthic.

Introduction

Of the systemic fungicides, the benzimidazole compounds and thiophanates are undoubtedly the most widely known, owing to their excellent sys-

temic properties, their great efficacy in controlling important plant diseases, but, unfortunately, also to the disappointment and problems they evoke when resistance develops in target fungi. The general biocidal properties of the benzimidazole compounds, the current knowledge of their mode of action and the biochemical basis of their selectivity and the resistance will be reviewed in this contribution.

Biocidal properties of benzimidazole compounds

At present various benzimidazole compounds are being used in the therapy of a number of diseases of plants, animals and man. The first compound developed was thiabendazole, which was introduced in 1961 as an anthelminthic. Later several other biocidal benzimidazole compounds came into use. Benomyl (Delp & Klöpping, 1968), carbendazim or MBC (Hampel & Löcher, 1973), fuberidazole (Schumann, 1968) and also thiabendazole (Staron & Allard, 1964) are well known as systemic fungicides. Thiophanates, introduced in 1971 (Aelbers, 1971) are often placed in this class of fungicides, since under natural conditions they are converted to benzimidazole compounds (Selling et al., 1970). Fenbendazole, mebendazole, oxibendazole and parbendazole have anthelminthic properties and are used in veterinary medicine. Nocodazole has been described as being active against tumors in mammals, including man. Structural formulae of these compounds are given in Figure 1.

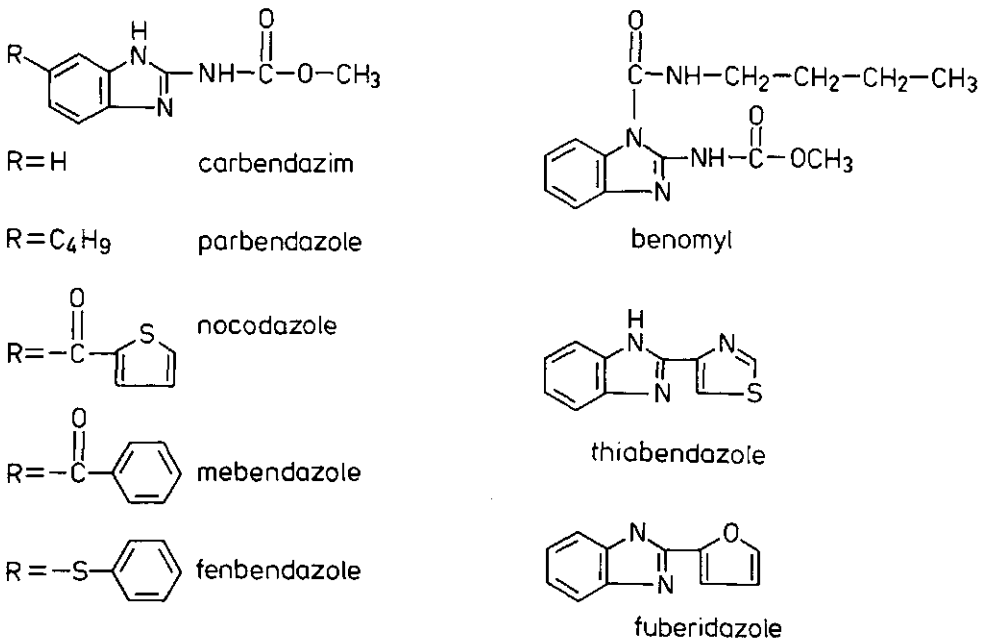

Figure 1. Structural formulae of biocidal benzimidazole compounds.

Development of resistance to these compounds is frequently encountered in crop protection, as well as in veterinary medicine. In a recent paper, Delp (1980) mentions that up to 1979 resistance to benzimidazole compounds has been found in fungi belonging to 16 different genera. According to Webb et al. (1978, 1979), thiabendazole-resistant strains of *Haemonchus contortus*, a sheep parasite, are present on over 50% of the farms in the Northern Tablelands of New South Wales, Australia.

Mode of action

Optical and electron microscope studies have contributed considerably to the understanding of the mode of action of benzimidazole compounds. Benomyl and its conversion product carbendazim have been shown to interfere with mitosis in fungi (Davidse, 1973; Hammerschlag & Sisler, 1973; Künkel & Hadrich, 1977; Richmond & Phillips, 1975). Borck (1973) and Howard & Aist (1977, 1980) described the disorganization of the fine structure of fungal hyphae induced by benomyl and carbendazim. In particular, the dislocation of the Spitzenkörper, which is presumed to function in hyphal linear elongation, was noticed in both studies. Mitosis in plants (Boyle, 1973; Marco Moll et al., 1973; Richmond & Phillips, 1975) and mammalian cells in vivo (Styles & Garner, 1974; Seiler, 1976) and in vitro (Styles & Garner, 1974; de Brabander et al., 1976b) also appears to be affected by carbendazim. In all these cases interference of carbendazim with the formation or the functioning of microtubules is thought to have been responsible for the observed effects.

Biochemical studies support the idea of an anti-microtubule mode of action of benzimidazole compounds. A carbendazim-binding protein was demonstrated in cell-free mycelial extracts of *Aspergillus nidulans* (Davidse & Flach, 1977) with biochemical properties characteristic for tubulin, the building block of microtubules. These properties are summarized in Table 1. Binding of colchicine, the classical inhibitor of mitosis in

Table 1. Biochemical properties of the carbendazim-binding protein in fungi.

High affinity binding of carbendazim, nocodazole and thiabendazole at the same site. Binding is reversible and fast at 4 °C.
Low affinity binding of colchicine at the carbendazim-binding site.
Optimum pH for binding: 6.6 - 6.9.
Loss of binding activity in a first order manner with $t^{\frac{1}{2}}$ of 6.5 h. Stabilization of the binding activity by carbendazim, glycerol and sucrose.
Molecular weight of 110 000.
Purification by a standard procedure for mammalian brain tubulin: ammonium-sulphate fractionation (35-50 %) and DEAE[a]-ion-exchange chromatography.

a. DEAE = diethylaminoethyl.

animal and plant cells, to the carbendazim-binding protein unequivocally showed it to be identical with tubulin, because this compound specifically binds to tubulin and in this way prevents its assembly into microtubules. A review on this subject has been written by Wilson & Bryan (1974).

The carbendazim-binding protein also has a high affinity for nocodazole, comparable to that of mammalian tubulin (Hoebeke et al., 1976), which binds nocodazole at the colchicine-binding site. Experiments (Davidse & Flach, 1978) have shown that also thiabendazole is bound to fungal tubulin at the carbendazim-binding site. A direct determination of the dissociation constant of the thiabendazole-protein complex, proved to be difficult, however, because of unspecific adsorption of the labelled compound to other proteins. Therefore this value was estimated by measuring the inhibition constant for the ability of thiabendazole to inhibit binding of radio-labelled carbendazim. The value obtained is the concentration of thiabendazole that would saturate half of the carbendazim-binding sites and it is therefore a reliable estimate of the dissociation constant. Values obtained for thiabendazole and carbendazim in binding experiments with cell-free extracts of *Penicillium expansum* are given in Table 2. *Penicillium expansum* was used since it is more sensitive to thiabendazole than *Aspergillus nidulans*. Upon comparison of the dissociation constants with the ED_{50} values, it appears that the biochemical basis of the antifungal activity of thiabendazole is similar to that of carbendazim.

Binding studies with the anthelminthics fenbendazole, mebendazole, oxibendazole and parbendazole revealed that these drugs were bound to mammalian brain tubulin (Friedman & Platzer, 1978; Laclette et al., 1980). Binding of mebendazole and fenbendazole to nematode tubulin has recently been described by Friedman & Platzer (1980).

The biochemical studies described establish that the effects of benzimidazole compounds on cellular processes are directly due to interference with microtubule assembly.

Table 2. Sensitivity to carbendazim and thiabendazole, and the dissociation constants of the thiabendazole- and carbendazim-tubulin complex of a wild-type strain of Penicillium expansum.

	ED_{50} (radial growth on agar) (μM)	Dissociation constant[a] (μM)
carbendazim	0.4	0.9 ± 0.21 (3)
thiabendazole	7	34 ± 15.4 (4)

a. The dissociation constant of the thiabendazole-tubulin complex was measured as the inhibition constant for the ability of thiabendazole to inhibit carbendazim-binding (Davidse & Flach, 1978). Values are means ± standard deviation; the number of experiments is given in parentheses.

Selectivity and resistance

The microtubular system is present in all eukaryotes. It has been shown that a high degree of conservatism exists in the amino-acid sequences of tubulins from various sources (Luduena & Woodward, 1975). Nevertheless, benzimidazole compounds are rather selective in their activity to eukaryotes. Carbendazim is highly toxic to certain groups of fungi, whereas others are insensitive (Bollen & Fuchs, 1972). Sensitivity to carbendazim is not restricted to fungi, however, since it is also highly toxic to the earthworm *Lumbricus terrestris* (Stringer & Wright, 1976), to fresh-water organisms like the alga *Chlorella pyrenoidosa*, to the fish *Salmo gairdneri* and the crustacean *Daphnia magna* (Canton, 1976). Especially the reproductive capacity of *D. magna* appears to be very sensitive. The embryonal development of mites (Delp & Klöpping, 1968) and plant-parasitic nematodes (Rössner et al., 1976) is also disturbed at low doses. Higher plants and mammals are not visibly affected by carbendazim, although cytological studies have revealed effects at the cellular level (Richmond & Phillips, 1975; Seiler, 1976).

The curative action of mebendazole in animal and human helminthiases implies selectivity for host and parasite. Ultrastructural studies have shown that upon treatment of the host, microtubules in cells of the parasite are completely destroyed, whereas cytoplasmic and spindle microtubules of the host cells remain undisturbed, even though both types of cells have been exposed to identical concentrations of mebendazole (Borgers et al., 1975a, b).

Experiments in vivo on the effect of nocodazole on experimental and human neoplasms have shown that this compound specifically eliminates microtubules in dividing and non-dividing neoplastic cells, whereas microtubules of normal cells in interphase apparently remain intact. Microtubules in mitotic normal cells, however, are seriously affected (de Brabander et al., 1975). In comparison with carbendazim, nocodazole is less selective in its activity for fungi and animals (Table 3).

Binding studies with carbendazim in cell-free extracts of fungi, plants and mammalian brain have led to a better understanding of the biochemical

Table 3. Fungal and mammalian toxicity of carbendazim and nocodazole.

	Aspergillus nidulans ED_{50} (radial growth on agar) (mg/l)	Mice LD_{50} (acute toxicity intraperitoneal) (mg/kg)
Carbendazim	0.9	6400[a]
Nocodazole	0.2	135[b]

a. Source: Seiler (1975).
b. Source: Atassi & Tagnon (1975).

Table 4. Carbendazim-binding activity in extracts of fungal, plant and animal cells.

System	Binding activity[a] (dpm ^{14}C-carbendazim/ml extract)
Aspergillus nidulans 003 (wild type)	5 640
Aspergillus nidulans 186 (extra sensitive)	12 200
Aspergillus nidulans R (resistant)	320
Penicillium brevicompactum (sensitive)	4 140
Penicillium brevicompactum (resistant)	220
Penicillium corymbiferum (sensitive)	2 080
Penicillium corymbiferum (resistant)	140
Alternaria sp.	370
Pythium irregulare	250
pea root tips	250
porcine brain	210

a. Binding was determined with a Sephadex G-100 gel filtration assay (Davidse & Flach, 1977) at 2.8 µM ^{14}C-carbendazim (70 000 dpm/ml) with fungal and plant extracts and at 6.1 µM ^{14}C-carbendazim (153 000 dpm/ml) with porcine brain extract.

basis of the selectivity. In extracts of carbendazim-resistant fungi no or only a low binding activity appeared to be present (Table 4) (Davidse & Flach, 1977). In strains of *Aspergillus nidulans*, the affinity of the binding protein, identified as tubulin, for carbendazim reflected the sensitivity of each strain (Table 5). The mutation responsible for the behaviour of the strains occured in one gene (van Tuyl, 1974, 1977), which has been identified as the structural gene for β-tubulin, one of the monomers of the tubulin molecule (Sheir-Neiss et al., 1978). Most of the strains carrying a mutation in this gene that leads to carbendazim resistance have electrophoretically abnormal β-tubulins. A correlation between affinity of tubulin for carbendazim and sensitivity was also found with strains of *Penicillium expansum* (Table 6). It was not possible to determine the genetic background, but it can be assumed that also here a mutation in one gene is involved (van Tuyl, 1977). The correlation between degree of sensitivity and the magnitude of the affinity constant in strains of *Aspergillus nidulans* and *Penicillium expansum* and the absence of carbendazim-binding activity in cell-free extracts of naturally resistant fungi (Table 4) suggests that the selectivity of carbendazim within fungi is based on a differential affinity of fungal tubulins to this ben-

Table 5. Sensitivity to carbendazim and the dissociation constant of the carbendazim-tubulin complex in strains of Aspergillus nidulans.

Strain	ED_{50} (radial growth on agar) (µM)	Dissociation constant[a] (µM)
003 (wild type)	4.5	2.2
186 (extra sensitive)	1.5	0.6
R (resistant)	95	27

a. Dissociation constants were determined with a charcoal binding assay (Davidse & Flach, 1977).

Table 6. Sensitivity to carbendazim and the dissociation constants of the carbendazim-tubulin complex in strains of Penicillium expansum[a].

Strain	ED_{50} (radial growth on agar) (μM)	Dissociation constant[b] (μM)
S (wild type)	0.4	0.9 ± 0.21 (3)
SS (extra sensitive)	0.07	0.2 ± 0.06 (3)
R (resistant)	2 500	~

a. Strains have been isolated and described by van Tuyl (1977).
b. The dissociation constant of the thiabendazole-tubulin complex was measured as the inhibition constant for the ability of thiabendazole to inhibit carbendazim-binding (Davidse & Flach, 1978). Values are means ± standard deviation; the number of experiments is given in parentheses.

zimidazole compound. That no differences could be found between the *Aspergillus nidulans* strains in rates of uptake and metabolism of carbendazim (Davidse, 1976) strengthens this supposition. The existence of other mechanisms of resistance should not be entirely excluded, however. A differential uptake has been suggested to be involved in the resistance of *Botrytis cinerea*, *Verticillium malthousei* and *Sporobolomyces roseus* to carbendazim (Gessler, 1976; Lambert & Wuest, 1975; Nachmias & Barash, 1976). Also in *Aspergillus nidulans*, other mechanisms of resistance might operate, since in addition to mutations in the β-tubulin gene other mutations that give a low degree of carbendazim-resistance have been found in two more loci (van Tuyl, 1977).

In cell-free extracts of pea root tips no carbendazim-binding activity has been demonstrated (Table 4). However, the presence of plant tubulin in these extracts has not been proven. The colchicine-binding assay, which was used as a diagnostic test, gave a negative result, but this might be due to a low affinity of plant tubulin for colchicine (Burns, 1973). Tubulin has been isolated from plant tissue (Rubin & Cousins, 1976) by a procedure not significantly different from the one which was used in these experiments, so it might be assumed that tubulin was present. So absence of carbendazim-binding activity could mean that plant tubulin has a low affinity to this benzimidazole compound. That low affinity is in agreement with the observation of Richmond & Phillips (1975) that mitosis in plant cells was only affected at relatively high doses of carbendazim. Since the plasmalemma of the plant cell is fully permeable for carbendazim (Peterson & Edgington, 1976), the insensitivity of higher plants might be primarily due to a low affinity of their tubulins for this compound. The affinity of mammalian brain tubulin for carbendazim is also low, since no binding could be detected (Table 4). The preparation showed considerable colchicine-binding activity, which indicates the presence of native tubulin. The selectivity of the various benzimidazole compounds is also expressed in their ability to inhibit microtubule polymerization in vitro. Nocodazole, fenbendazole, mebendazole, oxibendazole

and parbendazole are very potent inhibitors, whereas carbendazim and thiabendazole only slightly affect this process (Freedman & Platzer, 1978; Hoebeke & van Nijen, 1975; Laclette et al., 1980) (Figure 2). However, the low affinity of mammalian tubulin for carbendazim, might not be solely responsible for the non-toxicity of this compound to mammals, since carbendazim is rapidly hydroxylated to the 5-hydroxy derivative, which is excreted from the organism as a glucuronide or a sulphate conjugate (Gardiner et al., 1974). The low affinity of mammalian tubulin together with the detoxication mechanism might explain the relative insensitivity of mammals to carbendazim. The selectivity of the anthelminthic benzimidazoles is probably also based on a differential binding affinity between nematode tubulin and mammalian tubulin for these compounds (Friedman & Platzer, 1980). There has been no research done on whether a differential affinity is also involved in the resistance of helminths to thiabendazole.

The selectivity of nocodazole with respect to its effect in vitro on microtubules in interphase malignant cells and non-malignant cells is an interesting phenomenon. Since microtubules in mitotic normal cells are affected it is doubtful that a differential affinity of the tubulins of

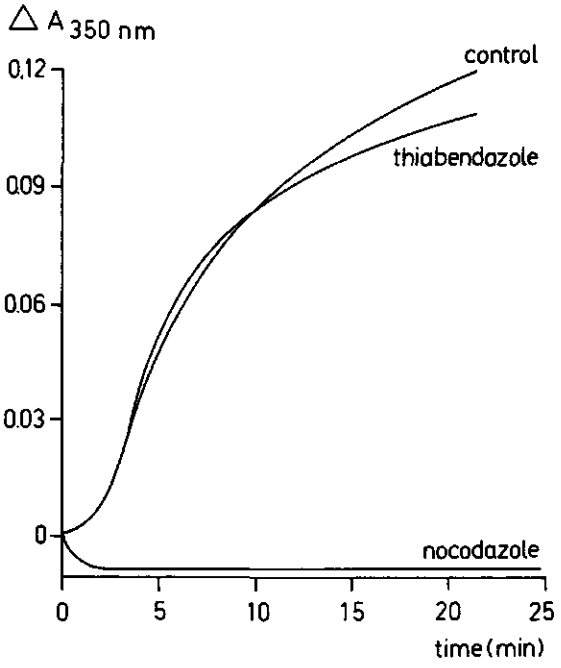

Figure 2. Effect of nocodazole and thiabendazole on microtubule assembly in vitro. Porcine brain tubulin, purified by two cycles of assembly and disassembly according to a modified procedure of Shelanski et al. (1973) was incubated at 37 °C with nocodazole or thiabendazole, both at a concentration of 50 µM. The compounds were added in dimethylsulfoxide (DMSO), final concentration 10 µg/ml. The control contained only DMSO. Assembly was measured by the increment in radiant absorbance (A) at 350 nm.

the different cell types is involved. It is more likely that the selectivity is based on a differential biochemical stability of the microtubules to the depolymerization action of nocodazole (de Brabander et al., 1975).

The difference in affinity to carbendazim between mammalian and fungal tubulin is probably due to minor differences in molecular structure, as it has been shown that tubulins from both sources can copolymerize in vitro (Davidse, 1975; Sheir-Neiss et al., 1976; Water & Kleinsmith, 1976). The mutations leading to resistance to carbendazim in *Aspergillus nidulans* and *Penicillium expansum*, which involve changes in affinity of tubulin to carbendazim, apparently do not impair the normal functioning of the microtubular system, as the growth rate or sporulation of the strains does not change. This indicates normal functioning of the microtubular system. Evidently the ability of the altered tubulin molecules to assemble has not been changed. This is an example of how a protein in spite of its conservatism can be altered when the survival of the organism is threatened.

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Resistance to ergosterol-biosynthesis inhibitors
I. Chemistry and phenomenological aspects

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Abstract

The ergosterol-biosynthesis-inhibiting fungicides comprise a heterogeneous group of chemical substances that despite their heterogeneity have three characteristics in common: they all have at least one nitrogen-containing ring; with some exceptions, they contain at least one asymmetric carbon atom; and they nearly all interfere with ergosterol synthesis by inhibiting the C-14 demethylation step. On the basis of the chemical structure of their nitrogen-containing ring, they can be classed as piperazines, morpholines, pyridines, pyrimidines, imidazoles and triazoles. Though limited, there is evidence to show that different stereoisomeric forms may differ significantly in fungitoxicity. Most ergosterol-biosynthesis-inhibiting fungicides are broad-spectrum fungicides, toxic to representatives of the Ascomycetes, Deuteromycetes and Basidiomycetes. Resistance to ergosterol-biosynthesis-inhibiting fungicides has so far not been observed under practical conditions. However under laboratory conditions resistant mutants can be readily obtained, though they generally have reduced fitness with respect to spore germination, germ-tube elongation, mycelial growth or sporulation. Reduced fitness is almost invariably reflected by reduced pathogenicity, so development of resistance to this type of fungicide under practical conditions is unlikely.

Keywords: azasterols, cross-resistance, diastereomers, dicarboximides, enantiomers, ergosterol-biosynthesis-inhibiting fungicides, fitness of resistant mutants, hypocholesteremic agents, imidazoles, iprodione, morpholines, piperazines, pyridines, pyrimidines, stereoisomerism, triazoles.

General

Among the systemic fungicides, the ergosterol-biosynthesis inhibitors, for various reasons, occupy a special place. As distinct from virtually all other fungicides dealt with in this book, the ergosterol-biosynthesis inhibitors are not classified on the basis of a common chemical structure or structural moiety, but according to a common mechanism of action. They comprise a large number of chemically very diverse compounds that in some way interfere with ergosterol biosynthesis. Not all of them are used against plant-pathogenic fungi. This means that the term 'ergosterol-biosynthesis inhibitors' has a wider connotation than the term 'ergosterol-biosynthesis-inhibiting fungicides'. The ergosterol-biosynthesis inhibitors are part of a still larger group of compounds, the inhibitors of lipid synthesis (Ragsdale, 1977), which encompass inhibitors of the biosynthesis of sterols, gibberellins, carotenoids, sex hormones and fatty acids.

This contribution will only cover the inhibitors of the biosynthesis of sterols, among which ergosterol is predominant in fungi. Apart from the ergosterol-synthesis-inhibiting fungicides used in agriculture, there are other sterol inhibitors, all of which are well known for their interference with a great variety of biological functions, and thus affect virtually all biological systems and living organisms. This may explain why in the past many of the so-called hypocholesteremic compounds, used in clinical medicine to lower serum levels of cholesterol, have been assayed for antifungal activity. These include chemicals like AY-9944 (*trans*-1,4-bis(2-chlorobenzylaminomethyl)-cyclohexane dihydrochloride) (Elliott, 1969; Matolcsy et al., 1973), SKF 525-A (β-diethylaminoethyldiphenylpropyl acetate hydrochloride), SKF 2314 (2,2-diphenyl pentanoic acid), SKF 3301 A (2,2-diphenyl-1-(β-dimethylaminoethoxy) pentane hydrochloride), SKF 7732-A_3 (tris(2-dimethylaminoethyl)phosphate trihydrochloride), SKF 7997-A_3 (tris(2-diethylaminoethyl)phosphate trihydrochloride) and SKF 16467-A (N-dimethylaminoethyl-α,α-diphenyl valeramide hydrochloride) (Nelson et al., 1967; Elliott, 1969); and triparanol, a compound that has some structural resemblance to ancymidol, fenarimol and triarimol (Ragsdale & Sisler, 1975). These compounds are slightly fungitoxic to such fungi as *Cochliobolus carbonum* (Nelson et al., 1967), *Phytophthora cactorum*, *Sordaria fimicola* (Elliott, 1969), *Alternaria tenuis*, *Aspergillus niger*, *Botrytis cinerea*, *Fusarium moniliforme*, *Helminthosporium sativum*, *Rhizoctonia* spp. (Matolcsy et al., 1973) and *Ustilago maydis* (Ragsdale & Sisler, 1975).

Hypocholesteremic agents are also known to affect growth of yeast cells. For instance, trifluperidol, a piperidine derivative (Figure 1), and triparanol both mildly inhibit multiplication of *Saccharomyces cerevi-*

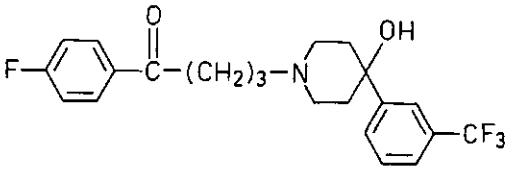


Figure 1. Structural formula of the piperidine trifluperidol.

siae cells (Aaronson, 1971; Sobus et al., 1977). Present knowledge suggests that the major step in the sterol biosynthetic pathway blocked by the hypocholesteremic agents is $\Delta^8 \rightarrow \Delta^7$ isomerization (Sobus et al., 1977; cf. Schmitt & Benveniste, 1979a).

Recently, another group of compounds, the azasteroid antifungal antibiotics from *Geotrichum flavo-brunneum* (Michel et al., 1975; Gordee & Butler, 1975), of which A25822B (15-aza-24-methylene-D-homocholesta-8, 14-dien-3β-ol) is the most active component, was reported to interfere with ergosterol biosynthesis, in this case by inhibition of the reduction of the Δ^{14} bond (Woloshuk et al., 1979). Fungi affected are *Aspergillus nidulans, Blastomyces dermatitidis, Cladosporium cucumerinum, Histoplasma capsulatum, Microsporum gypseum, Sporotrichum schenckii, Trichophyton mentagrophytes* and *Ustilago maydis*; azasterols are also active towards the yeast species *Candida albicans, Cryptococcus neoformans* and *Saccharomyces cerevisiae* (Gordee & Butler, 1975; Bailey et al., 1976; Woloshuk et al., 1979).

Both hypocholesteremic agents, namely AY-9944, and azasterols, namely A25822B, also interfere with sterol biosynthesis in higher plants, as has been illustrated with bramble cells. For both types of agents, the main sites of inhibition are identical to those reported for yeasts and fungi (Schmitt & Benveniste, 1979a; Schmitt et al., 1980). The same holds true for fenarimol, which in bramble cells, as in fungi, probably interferes with C-14 demethylation (Schmitt & Benveniste, 1979b). It is noteworthy that with all three sterol-biosynthesis inhibitors, stable bramble lines were obtained that were able to grow permanently in inhibitor-supplemented media. A similar phenomenon has been observed in lettuce, in which resistance to triforine, another ergosterol-biosynthesis-inhibiting fungicide, appears to be determined by a single recessive gene (Globerson & Eliasi, 1979; Maxon Smith, 1979). Thus higher plants appear to readily develop resistance to these compounds.

The ergosterol-biosynthesis-inhibiting fungicides used in agriculture

Although they are potentially useful in agriculture, most of the ergosterol-synthesis inhibitors dealt with so far are not used to control plant diseases. However, other ergosterol-synthesis-inhibiting fungicides

are rather extensively used in agriculture. Although they comprise a very heterogeneous group of structurally unrelated chemicals, they have three characteristics in common - be it with some exceptions. First, they all have at least one nitrogen-containing ring; they share this characteristic with innumerable compounds, among them systemic fungicides with an entirely different mode of action, as well as trifluperidol and the azasterol antifungal antibiotics, which, though fungitoxic, have not been used in agriculture so far. Second, with seven exceptions (buthiobate[1], clotrimazole[2], dodecyl-imidazole, EL-241, fluotrimazole[1], prochloraz[1], and XE 326), they contain at least one asymmetric carbon atom. This too is a structural feature occurring in an infinite number of chemicals, among them triparanol and the azasterols. Third, all ergosterol-biosynthesis inhibitors, with the known exception of tridemorph, and probably the other so-called morpholines (Kato et al., 1980; Kerkenaar, 1980), interfere with the ergosterol synthetic pathway by inhibition of C-14 demethylation. In addition, other steps in the biosynthetic pathway are blocked by some, but not all, of them. For triarimol, for instance, at least three sites of inhibition are now known (Ragsdale, 1975, 1977).

On the basis of the chemical nature of the nitrogen-containing ring present these ergosterol-biosynthesis-inhibiting fungicides can be divided as follows (for structural formulae see Figures 2-7):

- With a saturated ring system
. with two nitrogen atoms, piperazines (triforine[1])
. with one nitrogen and one oxygen atom, morpholines (dodemorph[1], fenpropimorph[1], tridemorph[1])
- With an unsaturated ring system
. with one nitrogen atom in a six-membered ring, pyridines (buthiobate[1], EL-241)
. with two nitrogen atoms in a six-membered ring, pyrimidines (ancymidol[3], fenarimol[1], nuarimol[1], triarimol)
. with two nitrogen atoms in a five-membered ring, imidazoles[5] (clotrimazole[2], dodecyl-imidazole, econazole[2], imazalil[1], ketonazole[2,4], miconazole[2], phenapronil[1], prochloraz[1], XE 326)

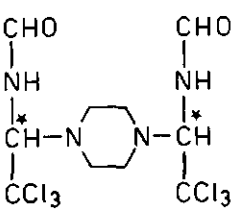
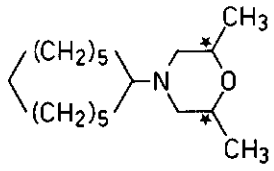
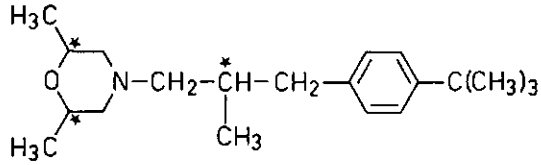


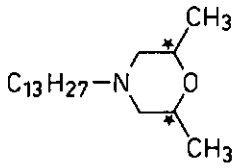
Figure 2. Structural formula of the piperazine triforine; star indicates asymmetric carbon atom.



dodemorph

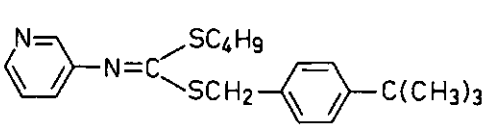


fenpropimorph

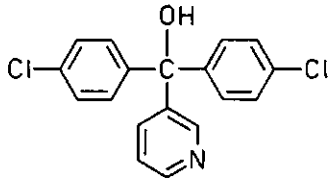


tridemorph

Figure 3. Structural formulae of morpholines; star indicates asymmetric carbon atom.

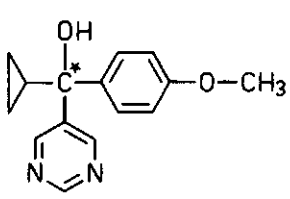


buthiobate

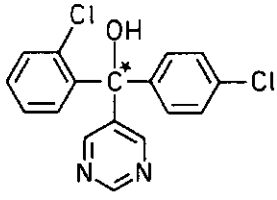


EL-241

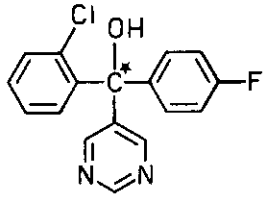
Figure 4. Structural formulae of pyridines.



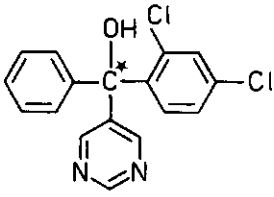
ancymidol



fenarimol

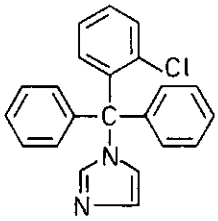


nuarimol

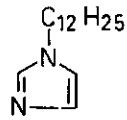


triarimol

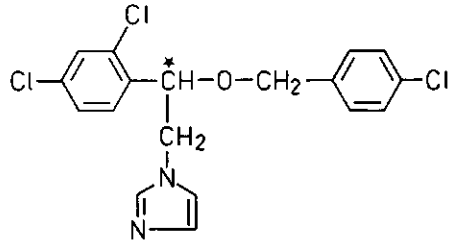
Figure 5. Structural formulae of pyrimidines; star indicates asymmetric carbon atom.



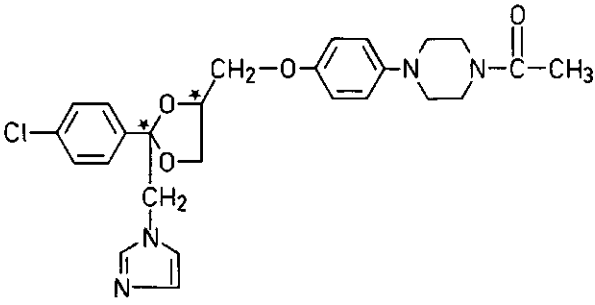
clotrimazole



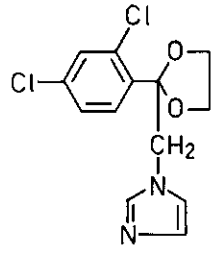
N-dodecylimidazole



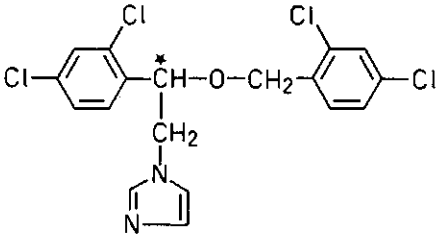
econazole



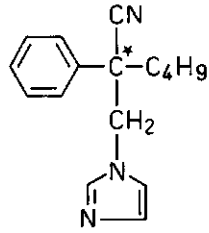
ketoconazole



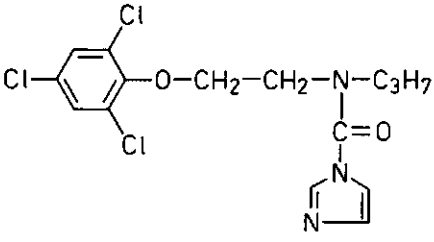
XE 326



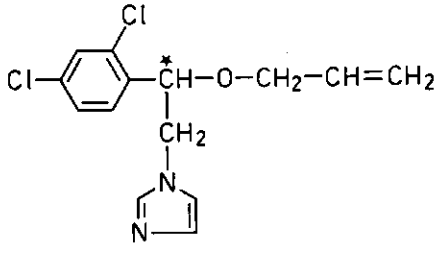
miconazole



phenapronil

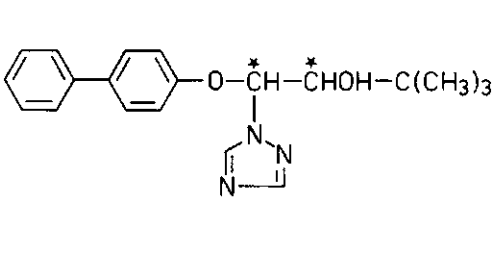


prochloraz

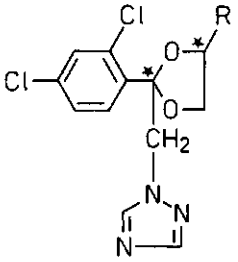


imazalil

Figure 6. Structural formulae of imidazoles; star indicates asymmetric carbon atom.

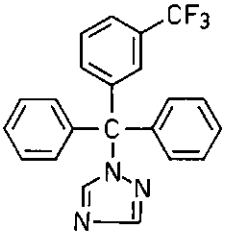


bitertanol

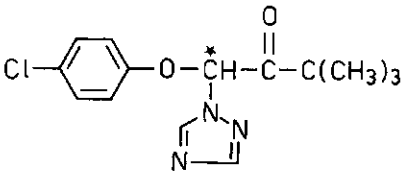


CGA 64250 R = n-C_3H_7

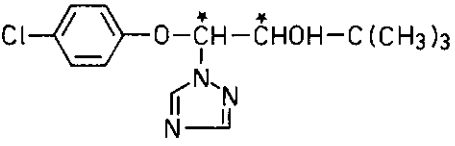
CGA 64251 R = C_2H_5



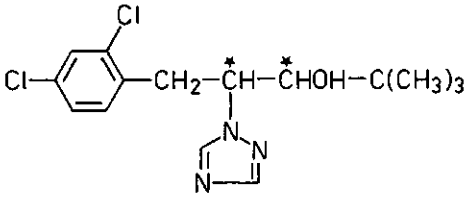
fluotrimazole



triadimefon



triadimenol



diclobutrazol

Figure 7. Structural formulae of triazoles; star indicates asymmetric carbon atom. N.B. The name diclobutrazol only applies to the 2R3R and 2S3S (threo) configuration, but not to the corresponding 2R3S and 2S3R (erythro) form.

. with three nitrogen atoms in a five-membered ring, triazoles (bitertanol[1], CGA 64250[1,6], CGA 64251[1,6], diclobutrazol[7], fluotrimazole[1], triadimefon[1], triadimenol[1]).

1. Released or proposed for agricultural use.
2. Released or proposed for clinical use against human fungal pathogens.
3. Ancymidol, primarily known for its growth regulatory activity in higher plants, is slightly fungitoxic to Cladosporium cucumerinum (Sherald et al., 1973).
4. The ketoconazole molecule also contains a piperazine ring (Thienpont et al., 1979).
5. In addition, a large number of imidazoles, among them butoconazole, isoconazole, sulconazole and tioconazole, are still in the experimental phase (cf. Heeres & van den Bossche, 1980).
6. See van Gestel et al. (1980) for related compounds.
7. This name applies to the threo configuration only (Bent & Skidmore, 1979).

This list not only contains agricultural fungicides, but also antimycotics used in medicine. Some experimental fungicides have been listed that chemically belong to the more well-known ergosterol-synthesis-inhibiting fungicides used in agriculture, but which may never be applied in practice. The list shows once more that it is virtually impossible to delimit these fungicides unequivocally, which makes it difficult to predict specifically the areas of application for prospective ergosterol-biosynthesis inhibitors.

The implications of stereoisomerism

Due to the presence of one or more asymmetric carbon atoms, most of these chemicals occur in different stereoisomeric forms, which may often differ significantly in fungitoxicity. For instance, the (-) enantiomer of triforine is much more active than its (+) analogue (Ost, 1976). Diclobutrazol (Bent & Skidmore, 1979), fenpropimorph (Pommer & Himmele, 1979) and triadimenol (Buchenauer, 1979b) are three other compounds whose activity depends on the stereoisomer involved.

Apart from the stereoisomerism due to the presence of the asymmetric carbon atoms in the side chains, in the triforine molecule another form of stereoisomerism originates from the nature of the piperazine ring. The chair form of the piperazine ring permits the 1-formamido-2,2,2-trichloroethyl side chains to occur in two different orientations at each of the ring nitrogen atoms, equatorial and axial. According to Josepovits & Gasztonyi (1976), with one side chain equatorial and the other axial, hydrogen bonding can be assumed to occur between the formamide group in one chain and the carbonyl group in the other. The alleged higher water solubility of this stereoisomer compared to the stereoisomer with both side chains oriented equatorially is believed to be responsible for its greater biological activity.

Though widely recognized in pharmacology, the implications of stereoisomerism, especially enantiomerism, have been almost neglected with respect to agricultural fungicides. In pharmacology, stereoselective effects of enantiomers, especially of representatives of so-called congeneric series of compounds, are nowadays taken into consideration by controlling drug geometry in drug design (cf. Witiak et al., 1978). Direct comparison of the isomeric activity ratio (A/B) with the activity (A) of the most potent member of each pair of enantiomers in such a congeneric series (B denotes the activity of the lesser active member) led Ariëns and co-workers (Lehmann F. et al., 1976; Lien et al., 1976) to conclude that lg A/B is a linear function of lg A. When the slope of this function is zero, the chiral centre (asymmetric carbon atom) of the enantiomers is not critically involved in the binding of the enantiomers to the target site: i.e. chirality is non-critical to stereoselectivity. This means that in such cases the asymmetric centre does not play a selective role

in the differential activity of the two members of the enantiomeric pair. In other instances (when the slope of the function is not equal to zero), however, stereoselectivity wholly or partially depends on chirality.

Two congeneric series of ergosterol-biosynthesis inhibitors, the imidazoles and the triazoles, seem to be outstanding 'tools' with which to study the effects of stereoselectivity in agricultural fungicides; perhaps the morpholines and the pyrimidines can also be used to examine these phenomena, which might provide a better insight into the lack of cross-resistance between structurally related fungicides and the mechanisms of resistance (p.87-100). Further, more knowledge about the effects of the formulation used on the ratio in which the stereoisomers are present and their fungitoxic activity, as well as about differential metabolism of stereoisomers (cf. Miyano et al., 1980) seems a prerequisite for full comprehension of the significance of stereoisomerism in the mode of action of ergosterol-biosynthesis inhibitors. It may be essential also for the optimization of bioactivity in congeneric series of these fungicides (cf. Verloop & Tipker, 1977).

Phenomenological aspects of resistance

Introductory remarks

In general ergosterol-biosynthesis inhibitors are broad-spectrum fungicides, toxic to representatives of Ascomycetes, Deuteromycetes and Basidiomycetes (Fuchs & Drandarevski, 1973; Buchenauer, 1979a); however Oomycetes and Zygomycetes are much less sensitive (Siegel et al., 1977; Buchenauer & Röhner, 1979; de Waard & Ragsdale, 1979). However, the reported sensitivities of fungi towards these fungicides - which even among taxonomically closely related fungal species often differ considerably - may depend on the predominant stereoisomer present in the formulations used (cf. Ost, 1976; H. Buchenauer, personal communication, 1981).

Generally, the ergosterol-biosynthesis-inhibiting fungicides used in agriculture have little effect on spore germination, in contrast to germ-tube elongation. Initial spore germination may be normal, but germ-tubes may become heavily distorted, resulting in abnormal growth. Hyphae are frequently swollen and/or excessively branched. Nevertheless, dry weight increase, respiratory activity, mitosis and nucleic acid and protein synthesis are hardly or not at all inhibited. However, in cultures treated with these fungicides, accumulation of free fatty acids often occurs, together with a decrease in C-4 desmethyl sterols (primarily ergosterol) and an increase in methyl and dimethyl sterols. Therefore inhibition of C-14 demethylation in the ergosterol biosynthetic pathway is believed to be their primary site of action, although additional sites of attack are possible (cf. Ragsdale, 1977).

Fitness and virulence of resistant mutants

So far, practical application of fungicides that inhibit ergosterol biosynthesis has not led to any confirmed case of resistance. Recently, however, Walmsley-Woodward et al. (1979a) reported increased levels of resistance of *Erysiphe graminis* f. sp. *hordei* to tridemorph in both glasshouse and field experiments. Whether tridemorph exerted a mutagenic effect, raising the low frequency of resistance existing in wild-type populations to the levels observed, or whether the reported changes were solely due to selection pressure, is not known. However, it is likely that once the fungicide ceases to be used, sensitive wild-type strains will eventually predominate, because of their greater competitiveness in the absence of the fungicide. A lower degree of fitness in the resistant pathogen population may be the reason why, so far, there have been no reports of widespread failure of tridemorph.

Attempts to obtain resistance to triadimefon in *Erysiphe graminis* ff. sp. *tritici* and *hordei*, *Uromyces appendiculatus* and *Uromyces fabae* by transferring these obligate parasites every fortnight onto triadimefon-treated plants did not result in a change in sensitivity (Kovacs & Tüske, 1980). This agrees with earlier findings (Drandarevski & Schicke, 1975) that successive transfers of bean-rust uredospores to bean plants treated with sub-lethal concentrations of triforine did not yield resistant strains; invariably, there was a decline in infection level and spore production rate and after a few passages no more uredospores were produced.

In contrast, resistant mutants can often be readily obtained in vitro, by selection of untreated or mutagen-treated conidia on agar media with lethal concentrations of any of the ergosterol-biosynthesis-inhibiting fungicides. Usually such resistant mutants are cross-resistant to most other ergosterol-biosynthesis inhibitors. A list of fungal species for which cross-resistance in vitro has been obtained so far is given on p.92.

Resistance in fungi to ergosterol-biosynthesis-inhibiting fungicides is generally accompanied with a decrease in fitness or pathogenicity. This was demonstrated first with triarimol- and triforine-resistant mutants of *Cladosporium cucumerinum*: the degree of resistance was found to be inversely proportional to pathogenicity (Fuchs & Viets-Verweij, 1975; Fuchs et al., 1977) (Figure 8). This relation has been since confirmed in experiments with the same and other fungal species (Buchenauer, 1977; van Tuyl, 1977), although in *Penicillium expansum*, imazalil resistance was not always coupled with decreased pathogenicity (van Tuyl, 1977). Mutants of *Aspergillus nidulans* with identified genes for resistance have displayed varying degrees of reduced fitness with respect to spore germination, germ-tube elongation (Figure 9), mycelial growth (Figure 10) and sporulation (de Waard & Sisler, 1976; de Waard & Gieskes, 1977). The rate of

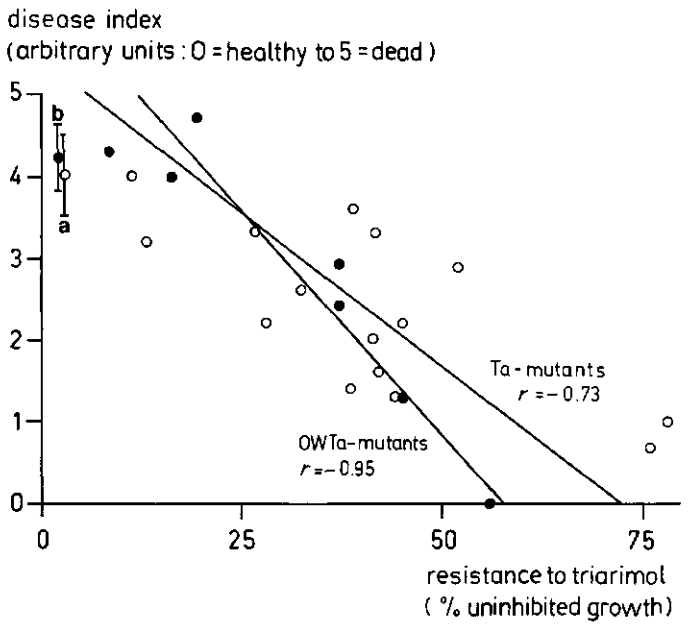


Figure 8. Relation of pathogenicity to degree of resistance to triarimol (2.5 µg/ml) for triarimol (Ta)-resistant Cladosporium cucumerinum mutants in a cucumber seedling test. a: green wild-type strain. b: off-white (OW) 'parent' strain (Source: Fuchs et al., 1977).

germ-tube elongation appeared to be negatively correlated with the degree of resistance, and all mutant strains resembled each other in their impaired ability to produce spores. Mutant strains of *Cladosporium cucumerinum* also produced less spores, which were often not viable (Sherald & Sisler, 1975; Fuchs & Drandarevski, 1976). Reduced fitness of resistant isolates has also been observed with obligate parasites. For instance, in tridemorph-resistant strains of *Erysiphe graminis* f. sp. *hordei* pathogenicity and resistance were found to be negatively correlated (Walmsley-Woodward et al., 1979b, 1980).

Practical implications of resistance

The observations on fitness and virulence suggest that resistance in pathogenic fungi to ergosterol-biosynthesis-inhibiting fungicides might have significant epidemiological implications, since the chance of such strains surviving in the absence of selection pressure by the fungicide is severely reduced. Fuchs & Drandarevski (1976) concluded on similar evidence that development of resistance to this type of fungicide under practical conditions is rather unlikely. The danger of development of resistant populations is also reduced as most resistant strains can still be controlled with normal fungicide application rates (see, for instance Brown & Hall, 1979).

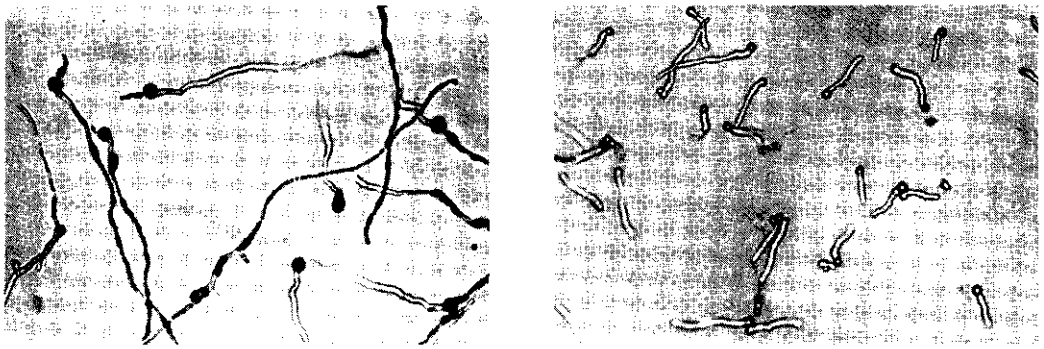


Figure 9. Spore germination and germ-tube elongation of Aspergillus nidulans after a 12 h incubation on a glucose-nitrate agar. Left: wild-type strain 003. Right: fenarimol-resistant mutant strain 193 (for details on strains see: de Waard & Gieskes, 1977).

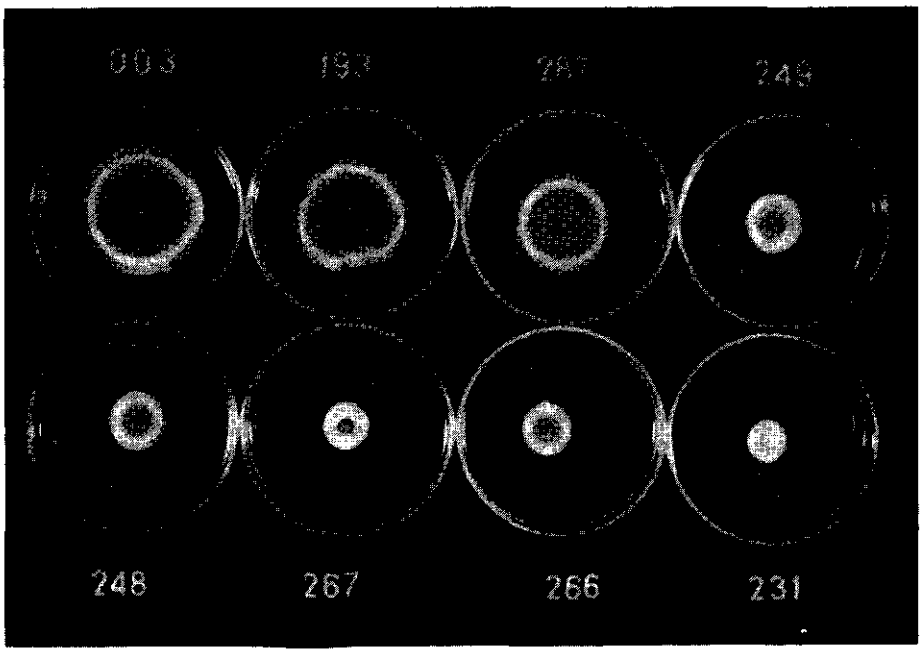


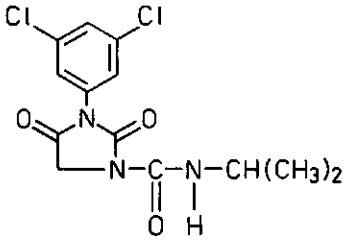
Figure 10. Mycelial growth of some Aspergillus nidulans strains on malt agar: 003 is the wild-type strain; others are fenarimol-resistant mutant strains (for details on strains see: de Waard & Sisler, 1976).

Iprodione: a special case

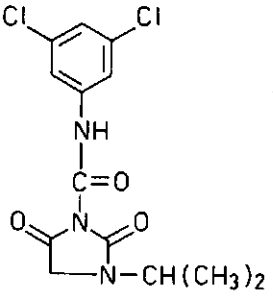
One more fungicide is worth special mention: iprodione (Figure 11), a compound usually referred to as a dicarboximide fungicide, which contains a hydantoin ring that is a saturated five-membered ring with three nitrogen atoms. Unlike the other dicarboximides, it has been implied that

iprodione interferes with ergosterol biosynthesis; the accumulation of 4,4-demethylsterols indicates that it inhibits at a site most probably different from that of C-14 demethylation (Pappas & Fisher, 1979). Controversial data on fungal resistance to this fungicide (p.101-117) may be due to structural rearrangement of iprodione in the (m)ethanolic solutions often used to dissolve the fungicide. Under these conditions iprodione rearranges to form an isomer (Figure 11), which, like the parent compound, is a dicarboximide with an identical ring system, but which almost entirely lacks fungitoxicity (Cooke et al., 1979). The suggestion that the so-called anomalous behaviour of *Botrytis cinerea* isolates on iprodione-containing media, observed by Leroux et al. (1977), is explained by this structural rearrangement seems rather unlikely. Both Leroux et al. (1977) and Schüepp & Küng (1978) obtained resistance levels (ED_{50}s of up to about 300 µg/ml, as compared to 0.3 µg/ml for the wild-type strains) that were comparable with those for the other dicarboximides. In both instances controls were included and precautions taken that should have precluded possible effects of the assumed isomerizations. However the resistance levels obtained by Dennis & Davis (1979), who found that their isolates were able to grow on potato-dextrose agar containing iprodione at a concentration of 10 mg/ml - 30 times higher than the level obtained by Leroux et al. and Schüepp & Küng - might be entirely or at least partially ascribable to the suggested molecular rearrangement to the less toxic isomer.

It is of utmost importance to verify the assumptions of Pappas & Fisher (1979) that inhibition of sterol biosynthesis is probably the primary action of iprodione, and to investigate whether cross-resistance to the established ergosterol-synthesis inhibitors is possible. Preliminary observations with *Aspergillus nidulans*, *Cladosporium cucumerinum* and *Penicillium italicum* have revealed only low levels of cross-resistance of



iprodione



rearrangement product of iprodione

Figure 11. Structural formula of iprodione and its structural rearrangement product (cf. Cooke et al., 1979).

two fenarimol-resistant *Cladosporium cucumerinum* isolates to iprodione and vinclozolin, and no cross-resistance whatsoever to procymidone (Fuchs & de Waard, unpublished data). Yet caution seems necessary, if only to prevent 'disease control agents with proven effectiveness being unnecessarily lost to resistance' (Delp, 1980).

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Resistance to ergosterol-biosynthesis inhibitors II. Genetic and physiological aspects

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Abstract

To date, development of resistance to fungicides that inhibit sterol biosynthesis has not been observed under practical conditions. Yet, a large number of mutants of different fungi with resistance to these fungicides have been isolated in vitro and characterized. Resistance to these fungicides has, at least in *Aspergillus nidulans*, been shown to be based on a multigenic system. Cross-resistance between sterol-biosynthesis inhibitors is the rule, even when they are chemically not related to each other. Resistance may be accompanied with pleiotropic effects such as increased sensitivity or resistance to unrelated toxicants and, possibly, reduced fitness. The mechanism of resistance can vary in different fungi. Resistance to fenarimol in *A. nidulans* is probably based on decreased uptake of the fungicide by resistant mutants as compared with the wild-type strain. Decreased uptake is due to enhanced energy-dependent efflux activity, the result of which is that the fungicide probably does not saturate the site of action. The genetic and physiological data on resistance are discussed in relation to their significance for development of resistance in practice.

Keywords: ergosterol-biosynthesis inhibitors, imazalil, fenarimol, genetics of imazalil resistance, mechanism of resistance to fenarimol, fungicide resistance, cross-resistance.

Introduction

Systemic fungicides with specific activity towards ergosterol biosynthesis are likely to become the most prominent group of chemotherapeutics in the near future. To date at least 17 chemicals of this type have been introduced for agricultural use (Table 1). Some of them have been used already for a significant period of time, others have been introduced only recently and have not yet been released for practical application.

Table 1. Agricultural fungicides that inhibit ergosterol biosynthesis[a].

Chemical group	Trivial name	Trade name(s)	Year of introduction
Piperazines	triforine	Funginex/Saprol	1969
Morpholines	dodemorph	Meltatox	1967
	tridemorph	Calixin	1969
	fenpropimorph	Corbel	1980
Pyridines	buthiobate	Denmert	1975
Pyrimidines	fenarimol	Rubigan/Rimidin	1975
	nuarimol	Trimidal/Triminol	1975
Imidazoles	imazalil	Fungaflor/Fecundal	1972
	prochloraz	(BTS 40542)	1977
	phenapronil	Sisthane	1978
Triazoles	fluotrimazole	Persulon	1973
	triadimefon	Bayleton	1974
	triadimenol	Baytan	1977
	bitertanol	Baycor	1979
	diclobutrazole	Vigil	1979
	propiconazole[b]	Tilt (CGA 64250)	1979
	etaconazole[b]	(CGA 64251)	1979

a. For structural formulae see p.71-86.
b. Proposed trivial name.

Wide-scale use of site-specific fungicides may lead to development of resistance within one to two years, as has been demonstrated for benzimidazoles (Schroeder & Provvidenti, 1969) and acylalanines (Davidse et al., 1981). But prolonged practical use of fungicides that inhibit ergosterol biosynthesis has not so far led to any confirmed case of resistance (p.71-86). For instance, in the Netherlands, tridemorph and triforine have been used in glasshouses against resistance-prone fungi such as powdery mildews for at least five years without apparent development of resistance. In contrast, resistance in vitro to sterol-biosynthesis-inhibiting fungicides can be readily obtained. These mutant strains often display reduced fitness or pathogenicity (see p.71-86). Probably, reduced pathogenicity and a general low degree of resistance account for resistance not yet being apparent in practical agriculture.

This contribution describes in detail some fundamental aspects of resistance to fungicides that inhibit sterol biosynthesis. Attention is paid to: the genetic background of resistance; cross-resistance and pleiotropic effects associated with mutations for resistance; and biochemical mechanisms of resistance. Knowledge of these phenomena is necessary to understand the behaviour and characteristics of resistant mutants under practical conditions.

Genetics of resistance

Resistance to sterol-biosynthesis inhibitors may be permanent or transient. Transient, or so-called phenotypic, resistance may be obtained by subculturing fungi on fungicide-containing media. Such resistance is adaptive and not due to expression of genes for resistance. Transient resistance is lost upon subculturing on fungicide-free media (Fuchs & Viets-Verweij, 1975). Transient resistance may also develop in stationary phase cultures, as shown for *Candida albicans* to miconazole (Cope, 1980a, b; Gale et al., 1980).

Permanent resistance in vitro to ergosterol-biosynthesis-inhibiting fungicides has been frequently reported (cf. Fuchs et al., 1977). Generally, permanent resistance is considered to be of a genetic nature, when decreased sensitivity is stable upon repeated subculturing on fungicide-free media. Determination of mutation frequencies and genetic analysis of genes for resistance have so far only been reported for imazalil resistance in *Aspergillus nidulans* (van Tuyl, 1977). From this work, it is evident that both spontaneous and ultra-violet (UV) and N-methyl-N'-nitro-N-nitrosoguanidine (NG)-induced mutations for resistance to imazalil occur rather frequently in comparison with mutations for resistance to other fungicides, like benomyl and carboxin (Table 2). However, the degree of resistance to these two fungicides is significantly higher than to imazalil (Table 3). Comparable data for other fungi have been published by van Tuyl (1977).

Imazalil resistance in *A. nidulans* is based on a multigenic system (van Tuyl, 1977). Genetic analysis of 21 imazalil-resistant mutants resulted in the identification of eight loci allocated to six different linkage groups. The loci and linkage groups, in parentheses, involved are *imaA* (VII), *imaB* (V), *imaC* (II), *imaD* (VIII), *imaE* (II), *imaF* (I),*imaG* (III), and *imaH* (III). The large number of linkage groups indicates that

Table 2. Mutation frequency of resistance to various fungicides in Aspergillus nidulans.

Mutagenic treatment[a]	Proportion (%) original population surviving mutagenic treatment	Mutation frequency[b]		
		benomyl[c]	carboxin[c]	imazalil[c]
SP	100	0.02	0.25	4
UV	50	7	12	160
NG	50	22	80	250

Source: van Tuyl (1977).

a. SP: spontaneous mutation; UV: ultra-violet light;
NG: N-methyl-N'-nitro-N-nitrosoguanidine.
b. Number of mutants per 10^7 surviving conidia.
c. Selection medium containing benomyl, carboxin or imazalil at concentrations of 2 µg/ml, 10 µg/ml or 2 µg/ml, respectively.

Table 3. Highest degree of induced resistance in Aspergillus nidulans to three fungicides.

Strain	ED_{50} (µg/ml)		
	benomyl	carboxin	imazalil
Wild type	0.7	1.5	0.4
Mutant	30	200	4

Source: van Tuyl (1977).

the loci conferring resistance are not clustered but distributed over the fungal genome. Mutations at the *imaA* and *imaB* loci occur most frequently, since of 202 resistant strains tested, 113 were found to be of the *imaA* type and 52 of the *imaB* type. The mutation at the *imaB* locus appeared to be allelic to *camD*, a mutation for resistance to chloramphenicol (Gunatilleke et al., 1975), and to a mutation determining fenarimol resistance (de Waard & Gieskes, 1977). Similarly, *imaH* was found to be allelic to *actC*, which confers resistance to cycloheximide. Two other genes for resistance to cycloheximide, *actD* and *actE* also conferred imazalil resistance, but in contrast to the *actC* locus, they were not picked up among mutants directly selected for imazalil resistance. This means that at least 10 different genes are involved in resistance to imazalil.

Allelic mutations in a certain locus for resistance to imazalil do not always result in the same degree of resistance. For instance, mutations at the *imaA* or *imaB* locus may be reflected by different minimum inhibitory concentrations (MIC), which implies that mutations in a particular gene may be dissimilar (Table 4). Mutations in all other loci only confer a low level of resistance (MIC of imazalil 2.5 - 5.0 µg/ml), suggesting that mutations in different genes are of variable physiological consequence (van Tuyl, 1977). Studies with heterozygous diploid imazalil-resistant strains of *A. nidulans* have shown that *imaA*, *imaB*, *imaC*, and *imaD* mutations are semi-dominant. This implies that the sensitivity of such heterozygous strains is intermediate between sensitive diploid and resistant haploid strains (van Tuyl, 1977).

Table 4. Degree of resistance of Aspergillus nidulans mutants with allelic imaA and imaB mutations for imazalil resistance.

Mutation	Number of strains tested	Number of strains for MICs (µg/ml) as indicated			
		1.0-2.5	2.5-5.0	5.0-8.0	8.0-12.0
None	1	1	0	0	0
imaA	11	0	3	7	1
imaB	4	0	0	3	1

Source: van Tuyl (1977).

Table 5. Degree of resistance of Aspergillus nidulans recombinant strains to imazalil.

Mutation	MIC (μg/ml)
None	2
imaA	20
imaB	10
imaA, imaB, M[a]	200

Source: van Tuyl (1977).

a. Modifier gene that fortifies the action of the imaA gene.

By genetic techniques it is possible to combine different single-gene mutations for resistance to imazalil in the same strain. This has been done with the *imaA* and *imaB* genes and a modifier gene *M* (van Tuyl, 1977). The recombinant strain possesses a relatively high degree of resistance, which indicates positive or additive interaction (Table 5).

Cross-resistance and pleiotropic effects

Cross-resistance can be defined as resistance to two or more toxicants that is caused by the same genetic factor (Georgopoulos, 1977). This implies that mutants selected on one toxicant are usually also resistant to toxicants that are chemically related or have a similar mechanism of action. Cross-resistance is often reciprocal, which means that mutants selected in the presence of a certain toxicant are also resistant to the other toxicant concerned, and vice versa. This also applies to mutants selected on media containing one of the ergosterol-biosynthesis inhibitors (Table 6), which, although often unrelated chemically, probably share a similar mechanism of action (cf. Ragsdale, 1977). Cross-resistance has been demonstrated also to fungitoxicants with a similar mechanism of action, e.g. ancymidol, clotrimazole, EL-241 and miconazole (Sherald et al., 1973; de Waard & Sisler, 1976; Barug & Kerkenaar, 1979).

Data in Table 6 indicate that cross-resistance between ergosterol-biosynthesis inhibitors is not always extant. For instance, cross-resistance in imazalil-resistant *A. nidulans* strains to fenarimol was almost absent in a strain carrying the *imaA* gene (de Waard & Gieskes, 1977). Similar results were found for imazalil-resistant strains of *Cladosporium cucumerinum* (Fuchs et al., 1977; van Tuyl, 1977) and *Phialophora cinerescens* (van Tuyl, 1977). Also in *Ustilago maydis*, cross-resistance was not always reciprocal (Barug & Kerkenaar, 1979). Perhaps cross-resistance does exist, but just has not been identified yet due to a relatively low mutation frequency of the genes involved, or to differences in dosage response. Such mutants may be missed when a limited number of mutants are tested.

Table 6. Cross-resistance of fungal mutants to fungicides that interfere with ergosterol biosynthesis.

Fungus	Fungicide used for selection	Cross-resistance to some fungicides[a]									Reference
		but	fen	ima	nua	pro	triar	trid	trif	triad	
Cladosporium cucumerinum	triarimol	+							+		Sherald et al., 1973; Sherald & Sisler, 1975
Aspergillus fumigatus	triarimol[b]								+		Sherald & Sisler, 1975
Cladosporium cucumerinum	triarimol[b]	+	+	+					+	+	Fuchs & Viets-Verweij, 1975
Cladosporium cucumerinum	triforine[b]	+	+	+			+[c]			+	Fuchs et al., 1977
Cladosporium cucumerinum	triadimefon								+	+	Buchenauer, 1976
Cladosporium cucumerinum	triadimefon			+			+		+		Leroux & Gredt, 1976
Cladosporium cucumerinum	triforine[b]			+			+			+	Leroux et al., 1976
Botrytis cinerea	triarimol[b]			+					+	+	Leroux et al., 1976
Ustilago maydis	triadimefon			+			+				Leroux & Gredt, 1976
Ustilago maydis	triarimol[b]			+						+	Leroux et al., 1976
Aspergillus nidulans	imazalil[b]		+[c]							+	van Tuyl, 1977; de Waard & Gieskes, 1977
Aspergillus nidulans	triarimol[b]		+	+						+	de Waard & Gieskes, 1977
Aspergillus niger	imazalil		+								van Tuyl, 1977
Cladosporium cucumerinum	imazalil		-								van Tuyl, 1977
Cladosporium cucumerinum	imazalil		-						-		Fuchs et al., 1977
Phialophora cinerescens	imazalil		-								van Tuyl, 1977
Botrytis cinerea	triadimenol		+	+	+[c]					+	Buchenauer, 1978
Cladosporium cucumerinum	triadimenol		+	+	+[c]					+	Buchenauer, 1978
Ustilago maydis	triadimenol		+	+	+[c]					+	Buchenauer, 1978
Ustilago maydis	fenarimol[b]			+	+	+		+[c]		+	Barug & Kerkenaar, 1979
Ustilago maydis	imazalil[b]		+		+	+		+[c]		-	Barug & Kerkenaar, 1979
Ustilago maydis	nuarimol[b]		+	+		+		-		+	Barug & Kerkenaar, 1979
Ustilago maydis	triadimefon[b]		+	+	+	+		+[c]			Barug & Kerkenaar, 1979
Ustilago maydis	tridemorph[b]		+[c]	+[c]	+	+[c]				-	Barug & Kerkenaar, 1979

a. Abbreviations: but = buthiobate; fen = fenarimol; ima = imazalil; nua = nuarimol; pro = prochloraz; triar = triarimol; trid = tridemorph; trif = triforine; triad = triadimefon; + = cross-resistance, - = no cross-resistance.
b. Strains with varying degrees of resistance tested.
c. Cross-resistance present in part of the strains tested.

Negatively correlated cross-resistance to ergosterol-biosynthesis-inhibiting fungicides has not been reported. Cross-resistance in *U. maydis* to unrelated fungicides like carbendazim and carboxin was absent (Leroux et al., 1976), and cross-resistance in *A. nidulans* to pimaricin, a polyene antibiotic with high affinity to ergosterol, could not be demonstrated either; some of the fenarimol-resistant strains were even slightly more sensitive to this antibiotic than the wild-type strain (de Waard & Sisler, 1976).

A pleiotropic mutation is defined as a single-gene mutation affecting more than one characteristic. Such a mutation may not only cause resistance to a toxicant but may also affect a more general cell property (Georgopoulos, 1977), which may result in, for example, positively- or negatively correlated cross-resistance to unrelated toxicants. For instance, triarimol-resistant mutants of *Botrytis cinerea* have an increased sensitivity to cycloheximide (Leroux et al., 1976). Mutations for resistance to imazalil in *A. nidulans* also give rise to pleiotropic effects such as resistance or increased sensitivity to unrelated toxicants like acriflavine, cycloheximide, chloramphenicol, and neomycin (Table 7). The *imaG* mutation was also correlated with cold sensitivity (van Tuyl, 1977). In addition, some imazalil- or fenarimol-resistant mutants showed pleiotropic effects with respect to non-related fungicides, such as 8-azaguanidine, p-fluorophenylalanine, D-serine, and thiourea (de Waard & van Nistelrooy, 1979), as well as with respect to fitness (de Waard & Gieskes, 1977). Reduced pathogenicity of pathogenic fungi (see p.71-86) might also be a pleiotropic effect of mutations for resistance.

Table 7. Pleiotropic effects of imazalil-resistant mutants of various fungi.

Fungus	Mutants or number of strains	Resistance[a]				Increased sensitivity[a]		
		acr	act	cam	neo	acr	act	neo
Aspergillus nidulans	imaA	+	-	-	+	-	-	-
	imaB	-	-	+	-	+	+	+
	imaC	-	+	+	-	-	-	-
	imaD	-	-	-	-	-	+	+
	imaE	-	+	+	+	-	-	-
	imaF	-	-	-	-	-	+	+
	imaG	-	+	+	+	-	-	-
Aspergillus niger	1	-	-	-	-	+	+	-
	3	-	+	-	-	+	-	-
Cladosporium cucumerinum	2	-	-	-	-	-	-	-
Phialophora cinerescens	1	-	-	-	-	-	-	-

Source: van Tuyl (1977).

a. Abbreviations: acr = acriflavine; act = cycloheximide; cam = chloramphenicol; neo = neomycin; pleiotropic effect: + = present; - = absent.

Mechanism of resistance

Resistance to fungicides with a specific mechanism of action may be based on decreased affinity of the target site for a toxicant. Such a mechanism of resistance has been described for benzimidazoles and oxathiins (cf. Georgopoulos, 1977). Resistance to ergosterol-biosynthesis-inhibiting fungicides might be based on decreased affinity of the target enzyme(s) involved in ergosterol biosynthesis for these fungicides. To date it has not been feasible to test this hypothesis experimentally. Alternatively, resistance to polyene antibiotics may be based on replacement of ergosterol by more 'primitive' sterols (cf. Hamilton-Miller, 1974). Such a mechanism appears not to be involved in the resistance to sterol-biosynthesis inhibitors, as mutants of *A. nidulans*, *C. cucumerinum* and *U. maydis* have almost normal concentrations of ergosterol (Sherald & Sisler, 1975; Ragsdale & de Waard, 1977; Leroux & Gredt, 1978).

Factors that influence the intracellular concentration of a fungicide, such as decreased uptake and increased detoxification, may also result in decreased sensitivity. Both factors may play a role in natural resistance to triforine (Gasztonyi & Josepovits, 1975). Transient resistance of *C. albicans* to miconazole in stationary-phase cultures is probably also due to detoxicification. This may be the result of unspecific accumulation of miconazole in cell walls of stationary-phase cells, which depletes the medium and prevents miconazole from reaching the plasma membrane. The resulting decreased sensitivity to miconazole is correlated with decreased release of K^+ by the cells. This mechanism, rather than interference with ergosterol biosynthesis, is thought to be the primary mechanism of action of miconazole (Cope, 1980a; Gale et al., 1980).

Resistance to fenarimol in *A. nidulans* could not be ascribed to increased metabolism (de Waard & Ragsdale, 1979), but rather to a differential uptake of the fungicide by wild-type and resistant strains (de Waard & van Nistelrooy, 1979). Uptake of fenarimol by the wild-type strain and the three genetically defined strains J146 (*imaB*), M196 (*imaB*), and R264 (*imaA, imaB, M*) is presented in Figure 1. Uptake by the wild-type strain was characterized by a rapid initial accumulation during the first 10 minutes of incubation and a subsequent gradual release; uptake by the mutant strains was nearly always at a constant low level.

Uptake by the wild-type strain appeared to be the resultant of influx and efflux of fenarimol. Influx could be inhibited by neither low temperature, anaerobiosis, starvation of mycelium nor incubation with several respiratory inhibitors and is therefore a passive process. Under identical test conditions efflux activity was severely inhibited and should thus be regarded as an energy-dependent process. Examples of such effects are given in Figures 2 and 3. After prolonged incubation (90 minutes), an equilibrium between influx and efflux was reached, resulting in an energy-de-

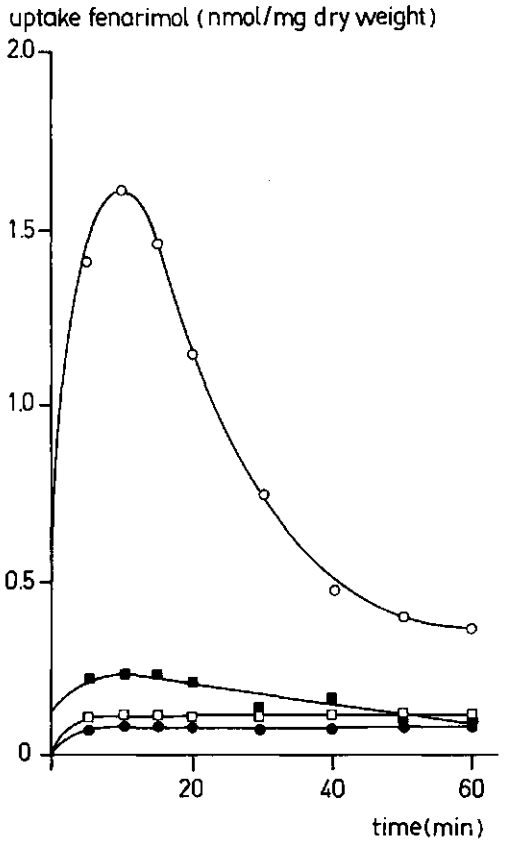

Figure 1. Uptake of fenarimol (30 μM) by mycelium of wild-type strain 003 (○) and fenarimol-resistant mutants J146 (●), M193 (□), and R264 (■) of Aspergillus nidulans in 23.4 mM K-phosphate buffer pH 6.0 containing 0.1 mM $CaCl_2.H_2O$ and 0.01 g/ml glucose (de Waard & van Nistelrooy, 1980).

pendent steady state, the efflux 'component' of which could be inhibited by addition of oligomycin or N,N'-dicyclohexylcarbodiimide, which instantaneously enhanced uptake (Figure 4). The establishment of an equilibrium at a low level of uptake suggests that efflux activity is inducible (de Waard & van Nistelrooy, 1980).

Uptake by the genetically defined fenarimol-resistant mutants also appeared to be determined by influx and efflux. Uptake could be considerably enhanced by low temperature, anaerobiosis, starvation of mycelium and incubation with respiratory inhibitors. Low uptake by these strains can be attributed to an energy-dependent efflux activity for fenarimol that rather than being inducible, like in the wild-type strain, is most probably constitutive. Upon inhibition of the efflux activity, net uptake resulted from the remaining passive influx, which in that case might be as high as in the wild-type strain. Examples of these effects are also

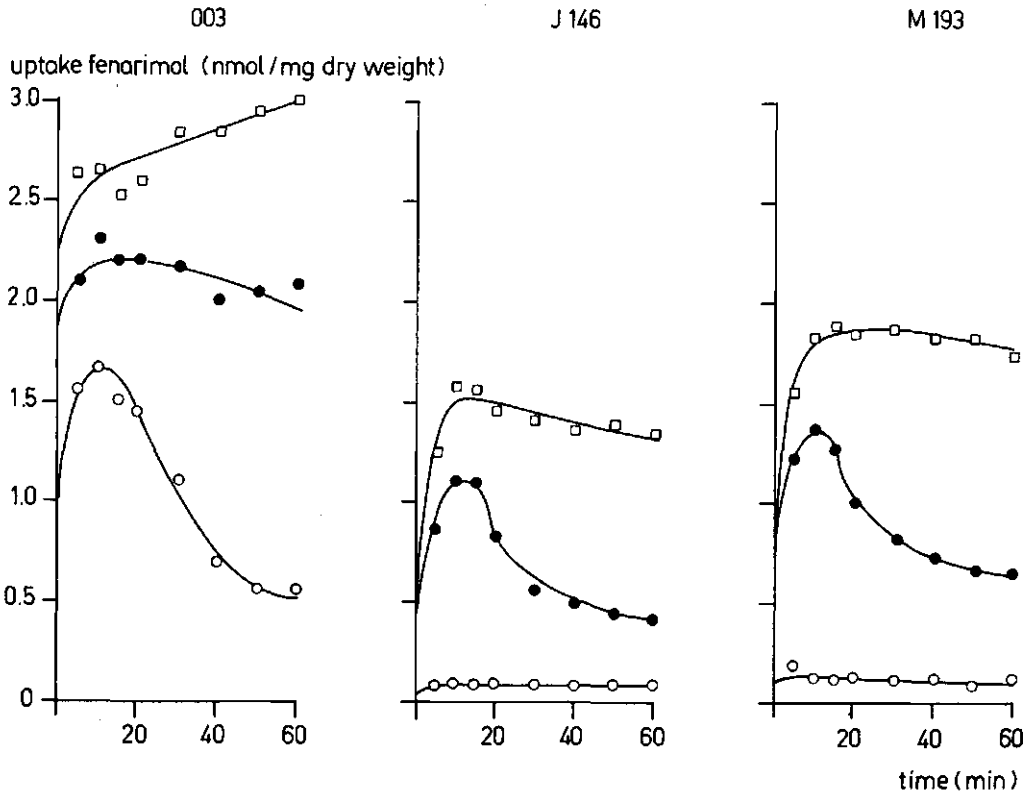

Figure 2. Effect of oligomycin on uptake of fenarimol (30 µM) by mycelium of wild-type strain 003 and fenarimol-resistant mutants J146 and M193 of Aspergillus nidulans. Treatments: control (○); 10 (●) µM and 100 (□) µM oligomycin (de Waard & van Nistelrooy, 1980).

given in Figures 2, 3, and 4.

The results presented in these figures suggest that wild-type and resistant strains only differ in their 'efficiency' to excrete fenarimol from the mycelium. The efflux rate of resistant strains seems to be high enough to maintain a low level of uptake immediately from the moment fenarimol is added, so the fungicide might not be able to reach the target site at a concentration sufficient for it to exert its fungistatic action. This high efflux rate may be associated with an alteration in the regulation of the *imaB* gene for resistance to imazalil, which all strains tested have in common. The altered energy-dependent efflux in resistant mutants may also account for their cross-resistance or collateral sensitivity to other chemicals. The regulation of the energy-dependent efflux mechanism in the fenarimol-sensitive wild-type strain may be such that there is an initial lag before the steady-state is reached. The amount of fenarimol, that accumulates during this initial lag or is present in the steady-state may be high enough to saturate the target site(s) in the

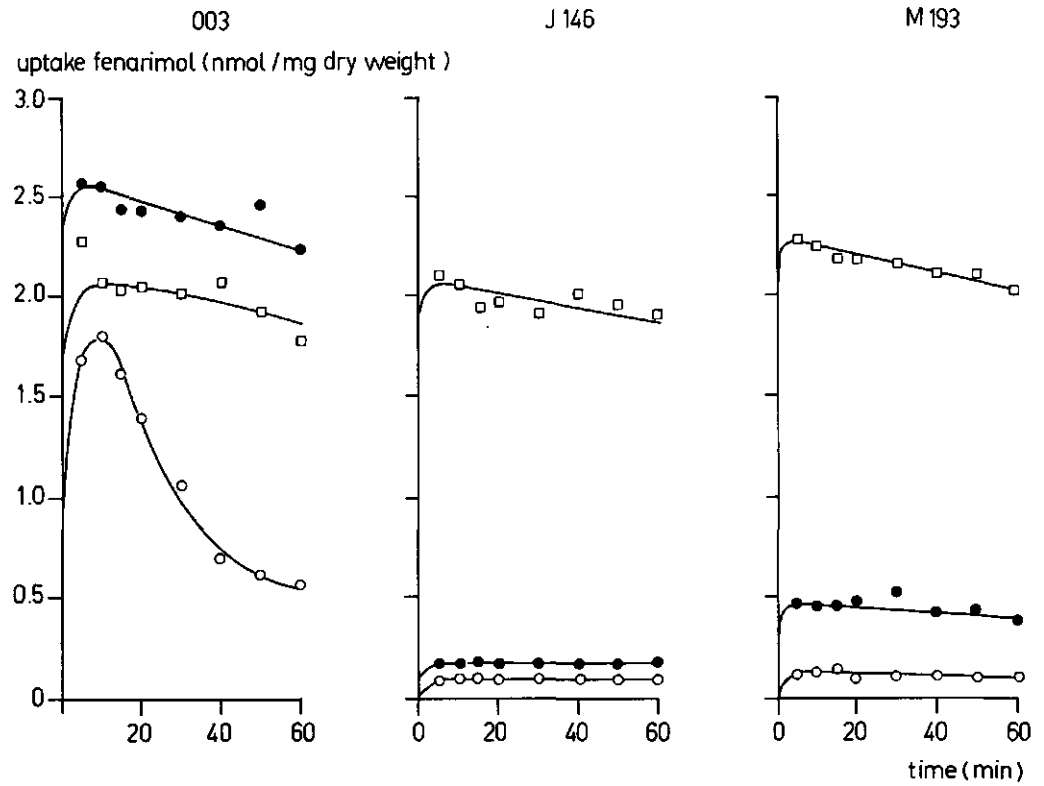

Figure 3. Effect of N,N'-dicyclohexylcarbodiimide (DCCD) on uptake of fenarimol-resistant mutants J146 and M193 of Aspergillus nidulans. Treatments: control (○); 20 (●) µM and 100 (□) µM DCCD (de Waard & van Nistelrooy, 1980).

fungus and consequently exert its fungistatic action (de Waard & van Nistelrooy, 1980).

Concluding remarks

The ergosterol-biosynthesis-inhibiting fungicides comprise a relatively new group of fungicides. It is not surprising that due to their specific mechanism of action, resistance to these fungicides may readily develop. However the degree of resistance is relatively low and it often seems to be correlated with adverse side-effects like decreased pathogenicity and fitness. This might be the reason why up till now development of resistant populations that cannot be controlled by normal fungicide application has not occurred under practical conditions. A crucial question is whether the adverse side-effects are really pleiotropic and thus coupled with the genes for resistance present, i.e. associated with the mechanism of resistance involved. A tentative, affirmative answer is supported by the mechanism of resistance to fenarimol in *A. nidulans*. The relatively high energy-

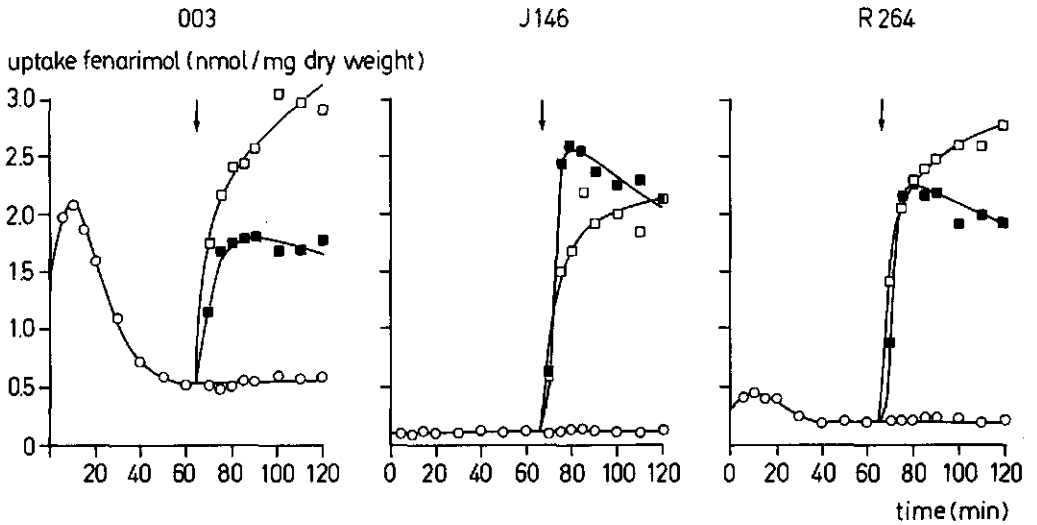

Figure 4. Effect of 100 µM oligomycin and N,N'-dicyclohexylcarbodiimide (DCCD) on uptake of fenarimol (30 µM) by mycelium of wild-type strain 003 and fenarimol-resistant mutants J146 and R264 of Aspergillus nidulans. Treatments: control (○); oligomycin (□) and DCCD (■), added 65 minutes after fenarimol (de Waard & van Nistelrooy, 1980).

dependent efflux activity of fenarimol in resistant mutants might be connected with constitutive membrane transport activities, which may be 'fuelled' with energy at the cost of other cell processes (e.g. germination, growth, and sporulation). However, it is not known whether the same mechanism of resistance operates in pathogenic fungi. Therefore, any conclusions about a negative correlation between resistance to sterol-biosynthesis inhibitors and fitness should not be too definite. This view is supported by the fact that field isolates of powdery mildews with decreased sensitivity to these fungicides have already been found (Walmsley-Woodward et al., 1979). At the Laboratory of Phytopathology of the Agricultural University, Wageningen, resistant mutants of *Penicillium italicum* have been isolated that do not show an appreciable loss in pathogenicity (unpublished data). Therefore, emergence in practice of mutant strains with normal pathogenicity may still be of concern since:

- multiple application of different fungicides in space and time will lead to increased selection pressure;
- strains with a relatively high degree of resistance may develop due to accumulation of genes for resistance in the same strain;
- resistance may not always be coupled with decreased pathogenicity;
- within resistant populations selection for the most competitive strain may take place;
- in isolated populations (glasshouses, packing houses) mutants with decreased pathogenicity may still build up resistant populations because of

a lack of competition from the wild-type strain.
These factors might lead to failure of disease control. Therefore, introduction and application of these fungicides in practice should be managed carefully. This is the more significant since in the near future at least 17 ergosterol-biosynthesis inhibitors (Table 1) may be used in agriculture, although not all will have the same application range. Negatively-correlated cross-resistance between these chemicals has not yet been reported. This implies once more the existence of a high selection pressure by fungicides that seem to be chemically unrelated. Such a phenomenon is important when one considers the possibility of using mixtures of fungicides or alternating spraying schedules to delay or prevent development of resistant populations.

The energy-dependent efflux mechanism for fenarimol in *A. nidulans* is also an interesting subject for further research. Comparable mechanisms may occur in pathogenic fungi. If so, one might be able to manipulate fenarimol uptake, for instance by inhibiting efflux activity with other chemicals. Such chemicals might enhance fungitoxicity of fenarimol and hence could be regarded as synergists. Experiments on this aspect of research are currently being carried out at the Laboratory of Phytopathology of the Agricultural University, Wageningen.

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Resistance to the dicarboximide fungicides

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Abstract

Resistance to the dicarboximide fungicides (dichlozoline, iprodione, procymidone, vinclozolin) is reviewed. Fungal variants resistant to the dicarboximides are usually cross-resistant to aromatic hydrocarbon fungicides. The mode of action of the fungicides, and the mechanism of resistance is unknown. Resistance in *Neurospora crassa* is multigenic, and resistant mutants are abnormally sensitive to media of high osmotic pressure. The genetic basis of resistance in *Botrytis cinerea* is not clear, but variants often show poorer sporulation and are less virulent than their parents. These two attributes probably account for the lack of extensive development of resistance in the field to date. Nevertheless variants showing substantial degrees of sporulation and virulence have been isolated and there are recent reports of loss of disease control by these fungicides in the field. Spraying programmes should not rely exclusively on these fungicides.

Keywords: fungicide resistance, dicarboximide fungicides, *Botrytis cinerea*, *Neurospora crassa*.

Introduction

The term dicarboximide fungicide has been loosely applied to anti-fungal compounds of the general formula shown in Figure 1. Structural formulae for the most important of the dicarboximides - vinclozolin ('Ronilan', BASF AG, F.R.G.), iprodione ('Rovral', Rhône-Poulenc Phytosanitaire, France; also called glycophene) and procymidone ('Sumisclex', 'Sumilex', Sumitomo Chemical Company Limited, Japan; also called dicyclidine) are given in Figure 2. The first dicarboximide fungicide to be developed, dichlozoline

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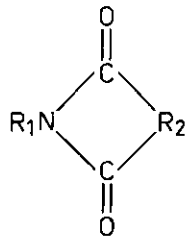
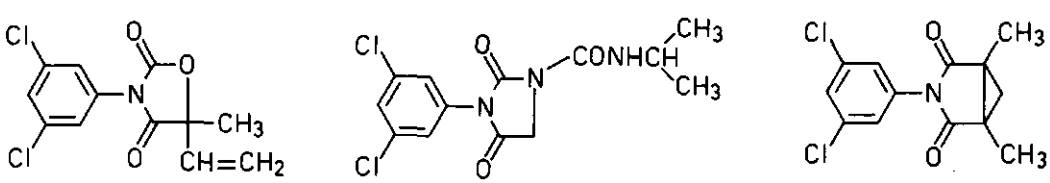


Figure 1. General structural formula of the dicarboximide fungicides; R_1 has invariably been a 3,5-dichlorophenyl group, but R_2 may have various structures.

('Sclex', Sumitomo Chemical Company Limited, Japan) (Menager et al., 1971) was withdrawn from the market soon after its introduction.

The presence of chlorinated benzene residues may explain why fungal variants resistant to the dicarboximide fungicides are frequently cross-resistant to compounds of the aromatic hydrocarbon group, such as dicloran and quintozene (Georgopoulos & Zaracovitis, 1967). The dicarboximide fungicides have been used widely to control diseases caused by the taxonomically related pathogens *Sclerotinia*, *Monilinia* and *Botrytis*; particularly important are grey mould of grape, strawberry and tomato (caused by *Botrytis cinerea*) and the brown-rot diseases of fruit (caused by *Monilinia* spp.). Iprodione has been used against a wider spectrum of diseases in the field than have the other dicarboximides (Burgaud et al., 1975). The introduction of the dicarboximide fungicides was particularly welcome because of the onset of resistance in *Botrytis* and *Monilinia* to the previously very effective carbendazim-generating fungicides.

Dichlozoline and vinclozolin are considered to be non-systemic (Menager et al., 1971; Eichhorn & Lorenz, 1978), but there is good evidence that procymidone is translocated in bean and cucumber (Hisada et al., 1977), and strawberry (Cooke et al., 1979b). Iprodione, initially reported to be non-systemic (Burgaud et al., 1975), is translocated in potato (Cayley & Hide, 1980).



vinclozolin iprodione procymidone

Figure 2. Structural formulae of some common dicarboximide fungicides.

Because of the emergence of resistant isolates of many pathogens to various types of fungicide, the question has been asked: 'Will the dicarboximide fungicides, too, encounter resistance problems in the field?'.

Field experience

The dicarboximide fungicides have been used extensively in the field for several years, and many workers have been active in monitoring natural populations for resistance. In addition, laboratory studies have been used to assess the probability of resistance development in the field. Resistant variants have been recovered from both the field and the laboratory (Tables 1 and 2). A lack of disease control following artificial inoculation of strawberry plants with resistant variants of *Botrytis cinerea* has been found (Hunter et al., 1979), but relatively few examples of loss of control of naturally-occurring disease have been reported. Maraite et al. (1981) described an instance in *Botrytis cinerea* on everbearing strawberries subjected to repeated dicarboximide applications; the lack of control was not marked, however. More recently there have been reports of a lack of control of *Botrytis cinerea* on *Hebe* spp. in a nursery (M.J. Griffin & C. Gay, personal communication, 1981), on glasshouse tomato and cucumber (W.F.T. Hartill, personal communication, 1981) and on glasshouse cyclamen (A.C. Pappas, personal communication, 1981). Holz (1979) attributed a local outbreak of *Botrytis cinerea* on grape flowers to the presence of resistant variants. However Lorenz (1980) found resistant variants were widespread in grape vines, both in the field and in trials, but detected no lack of control. Similarly iprodione-resistant variants of *Botrytis squamosa* on onion were controlled by iprodione in trials (Presly et al., 1980).

Mode of action

Despite intensive investigation, the mode of action of the dicarboximides remains elusive. Their chemical similarity, their similar effects on fungi and the cross-resistance patterns shown by resistant variants support the assumption that all fungicides in the group share a common mode of action. Some responses that suggest that iprodione differs from the others can be accounted for by the finding that iprodione rearranges to a much less active isomer in ethanolic or methanolic solutions, which are often used in biological testing (Cooke et al., 1979a).

Table 1. Reports of resistance to dicarboximide fungicides in variants isolated from the field.

Species	Origin	Reference
Botrytis cinerea	strawberry fruits from dicarboximide fungicide trials, 1978 (8 resistant isolates)	Davis & Dennis, 1979 Dennis & Davis, 1979
	grape flowers, severe infection	Holz, 1979
	strawberry fruit from iprodione trial, 1978 (1 resistant isolate)	Pappas et al., 1979
	field survey, 1978-1979 (14/101 isolates resistant)	Letham & Penrose, 1980
	field survey of grape vines treated with dicarboximine fungicides, 1978-1979 (resistant isolates common, 101 sites examined)	Lorenz, 1980
	grape-vine trial with dicarboximide funcides, 1979 (resistant isolates common)	Lorenz, 1980
	strawberry fruits from dicarboximide fungicide trials, 1977-1979 (3/799 isolates resistant)	Maraite et al., 1981
	everbearing strawberries after intensive dicarboximide fungicide treatment, 1979 (resistant isolates common)	Maraite et al., 1981
	tomato, carrot, and strawberry leaf litter, 1978	Davis & Dennis, 1981a
	field survey of different crops treated with dicarboximide fungicides, 1980 (5/804 isolates resistant)	Griffin & Gay, personal communication, 1981
Monilinia fructicola	field screening (3 isolates resistant out of a number tested)	Sztejnberg & Jones, 1978

Morphological effects

In contrast to many protectant fungicides, the dicarboximides affect spore germination less than mycelial growth (Buchenauer, 1976; Fritz et al., 1977; Pappas & Fisher, 1979). Germ-tube elongation of *Botrytis cinerea* in sucrose solution is stimulated by very low concentrations

Table 2. Reports of resistance to dicarboximide fungicides in variants isolated on fungicide-amended medium in vitro.

Species	Selection conditions [a]	Rate of resistance (per 10^7 spores)	Reference
Alternaria alternata	iprodione (100)		McPhee, 1980
Botrytis cinerea	vinclozolin or iprodione (30)	< 1-5 (30-140 with U.V. mutagenesis)	Leroux et al., 1977
	vinclozolin (30)	0-85 (0-70 with U.V. mutagenesis)	Gullino & Garibaldi, 1979
	procymidone (20-500)	1.7-34.7	Hisada et al., 1979
	vinclozolin (8.6)	< 0.1-20	Maraite et al., 1980
	iprodione (50)	0-8	Davis & Dennis, 1981a
	or		
	vinclozolin (50)	0.2-14.8	
Botrytis squamosa	iprodione (4-2500)		Presly et al., 1980
Botrytis tulipae	iprodione (50)	18	Chastagner & Vassey, 1979
	or		
	vinclozolin (50)	400	
Monilinia functicola	iprodione (30)	26	Sztejnberg & Jones, 1978
	or		
	vinclozolin (30)	18	
	or		
	procymidone (30)	770	
Penicillium expansum	iprodione (30-100)	2	Leroux et al., 1978
	iprodione (150)	53	Rosenberger et al., 1979
	or		
	vinclozolin (150)	31	
Ustilago maydis	iprodione (30-100)	(50-200 with U.V. mutagenesis)	Leroux et al., 1978

a. Value in brackets is the concentration of the named fungicide in mg/l.

of vinclozolin (0.143 mg/l), whereas at concentrations of 1.43 mg/l and above, elongation is restricted, and the germ-tubes become swollen and distorted (Buchenauer, 1976). However, even at a concentration of 143 mg/l, 26 % of the conidia germinated. Hisada & Kawase (1977) showed that if germination takes place in a liquid-complete medium containing procymidone, most germlings eventually burst. A similar distortion and bursting was found with hyphal cells after they were incubated in the presence of fungicide for 2 hours. Dry weight increase was not reduced over this period, although hyphal cell number decreased markedly (Hisada et al., 1978). Bursting of cells exposed to fungicide has also been reported with vinclozolin and iprodione (Eichhorn & Lorenz, 1978; Davis & Dennis, 1981a). With *Ustilago avenae*, Buchenauer (1976) found a much greater inhibition of sporidial multiplication than of dry-weight increase in the presence of vinclozolin, and the sporidia appeared swollen and branched. Protoplasts made from *Botrytis cinerea* mycelium were unaffected by a high concentration of procymidone and even regenerated abnormal cell walls after 24 hours of incubation (Hisada & Kawase, 1977). Similar effects, including conidial and hyphal bursting (Sharples, 1961; Georgopoulos et al., 1967), production of swollen, distorted hyphae (Macris & Georgopoulos, 1973; Threlfall, 1972), rapid inhibition of sporidial multiplication (Tillman & Sisler, 1973), and regeneration of cell wall by protoplasts in the presence of fungicide (Macris & Georgopoulos, 1973) have been reported for the aromatic hydrocarbon fungicides.

Although these observations indicate a disturbance of normal cell-wall synthesis, one or more of the responses, including stunting of apical growth, abnormal hyphal swelling and occasional bursting of cells are induced by diverse anti-fungal compounds, including the protein inhibitor cycloheximide (Sternlicht et al., 1973), the antitubulin carbendazim (Howard & Aist, 1977), hexose analogues such as sorbose (Moore, 1981) and the enigmatic cytochalasins (Betina et al., 1972; Allen et al., 1980). Thus these morphological effects, by themselves, provide little insight into the mode of action of the dicarboximides.

Metabolic effects

Dicarboximides have virtually no influence on respiration (Buchenauer, 1976; Fritz et al., 1977; Hisada et al., 1978; Pappas & Fisher, 1979). Membrane integrity, as judged by efflux of electrolytes (Buchenauer, 1976; Pappas & Fisher, 1979) or ^{14}C-labelled metabolites (Hisada & Kawase, 1977), is not affected, nor is uptake of ^{14}C-glucose (Hisada et al., 1978). Although some workers report little or no effect on synthesis of DNA, RNA or protein (Buchenauer, 1976; Pappas & Fisher, 1979), Fritz et al. (1977) and Hisada et al. (1978) found reduced rates of incorporation of radioactively-labelled uridine. Sterol metabolism is

little influenced by dicarboximides (Buchenauer, 1976; Fritz et al., 1977; Pappas & Fisher, 1979). Although most lipid fractions are unaffected by them, an increase in the free fatty-acid fraction has been reported a number of times (Buchenauer, 1976; Fritz et al, 1977; Pappas & Fisher, 1979).

Hisada et al. (1978) found procymidone at an inhibitory concentration stimulated cell wall synthesis, as judged by wall dry-weight increase, although there was no marked effect on wall composition. They reported an enhancement of the incorporation of radioactively labelled acetate, D-glucose and D-glucosamine into the cell wall, in contrast to Pappas & Fisher (1979), who found a slight reduction in incorporation of D-glucosamine and N-acetyl-D-glucosamine.

This brief survey indicates that despite some possible leads, no metabolic step has yet been identified that can be unequivocally linked with the site of action. A similar situation is found with the aromatic hydrocarbons (Tillman & Sisler, 1973; Threlfall, 1968).

Genetic effects

The ability of certain fungicides to stimulate somatic segregation of genetically-marked diploid strains of *Aspergillus nidulans* was suggested by Georgopoulos et al. (1976) as a criterion for grouping fungicides into those that do, and those that do not, interfere with the hereditary process. Antifungal compounds such as the chitin-synthase inhibitor polyoxin D, the protein-synthesis inhibitor cycloheximide and the respiratory inhibitor carboxin had no effect, whereas the DNA intercalator actinomycin D, the antitubulins griseofulvin and benomyl and fungicides in the aromatic hydrocarbon group all stimulated segregation. The mechanism of segregation may differ depending on the agent responsible: Kappas (1978) provided evidence that whereas benomyl causes non-disjunction, the aromatic hydrocarbons act primarily via a breakage-deletion mechanism, although Azevedo et al. (1977) suggest that the aromatic hydrocarbons act mainly via non-disjunction.

More recently, Georgopoulos et al. (1979) found that the dicarboximide fungicides, iprodione, procymidone and vinclozolin also stimulate somatic segregation and they concluded that 'their effect on the chromosomes, and possibly on the mitotic spindle, is a main reason for their fungitoxicity'. This conclusion seems premature, as such effects could be secondary, or at least reflect action at only one of a number of sites. Even benomyl, which is usually classified as a fungicide active on the nucleus (Georgopoulos, 1977; Leroux & Fritz, 1978), causes a rapid reduction in hyphal linear growth rate unrelated to its mitotic effect (Howard & Aist, 1977; 1980). Both nuclear and hyphal tip effects are secondary to the primary action of benomyl, which is to prevent microtubule assembly, a

process required for both production of the mitotic spindle and the cytoplasmic 'cytoskeleton'. Recently Bellincampi et al. (1980) reported that the antifungal polyenes amphotericin B and pimaricin, the sterol-synthesis inhibitor fenarimol, and miconazole, which is active on the plasma membrane ATPase (Dufour et al., 1980), can stimulate somatic segregation. Although these results cast some doubt on the specificity of the somatic segregation test, they cannot be satisfactorily reconciled with the earlier results of Georgopoulos et al. (1976), who found that the polyene nystatin and the sterol synthesis inhibitor triarimol were without effect.

Modes of resistance

The mechanisms whereby fungi become resistant to fungicides are diverse (Georgopoulos, 1977). In some instances the mechanism may involve the site of action, giving an insight into the mode of action, but in other instances it may be unrelated. Little is known of the resistance mechanisms to the dicarboximides. Leroux et al. (1978) found iprodione uptake by resistant variants of *Botrytis cinerea* and *Penicillium expansum* to be little different from that of sensitive isolates, although uptake of procymidone decreased with resistant variants. Pappas et al. (1979) found no difference in uptake of three dicarboximides between dicarboximide-resistant and sensitive isolates of *Botrytis cinerea*.

Recently Beever (unpublished data) found that some *osmotic* mutants of *Neurospora crassa*, in contrast to wild types, are resistant to fungicides in the dicarboximide and aromatic hydrocarbon group, i.e. these loci are in fact resistance loci. *Osmotic* mutants are characterised by their abnormal sensitivity to high osmotic pressure, being inhibited, for example, on minimal medium supplemented with NaCl with a concentration of 40 g/l (Mays, 1969). Some show the remarkable property of growing as protoplasts in liquid media of high osmotic pressure (Hamilton & Calet, 1964). The products of the *osmotic* genes are as yet unknown, but one suggestion is that they are involved in the supply of precursors to the cell-wall synthesizing enzymes (Mays, 1969), and, indeed, the strains do show some differences in overall cell-wall composition (Livingston, 1969). Their abnormal osmotic sensitivity could result from an inability to produce intracellular solutes, such as polyols (Jennings & Austin, 1973), at a concentration sufficient to counterbalance the external osmotic pressure.

Genetics of resistance

Mutants resistant to the aromatic hydrocarbons have been isolated in a number of fungi. In *Nectria haematococca* five chromosomal loci that can

confer resistance have been indentified (Georgopoulos, 1977), and in *Aspergillus nidulans* two chromosomal loci are known (Threlfall, 1968). Because variants resistant to the dicarboximides frequently exhibit cross-resistance to the aromatic hydrocarbons, these same loci will probably also be dicarboximide-resistance loci, but confirmatory tests need to be performed.

In the discussion of modes of resistance we noted that certain *osmotic* mutants of *Neurospora crassa* are resistant both to dicarboximide and aromatic hydrocarbon fungicides. There are six *osmotic* genes in *Neurospora crassa*, all mapping in linkage group I (Mays, 1969) and, so far, mutants of *os-1* and *os-4* have been tested and found to be resistant (R.E. Beever, unpublished data). In addition, three mutants selected as resistant to vinclozolin were found to be osmotically sensitive, but we do not know whether all osmotically-sensitive strains are resistant, or whether all resistant strains will prove to be osmotically sensitive. However, it is clear that more than one chromosomal gene can mutate to confer resistance in the fungus. As with other species, spontaneous resistant sectors readily arise on fungicide-containing media inoculated with plugs of *Neurospora crassa* mycelium. This can be reasonably accounted for by a combination of the fungistatic action of these fungicides, which allows development of a dense compact colony from which a resistant mutant nucleus can segregate in a rapidly growing sector, and by the number of loci that can mutate to give resistance.

Dicarboximide-resistant variants have been isolated from a number of plant pathogens (Tables 1 and 2). Although little is known of the genetic basis of resistance in these instances, some attention has been given to stability of the variants. With *Alternaria alternata*, resistant variants retained their resistance following a number of successive transfers in vitro, and single spore isolates retained the characteristics of their parents (McPhee, 1980). With *Botrytis cinerea*, most workers have found that resistant variants retain their resistance after subculture on a fungicide-free medium (Leroux et al., 1977; Schüepp & Küng, 1978; Lorenz & Eichhorn, 1978; Garibaldi & Gullino, 1979; Hisada et al., 1979). However Davis & Dennis (1979) reported that resistance was sometimes lost if newly isolated variants were not subcultured initially on a fungicide-containing medium. Garibaldi & Gullino (1979) found that less than 50 % of conidia from resistant colonies grown on a fungicide-free medium gave resistant colonies when newly isolated variants were used, but after five generations of single spore isolations most or all produced resistant colonies. Growing resistant variants on a fungicide-amended medium increased the proportion of resistant conidia produced (Gullino & Garibaldi, 1979, 1980). These results are reminiscent of those of Esuruoso & Wood (1971) for aromatic hydrocarbon fungicides. They found that only about half the spores produced by resistant variants in the absence of fungicide gave

resistant colonies, whereas all spores produced by these variants in the presence of fungicide vapour gave resistant colonies. Further testing of the sensitive colonies produced in the absence of fungicide showed that they gave only sensitive colonies, whereas second-generation resistant colonies gave a mixture. Such instability can be explained from the knowledge that *Botrytis cinerea* conidia are multinucleate (Epton & Richmond, 1980) and on the reasonable, but unproven, assumptions that resistance is chromosomal and dominant in heterokaryons. Newly isolated resistant variants are probably often heterokaryotic; growth in the presence of fungicides would increase the proportion of resistance nuclei in conidia produced. In some instances, resulting resistant strains may be homokaryotic for resistance nuclei (Webster et al., 1970).

The persistence of the resistance character differs when mixtures of conidia of sensitive and resistant isolates inoculated into fungicide-free media are subcultured. Hisada et al. (1979) found a rapid reduction in the proportion of resistant conidia, but they examined only one combination. Maraite et al. (1980) showed that although resistance is rapidly lost in some combinations, in other combinations a high proportion of conidia retained resistance; similar results were found for subcultures transferred on cucumber slices. These differences may in part reflect differences in sporulation of the different isolates, but they may also reflect differences in the extent of heterokaryon formation between the various pairs.

The cross-resistance patterns and the degree of resistance shown by resistant variants differ. Leroux et al. (1977) examined a range of *Botrytis cinerea* variants selected on iprodione or vinclozolin at concentrations of 30 mg/l, or on dicloran at 100 mg/l; they found that the variants could be classified into four groups, viz. a, b, c, d. Data for representative variants are summarized in Table 3. Later work by Leroux et al. (1978) showed that the behaviour of Groups c and d of the table was not stable; Group c variants became sensitive to dicloran and quintozene and Group d variants became resistant to these two fungicides. A distinctive feature of some variants is that their growth is stimulated by moderate concentrations of fungicide (Leroux et al., 1978; Dennis & Davis, 1979; Maraite et al., 1981).

The existence of variants of relatively low resistance to dicarboximides (e.g. Group c) could be of significance in the field (Maraite et al., 1980, 1981). By selecting for resistance at a low concentration (iprodione, 2 mg/l), Beever (unpublished data) has recovered a number of resistant variants of *Botrytis cinerea* with ED_{50} values for mycelial growth against iprodione in the range 2-10 mg/l. Although some showed high resistance to dicloran (ED_{50} > 100 mg/l), others were only slightly resistant (ED_{50} 2-10 mg/l).

Table 3. *Resistance pattern of various strains of* Botrytis cinerea.

Isolate [1] (group)	ED_{50} values of fungicides for linear growth of mycelium (mg/l)				
	dichlozolin	vinclozolin	iprodione	dicloran	quintozene
Culture S benomyl sensitive	0.1	0.15	0.3	0.7	2
Culture B benomyl resistant	0.2	0.25	0.4	1	1.5
SUG 2 (a)	> 1000	> 1000	1000	> 100	> 100
SOV 1 (b)	> 1000	> 1000	15	> 100	> 100
SOD 3 (c)	2	3	1.5	>100	> 100
SOG 1 (d)	> 1000	> 1000	900	1.5	1

Source: after Leroux et al., (1977, Tables 2 & 6).

1. Resistant cultures were selected in vitro from culture S; U designates isolates recovered after ultra-violet irradiation of conidia; O designates isolates not irradiated; G designates selection on glycophene (iprodione), V selection on vinclozolin and D selection on dicloran.

Benomyl-resistant variants from the field are usually sensitive to the dicarboximide fungicides (Buchenauer, 1976; Eichhorn & Lorenz, 1978), but variants exhibiting dual-resistance are readily isolated following their exposure to dicarboximides in vitro (Leroux et al., 1977).

Fitness of resistant variants

Are resistant variants ecologically less fit than sensitive strains? The field results reported to date (p.103) suggest that this may be so. Various attributes of resistant variants have been examined in the laboratory in the anticipation that these may reveal properties that could account for such reduced fitness. Similar studies of variants resistant to sterol inhibitors have indicated why resistance to these fungicides may never become a problem (Fuchs et al., 1977). There are very few data for species other than *Botrytis cinerea* and even interpretation of the data for this species is complicated by its well known variability (Grindle, 1979; Lorbeer, 1980). Many wild strains are naturally heterokaryotic (or heteroplasmons) and monoconidial isolates selected from such strains often differ markedly among themselves and from their parents. Much of the variation shown by resistant isolates may be a reflection of this variability, rather than a consequence of the presence of a particular resistance gene.

Morphological and physiological properties

Linear growth rates have now been measured for a considerable number of *Botrytis cinerea* variants, and values range from well below to well above those of the respective parents (Leroux et al., 1978; Hisada et al., 1979; Gullino & Garibaldi, 1979; 1980; Maraite et al., 1980). Conidial production on agar media is usually less than that of the parent; values for resistant variants, expressed as a proportion of their parents, range from 1 % to 30 % (Leroux et al., 1978) and 0 % to 34 % (Hisada et al., 1979). Gullino & Garibaldi (1979) report a mean value of 32 % for eight resistant variants. However some laboratory variants described by Maraite et al. (1980) showed conidiation well in excess of that of many wild isolates, and Maraite et al. (1981) note that conidiation of resistant variants from the field 'showed the same range of variation' as conidiation of sensitive ones. Conidial viability of resistant variants was greater than 90 % for all variants studied by Davis & Dennis (1981b). A reduction in number and branching of germ tubes was noted by Leroux et al. (1977).

Some resistant variants of *Botrytis cinerea* produce relatively few sclerotia, but others produce as many as their parents (Hisada et al., 1979; Gullino & Garibaldi, 1980). Resistant variants are apparently much more sensitive to copper oxychloride than wild strains, although only four variants have been tested (Garibaldi & Gullino, 1979). The dicarboximide resistance of the *osmotic* mutants of *Neurospora crassa* prompted the examination of the osmotic sensitivity of resistant variants of *Botrytis cinerea* selected in the laboratory. The growth of many resistant variants was indeed strongly inhibited on a malt-agar medium supplemented with NaCl with a concentration of 40 g/l (R.E. Beever, unpublished data), a property that could well be of significance in determining virulence.

Virulence

Workers have tested the virulence (relative pathogenicity) of variants in a number of ways. The tests can be grouped into those that use a conidial inoculum, which mimic the establishment of primary disease sites, and those that use mycelial plug inoculum, which mimic secondary spread of disease as, for example, from rotting fruit. We will consider first those tests that used a conidial inoculum. Hisada et al. (1979) measured lesion diameter on seedling leaves of cucumber and found that although many strains were non-virulent, or much less virulent than their parents, the most virulent strain had a value of 88 % of its parent. Rather similar results were reported by Garibaldi & Gullino (1979), who measured relative necrosis on broad bean leaves; although the virulence of resistant variants was always less than that of their parents, certain resistant

variants were more virulent than some of the sensitive strains. Maraite et al. (1980) found that, relative to their parents, higher numbers of conidia of resistant variants were needed to establish infection on cucumber slices.

The mycelial-plug technique has also been widely used. Measuring the degree of colonization of slices of cucumber fruits, Leroux et al. (1977; 1978) found that although many resistant variants had low virulence, others were at least as virulent as their parents. Maraite et al. (1980; 1981) reported that the rate of colonization by resistant variants was usually reduced, but some strains, including those isolates from the field, colonized rapidly. Similar results were reported by Schüepp & Küng (1978) with apple fruits. Gullino & Garibaldi (1979) measured weight loss from grapes and found that all variants incited much less weight loss than their respective parents, except in one instance where the parent itself was almost non-virulent; three of the variants were non-virulent. It would be interesting to determine whether there is a correlation between osmotic sensitivity, as judged by inhibition on agar media, and virulence. As grapes lose weight their content of soluble sugars can reach very high concentrations. The variants studied by Davis & Dennis (1981a) were all able to infect and colonize strawberry fruits and tomato leaves, from a mycelial inoculum, but colonization of either whole carrots, or carrot discs, was very restricted.

In conclusion, it appears that while resistant variants are usually less virulent than their sensitive parents, where these are available for comparison, the range of virulence shown by both groups is broad and can overlap.

Field performance and the disease cycle

Whether resistant variants will cause significant epidemics in the field, in either the presence or absence of the selective fungicide, depends on many factors, including the variant's ability to overwinter, produce conidia abundantly and infect and colonize tissue efficiently. A poor performance at any phase of the disease cycle will reduce a pathogen's overall effectiveness.

There are many difficulties in extrapolating laboratory tests to the field, but only a few workers have carried out field trials. By spraying strawberry flowers in the field with conidial suspensions, Hunter et al. (1979) and Davis & Dennis (1981b) showed that resistant variants of *Botrytis cinerea* can cause disease on the fruit; treatment with the dicarboximides increased the proportion of rots containing the dicarboximide-resistant strains. Resistant variants have also been shown to persist for many months on strawberry-leaf debris in the field (Hunter et al., 1979; Maraite et al, 1981; Davis & Dennis, 1981b). Two factors identified in

the laboratory tests merit consideration as major reasons for the reduced fitness of resistant variants. First is the reduced sporulation ability. Undoubtedly this can account for the poor performance of many variants, including those in the field trials of Davis & Dennis (1981b). However certain resistant variants do show high sporulation rates and data indicating spread of resistance in the field have been obtained (Maraite et al, 1980, 1981). The second factor is the decreased virulence of resistant variants, although a slight reduction in virulence might still give variants capable of inciting epidemics, especially in the presence of selective fungicides.

Such considerations, and the recent reports of breakdowns of disease control in the field, make it unwise to rely on the sole use of the dicarboximide fungicides in a spraying programme. The prudent approach must be to try to prevent resistance development by adopting programmes that alternate or mix fungicides for which fungi do not show cross-resistance (Griffin, 1980).

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Acylalanines: resistance in downy mildews, Pythium and Phytophthora spp.

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Abstract

In laboratory experiments selection for resistance in fungi to the acylalanines appears to be quite easy. Isolates with a high level of stable resistance and unimpaired virulence could be obtained from *Phytophthora capsici*, *P. megasperma* f. sp. *medicaginis* and *Pythium ultimum*. Isolates with transient resistance or reduced virulence, or both, were frequently found. Resistance in vivo does not always correlate with resistance in vitro, and experiments with *P. infestans* yielded only isolates that were resistant in vitro. In practice, development of resistance in *P. infestans* occurred quite rapidly after exclusive use of metalaxyl under heavy disease pressure, and also in *Pseudoperonospora cubensis*, the causal organism of downy mildew of cucumber. The importance of laboratory experiments to evaluate the potential of target fungi to develop resistance to fungicides is emphasized. Such results can be of great value for the development of use strategies for new fungicides.

Keywords: acylalanines, metalaxyl, furalaxyl, milfuram, *Phytophthora capsici*, *Phytophthora infestans*, *Phytophthora megasperma* f. sp. *medicaginis*, *Pythium ultimum*.

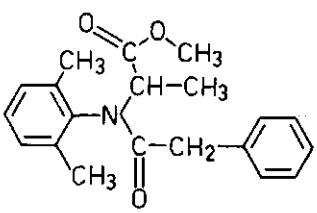
Introduction

The acylalanines have become in a short time increasingly important in the control of several important plant pathogenic fungi belonging to the order Peronosporales. Damaging pathogens such as downy mildews of a number of crops, potato late blight, blue mold of tobacco and soil-borne *Pythium* and *Phytophthora* spp. are among the main target fungi. The systemic behaviour of the acylalanines makes them excellent for control of downy mildews of several crops in tropical areas, where rapid crop growth and heavy precipitation often requires very frequent application of conventional fungicides; with the acylalanines longer spraying intervals are

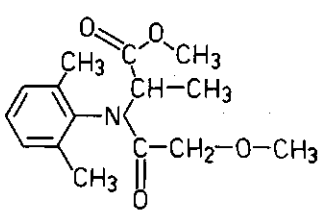
feasible.

This group of fungicides includes metalaxyl and furalaxyl, whose biological properties have been studied in detail (Bruck et al., 1980; Cohen et al., 1979; Hickey & Coffey, 1980; Staub et al., 1978, 1980; Staub & Young, 1980), together with a number of experimental chemicals. Structural formulae are given in Figure 1; the term acylalanine does not strictly cover all the compounds since three of them do not have an alanine moiety. Since it is difficult to find a more adequate name, and since the term acylalanine is established in the literature, this term will be used throughout this contribution.

Their selectivity and systemic behaviour suggest that these compounds act in a very specific way. Interference with RNA synthesis is a proposed mechanism of action (Fisher & Hayes, 1979; Davidse, 1981b; Kerkenaar, 1981).



'Galben'



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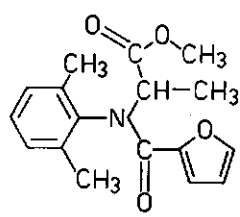

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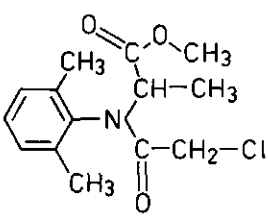

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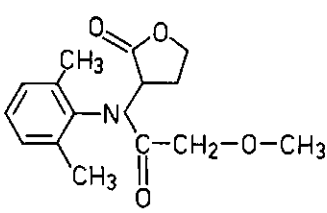
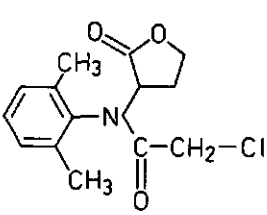
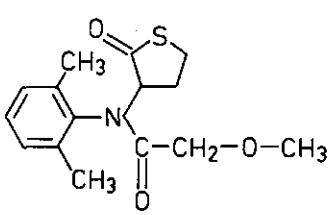


RE 26745



milfuram



RE 26940

Figure 1. Structural formulae of acylalanine-type fungicides.

Therefore, if the problems met with other specifically-acting fungicides are any example, development of resistance to the acylalanines is a potential threat to their long-lasting usefulness.

In this contribution the use and predictive value of laboratory experiments to evaluate the potential of target fungi developing resistance to acylalanines are described. Two examples of development of resistance in practice are also presented and aspects of resistance in vivo and in vitro discussed.

Development of resistance in laboratory experiments

Selection for resistance in acylalanines in a number of fungi has been attempted in several laboratories to estimate the risk of development of resistance under practical conditions before these compounds were introduced. Several approaches have been used. Staub et al. (1979) cultured isolates of *Phytophthora infestans* on media at sublethal concentrations of furalaxyl. Sporangia and zoospores from isolates showing reduced sensitivity were harvested and plated out on media at high concentrations. In this way several isolates were obtained which were, with respect to ED_{50} values, between two and more than 100 times less sensitive to furalaxyl than the original isolates. The isolates were cross-resistant to CGA 29212 and metalaxyl, and they maintained their resistance for at least 2 years with repeated subculturing on media without the fungicides. But these strains had either lost their virulence or they were still fully sensitive to treatments with acylalanine fungicides on tomato or potato plants. Hence in vitro resistance is not necessarily related to in vivo resistance. In growth room experiments in which a field population of *P. infestans* was exposed to sublethal dosages of metalaxyl on potato plants for 11 infection cycles, no isolates with a decreased sensitivity were obtained. Based on this study Staub et al. stressed the importance of including in vivo tests in studies dealing with resistance to acylalanines. Their results gave the impression - it was never stated - that resistance to acylalanines in *P. infestans* was unlikely to occur.

Bruin (1980) studied the development of resistance to metalaxyl in several fungi. Using more or less similar procedures as Staub et al.(1979), he obtained after 6-12 transfers isolates of *Phytophthora capsici* and several *Pythium* spp. that showed in terms of ED_{50} values 100 - to 3 600-fold decreased sensitivity to metalaxyl. UV irradiation of zoospores of *P. capsici* and mycelium of both *P. capsici* and *Pythium ultimum* also gave highly resistant strains. Some of the metalaxyl-resistant strains retained their high level of resistance during 8 months of subculturing in the absence of the fungicide; other strains slowly reverted to intermediate resistance levels or lost their resistance completely. All strains were usually (some exceptions occurred) cross-resistant to furalaxyl,

milfuram, RE 26745, RE 26940 and 'Galben'. The metalaxyl-resistant strains of *P. capsici* and *P. ultimum* appeared to be still pathogenic on peppers and peas, respectively, and insensitive to treatments with metalaxyl at dosages that completely controlled disease development caused by the original strains.

Like Staub et al. (1979), Bruin (1980) was not able to obtain metalaxyl-resistant isolates of *P. infestans* after several cycles of mass selection with sporangia on metalaxyl-treated potato-tuber tissue. A similar procedure with *Peronospora parasitica* spores, either untreated or treated with UV light, and cabbage seedlings also did not result in loss of sensitivity. Davidse (1980, 1981a) obtained metalaxyl-resistant isolates of *P. megasperma* f. sp. *medicaginis*, the causal organism of root rot of alfalfa, in several ways. Adaptation and mass selection from zoospores yielded 19 strains with a relatively low degree of resistance, all of which were less virulent than the original isolate in an alfalfa-seedling assay. Only one of those strains showed resistance in vivo. Mutagenic treatment of the zoospores with N-methyl-N'-nitro-N-nitrosoguanidine (NG) resulted in many highly resistant strains, however. Half of those were as virulent as the original isolate and a considerable number of that half were also highly resistant in vivo.

Metalaxyl at even 10 times the dosage needed to control root rot in mature alfalfa plants caused by a sensitive isolate failed to protect these plants from the disease when they were inoculated with resistant isolates. The resistance to metalaxyl appeared to be highly stable, since virulent resistant strains did not lose their resistance after 12 infection cycles in a seedling assay in the absence of the fungicide; neither did mixed populations of a resistant and a sensitive isolate under similar conditions.

Table 1 summarizes the results obtained in several laboratories. The data obtained in the laboratory unequivocally show that a number of target fungi of acylalanine fungicides have the potential to develop a high level of resistance in vitro as well as in vivo without any implications as to their virulence. This justifies the conclusion that development of resistance under practical conditions is likely to occur.

Development of resistance in practice

Pseudoperonospora cubensis

During the 1979-1980 growing season, cucumber growers from the southern Greek mainland, Crete and Israel reported loss of effectiveness of metalaxyl in controlling cucumber downy mildew caused by *Pseudoperonospora cubensis*. Growth-room experiments (Georgopoulus & Grigoriu, 1981; Katan & Bashi, 1981; Malathrakis, 1980; Pappas, 1980; Reuveni et al.,

Table 1. Development of resistance to acylalanines in laboratory experiments.

Organism	Method	Resistance factor[a] in vitro	Resistance in vivo
P. infestans [b]	adaptation and mass selection	<140	no
	selection in vivo (12 cycles on foliage)		no
P. infestans [c]	selection in vivo (12 cycles on tubers)		no
P. parasitica [c]	selection in vivo (12 cycles on seedlings with and without UV irradiation of spores)		no
P. capsici [c]	adaptation	500	yes
	mass selection from UV-irradiated spores	600	yes
Pythium spp. [c]	adaptation	14-3 600	
P. ultimum [c]	adaptation from UV-irradiated mycelium	50 000	yes
P. nicotinanae [c]	adaptation	>100	
	mass selection from NG-treated zoospores	>100	
P. megasperma [e] f. sp. glycinea	adaptation	>500	yes
	mass selection from NG-treated zoospores	>500	yes
P. megasperma [f] f. sp. medicaginis	adaptation	< 10	no
	mass selection from NG-treated zoospores	>500	yes

a. The resistance factor is the ratio of the ED_{50} value for the resistant strain to that for the wild-type strain.
b. Source: Staub et al. (1979).
c. Source: Bruin (1980).
d. Source: Dekker et al. (unpublished data).
e. Source: Davidse & Krüse (1981).
f. Source: Davidse (1980, 1981a).

1980) showed that the fungicide was almost completely inactive against isolates from these areas, even when the plants were sprayed at dosages several times higher than recommended. Inoculation of detached leaves floating on fungicide solutions demonstrated that a considerable difference existed in minimal inhibiting concentrations for sensitive and resistant isolates (Table 2). Resistance appeared to be very stable. Even maintaining the organism for 20 generations over a period of 7 months on untreated plants did not result in any loss of resistance.

Table 2. Activity of metalaxyl against sensitive (S) and resistant (R) isolates of Pseudoperonospora cubensis in a detached leaf assay [a].

Isolate	Average disease rating [b] at various metalaxyl concentrations (µg/ml)					
	0	10	50	100	250	500
1 (R)	9	10	6	1	1	0
2 (R)	10	10	5	2	0	0
5 (R)	10	9	5	4	0	0
6 (R)	9	10	10	7	1	0
9 (S)	10	0	0	0	0	0

Source: Pappas (1980).

a. Detached leaves were placed in petri dishes with the lower surface in contact with aqueous fungicide solutions containing benzimidazole as preservative (50 µg/ml). After 24 h each leaf was inverted and when the surface was dry, inoculated with 10 droplets of a sporangial suspension.

b. Average rating on a 0-10 scale: 0 = none; 10 = maximum infection. Rating made for three replicates five days after inoculation.

Phytophthora infestans

In the summer of 1980 Dutch potato farmers complained about the bad performance of metalaxyl in controlling potato late blight. Although the weather was very favourable for disease development only inadequate disease control was reported in fields sprayed with metalaxyl, and later on also from fields sprayed with a mixture of metalaxyl and mancozeb. Failure did not occur in fields treated with conventional fungicides only. To determine whether resistance had developed the sensitivities of several isolates were compared in a leaf-disc assay (Table 3) (Davidse et al., 1981). An up to 1 000-fold difference in sensitivity was noticed between the isolates. Isolates originating from fields where metalaxyl failed were usually highly resistant. Sensitive isolates were found in fields in other parts of the Netherlands where the disease incidence was low due to still adequate control by metalaxyl or conventional fungicides.

Resistant strains probably arose independently in several areas, because resistant isolates belonged to at least two different races of the pathogen. The failure of the mixture to control the disease adequately was probably a result of the presence of an almost entirely metalaxyl-resistant population. At the levels applied, mancozeb obviously was not able to withstand the high disease pressure.

The isolates maintained their high level of resistance for at least four months during weekly transfers on untreated potato tuber tissue. Resistant isolates were also resistant to furalaxyl, milfuram and 'Galben'. Their sensitivity to cymoxanil, fosetyl-Al and propamocarb did not change.

Discussion

Rapid development of resistance to acylalanines can be observed in the

Table 3. Activity of metalaxyl against sensitive (S) and resistant (R) isolates of Phytophthora infestans in a detached leaf assay [a].

Isolate	Average disease rating [b] at various metalaxyl concentrations (µg/ml)					
	0	0.01	0.1	1	10	100
19 (S)	3.4	3.6	0	0	0	0
24 (S)	3.2	3.0	0	0	0	0
29 (R)	4.0	4.0	4.0	4.0	4.0	1.2
52 (R)	4.0	4.0	4.0	4.0	4.0	0
84 (R)	4.0	4.0	4.0	4.0	4.0	4.0

Source: Davidse (1981a).

a. Potato leaf discs were placed upside down on fungicide solutions in petri dishes and inoculated with a droplet of a sporangial suspension.

b. Average rating on a 0-4 scale: 0 = none; 1, 2, 3 and 4 = 0-25, 25-50, 50-75 and 75-100 %, respectively, of the surface of the disc covered with sporangia. Rating made for five replicates.

laboratory and the field. In the laboratory, in addition to isolates with stable resistance and normal virulence, isolates with transient resistance and/or decreased virulence are fequently found. Resistance in vitro is not always correlated with resistance in vivo. This has also been observed for some field isolates of *P. infestans*, which although highly resistant in vitro displayed normal sensitivity to metalaxyl on tuber or leaf tissue (Bruin, 1980; Bruck et al., 1980).

An explanation for these phenomena can only be speculative. Bruin (1980) found that the ED_{50} value for growth inhibition of single spore or hyphal tip isolates originating from a single resistant strain of *P. capsici* varied between 0.1 and 450 µg/ml. It indicates a heterogeneous population of nuclei consisting of 'resistant' and 'sensitive' nuclei in the parent strain. Assuming a similar heterogeneity in originally sensitive isolates it might explain the ease with which *Phytophtora* and *Pythium* spp. adapt to metalaxyl in vitro. Adaptation in this case implies selection of nuclei. Whether or not these strains revert to sensitivity depends on the presence or absence of originally 'sensitive' nuclei in the adapted strain. A similar heterogeneous distribution of genes for virulence among the nuclei might result in a change in virulence during adaptation for sensitivity, the outcome depending on the distribution of these genes among 'sensitive' and 'resistant' nuclei. Variation in virulence among the asexual progeny is a frequently encountered phenomenon (Erwin, 1965). The 'resistant' nuclei undoubtedly originate by mutation, the rate of which can be enhanced by mutagens. Much of this explanation is based on circumstantial evidence that still has to be confirmed experimentally. No data are yet available about the biochemical mechanism of resistance, but its high level indicates that a decreased affinity at the target site might be involved. Bruin (1980) and Davidse (unpublished data) did not find indications of an enhanced metabolism or decreased uptake.

Although now there are enough of what appears to be conclusive results from laboratory experiments to predict the development of resistance under field conditions, that information was only partly or not at all available when the acylalanines were first introduced. On the contrary many data were available from field experiments showing the good performance of these compounds and without any sign of development of resistance, even under high infection pressure. The producer involved, the distributors of acylalanines and sometimes the local advisers were faced with the dilemma of deciding how to evaluate the results of the laboratory experiments and how much importance to give them in developing a use strategy. For some crops and some diseases there was almost no choice. In Greece and Israel, for instance, the growing of cucumbers in plastic houses during the winter season creates extremely favourable conditions for the development of *P. cubensis*, which was difficult to control before the acylalanines were introduced. The continuous and exclusive use of metalaxyl for nearly two years there led to the development of resistant populations of this fungus.

For a number of diseases that could already be controlled efficiently with conventional fungicides, however, several use strategies were imaginable. In such a case commercial motives often determine the strategy, and a sales strategy rather than a use strategy was sometimes followed. So the distributor of metalaxyl in the Netherlands recommended an almost exclusive use of this fungicide to control potato late blight. That recommendation proved to be disastrous and the use of metalaxyl for control of potato late blight in the Netherlands had to be discontinued little more than two years after it was first registered there.

Could mixtures of metalaxyl and conventional fungicides have delayed or prevented the development of resistance in *P. infestans* and *P. cubensis*, provided that other fungicides effective against the latter were available? For both pathogens a resistant population developed only after continuous and exclusive use of metalaxyl, admittedly under conditions very favourable for disease. If the conditions had been less favourable, or if only mixtures had been used, would resistance still have developed? No data are available to answer these questions. Circumstantial evidence indicates that mixtures affect the development of resistance in *P. infestans*. In the U.K., where in the summer of 1980 a mixture of metalaxyl and mancozeb was used to control potato late blight, no cases of resistance have been reported. However, in the same year in Ireland, where metalaxyl was used exclusively, resistance was found.

The use of mixtures to prevent or delay the development of resistance is controversial, because it is almost impossible to prove their effectiveness experimentally, and only mathematical models (Kable & Jeffery, 1980; Delp, 1980; this book, p.177-186) provide some insight into that effectiveness, but the fact that the companion fungicide has its own retarding

effect on the development of the disease may alone justify their use. Should resistance develop to one component of the mixture, the second might keep the resistant population low, ideally below the threshold level for crop losses. However, this approach requires a strong companion fungicide, for which there might be economical or registrational restraints.

Metalaxyl is now mixed with other fungicides to control foliar pathogens with a high reproduction rate (Staub & Schwinn, 1980). Another strategy may be to combine it with other fungicides but limit its use to only parts of a crop and use it for limited periods only.

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Can we estimate the fungicide-resistance hazard in the field from laboratory and greenhouse tests?

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Abstract

The fitness of the fungicide-resistant strains compared to that of sensitive wild-type strains is of paramount importance for the build-up of a resistant pathogen population. Parameters of fitness may be measured in laboratory and greenhouse tests. With some fungicides, resistance appears to be linked to reduced fitness. In such cases, the larger the difference between resistant and sensitive strains the more the build-up of a resistant pathogen population will be hampered. If the difference is very small or if a link does not exist, it will usually be difficult or even impossible to predict from laboratory or greenhouse tests whether resistance problems may arise in practice. The relation between resistance and fitness is reviewed for various groups of fungicides.

Keywords: fungicide resistance, fitness of resistant strains, prediction of resistance, testing for fitness, benzimidazoles, hydroxypyrimidines, carboxamides, organophosphates, antibiotics, ergosterol-biosynthesis inhibitors, acylalanines, dicarboximides, organic tin compounds.

Introduction

When failure of disease control occurs after repeated application of an originally quite effective fungicide, laboratory and greenhouse tests may reveal that this is due to the development of resistance to the compound concerned. However it is usually then too late to prevent crop losses. It would be valuable if, before the introduction of a chemical for control of a particular disease, information could be obtained from experiments with fungi in vitro, i.e. on an artificial medium, or on plants in the greenhouse about the chances of a resistance problem arising in practice. There are several in vitro tests with and without the use of mutagens, to assess the potential of fungi becoming resistant to a particular fungicide. Are these tests able to predict the occurrence of problems of resistance in the field?

Emergence of resistance in vitro

The capability of fungi to become resistant to a fungicide may be tested in the laboratory in several ways. A common method is the plating out of spores on an agar medium containing an amount of fungicide just greater than minimal inhibitory concentration (MIC). Only those cells that have acquired resistance by mutation (or in another way) will survive and form a colony. By counting the number of colonies the frequency of mutation for resistance can be calculated. Because the frequency of spontaneous mutation for resistance is low, usually between 10^{-4} and 10^{-9}, large numbers of spores are usually required for these experiments. Mutagenic chemicals or ultra-violet (UV) irradiation is often used to increase the number of mutants. Another method also often used is to transfer mycelium to an agar plate containing a growth-inhibiting but sublethal concentration of the fungicide. Development of resistance in cells of the mycelium may result in more rapidly growing sectors in the colony. These can be isolated and studied. After isolation of resistant colonies and exposure of the mycelium or the spores to a range of concentrations of the fungicide the degree of resistance can be assessed.

In studies with *Aspergillus nidulans* as a test fungus and UV irradiation as a mutagenic agent, van Tuyl (1977) compared a number of systemic fungicides on frequency of mutation towards resistance and the level of that resistance (Table 1). Results similar to those in Table 1 were also obtained in experiments with plant pathogenic fungi such as *Cladosporium cucumerinum* and *Ustilago maydis*. Although the frequency of mutation towards resistance and the level of resistance varies for different fungicides, in such in vitro experiments resistance to all systemic fungicides tested could be induced. These fungicides, and also a few of the non-systemic conventional ones, are socalled specific-site inhibitors: they act by interfering with the metabolism of the fungal cell at one particular

Table 1. Induction of resistance by UV treatment of conidia of Aspergillus nidulans.

	Number of resistant mutants per 10^7 survivors	ED_{50} (µg/ml)	
		wild-type fungus	most resistant mutant
Benomyl	5	0.7	30
Thiabendazole	9	8	125
Carboxin	2	9	200
Chloroneb	25	10	>1000
Cycloheximide	40	150	>4000
Imazalil	100	0.5	4
Pimaricin	1	1.5	6

Source: after van Tuyl (1977).

site. Genetic changes in fungi resulting in resistance to such specific-site inhibitors seem to occur readily, in contrast with multi-site inhibitors, which most conventional fungicides are.

Build-up of resistance in the field

The emergence in vitro of mutants resistant to a particular fungicide does not imply *a priori* that the use of this compound in the field also will lead to failure of disease control. That will only happen after a considerable proportion of the pathogen population has become resistant. The build-up of resistance within a population depends on various factors, particularly the fitness of the resistant mutants in relation to that of the wild-type pathogen - in both the absence and presence of the fungicide. Fitness is a comparative concept: one strain is more or less fit than another under conditions in vitro, in the greenhouse or in the field. Parameters of fitness in vitro are spore germination, mycelium growth on agar or in a liquid medium, and sporulation on agar medium. Important parameters of fitness on the host plant are the chance of infection, the speed of colonization of the host tissue and the degree of sporulation. Strains with higher fitness are more competitive.

To compare fitness, competition tests may be carried out between two or more strains, either in the presence or in the absence of the fungicide. In the presence of the fungicide the resistant strains have a clear advantage over the sensitive ones, which are strongly inhibited by the fungicide. Experiments in the absence of the fungicide may give an indication of the chance for survival of resistant mutants during spray intervals or when application of the fungicide is interrupted. In such experiments host plants are inoculated with a mixture of spores of the strains to be compared. When sporulating lesions appear, spores are harvested and used for inoculation of a second batch of plants. This procedure is repeated a number of times. The spores then harvested are tested for resistance, using a diagnostic fungicide concentration. The ratio of resistant to sensitive conidia is compared with that ratio at the beginning of the experiment. A decrease indicates that the resistant strain is less competitive under the conditions of the experiment.

As an example, the results of an experiment with benomyl-resistant and sensitive powdery mildew is given in Table 2. It appears that under the conditions of that experiment the benomyl-resistant pathogen is less competitive than the sensitive fungus. However, it does not follow that in general benomyl-resistant strains are less fit than the wild-type benomyl-sensitive fungus: there may be other resistant strains that do not have reduced fitness. Even if they occur at extremely low frequencies, the fungicide will select for these mutants, resulting in a rapid build-up of a resistant pathogen population. Only if repeated experiments with

Table 2. Competition between a benomyl-resistant and sensitive strain of cucumber powdery mildew in absence of the fungicide.

Inoculation mixture (sensitive: resistant)	Proportion of resistant colonies (%) after successive transfers of inoculum			
	1st	2nd	3rd	4th
90 : 10	4	7	3	0
50 : 50	32	34	17	0
10 : 90	45	41	23	5

fungicide-resistant strains invariably show a lower fitness can a link between resistance and reduced fitness be assumed. As the difference in fitness between the sensitive and the resistant pathogen becomes very large, the build-up of a resistant pathogen population will be seriously hampered or even not take place, depending on the selection pressure by the fungicide. But laboratory and greenhouse tests may not provide all information about fitness of strains under field conditions. Therefore field observations may be necessary for the full answer, especially if differences in fitness are so small that they cannot be detected in the greenhouse.

Resistance linked to reduced fitness

There is evidence that with some fungicides at least, increased resistance is linked to decreased fitness. This has been studied for pimaricin, an antifungal antibiotic belonging to the polyene macrolides (Dekker & Gielink, 1979a). This compound is used against certain fungal pathogens in man and to prevent moulding of freshly made cheese. Although pimaricin-resistant strains of yeasts and other fungi have been obtained in the laboratory, no resistance problems have been encountered in practice, i.e. in medicine and cheese packing houses. The antibiotic is also quite active against many plant pathogenic fungi. In laboratory experiments, pimaricin-resistant strains of *Cladosporium cucumerinum*, the cause of cucumber scab, and *Fusarium oxysporum* f.sp. *narcissi*, the cause of narcissus bulb rot, have been obtained. All resistant strains tested have shown reduced sporulation in vitro and strongly reduced pathogenicity.

On the basis of these data and the mechanism of action of polyene macrolide antibiotics, a link between resistance and reduced fitness has been postulated. Pimaricin and other polyene macrolides complex with ergosterol in the fungal membrane, which causes leakage and cell death. Studies with yeasts and nystatin, a polyene macrolide related to pimaricin, showed that resistant strains had an altered sterol pattern, namely, a strongly reduced amount of ergosterol in the membrane with an increase in sterols, which are biogenetically more primitive than ergosterol

(Lomb et al., 1975). The major sterols of resistant cultures of *Candida albicans* and *C. utilis* are with increasing resistance successively more primitive biogenic precursors of ergosterol (Fryberg et al., 1976). These precursors show a lower affinity to polyene antibiotics than ergosterol, so that the fungal membrane of the resistant strains contains fewer or weaker binding sites to the antibiotics, which results in decreased sensitivity. In addition, replacement of ergosterol by precursors appears to cripple the fungal cells, but it is not lethal.

Resistance to nystatin in yeast (Woods, 1971) and pimaricin in *Aspergillus nidulans* (van Tuyl, 1977) has been reported to be due to single gene mutations. It seems plausible that such a mutation, which leads to an altered sterol pattern, is responsible both for resistance and decreased fitness. This means that build-up of a resistant population is hampered. There are indications that also for other groups of systemic fungicides, resistance is linked to reduced fitness.

Type of fungicide

General

Information on the possibility of resistant strains emerging in vitro is available for most groups of new fungicides. Whether problems arise in practice depends on several factors, the competitive ability of resistant strains being an important one. However such information - obtained from greenhouse or field tests - is still very limited for most fungicides. Some fungicides have been used so long that the chances of a resistant population of a particular pathogen building up are known, but for very new fungicides the benefits of such experience are not yet available. The situation for specific-site inhibitors will be reviewed here. Note that with exception of the dicarboximides and the triphenyl tin compounds most of these fungicides are systemic.

Benzimidalozes and related compounds

Development of resistance to benomyl and other benzimidazoles has been reported for a large range of plant pathogens, both in the laboratory and the field (Dekker, 1977). Fitness of resistant strains appears to vary widely. Sometimes it has been reported to equal that of the sensitive pathogens, as was shown for benomyl resistance in *Cercospora beticola* in Greece (Dovas et al., 1976). For benomyl the conclusion seems justified that resistance is not absolutely linked to decreased fitness. Moreover, resistance to benzimidazole fungicides has been found stable with this and many other pathogens, which would aggravate the consequences should resistant strains that are fit occur.

Hydroxypyrimidines

Soon after the introduction of dimethirimol for control of cucumber powdery mildew in glasshouses, resistance developed in this pathogen. High selection pressure by the fungicide, favoured by the mode of application and probably also by the isolated environment provided by a glasshouse, contributed to this phenomenon; resistance was not reported from cucurbit fields in Mediterranean countries. After interruption of dimethirimol application the percentage of resistant individuals in the pathogen population gradually declined and practical application could be resumed to a limited extent p.219-230. It seems that under practical conditions the competitiveness of resistant strains is somewhat less than that of the sensitive fungus. This might also be true for ethirimol, which is used to control barley powdery mildew, by seed treatment or spraying the aerial plant parts. Although resistant isolates can be obtained from ethirimol-treated crops, the chemical continues to be effective. Strains with high resistance do not tend to increase in the presence of ethirimol because they are apparently less competitive (Shephard et al., 1975).

Carboxamides

Carboxin is used as a seed treatment against smut in wheat and barley. Although resistance in vitro in *Ustilago hordei* was obtained easily (Ben Yephet et al., 1975), there have been no reports of disease-control failure due to development of resistance. Oxycarboxin resistance resulting in control failure of *Puccinia horiana* on chrysanthemum has been reported by Abiko et al. (1977); the resistant strains appeared less competitive than the sensitive pathogen (Uesugi, 1978). But all carboxin-resistant mutants of *Ustilago maydis* (tested by artificial inoculation of corn seedlings in the greenhouse) did not show reduced pathogenicity (Georgopoulos et al., 1975). Georgopoulos (personal communication, 1981) has suggested that the low apparent infection rate might explain why no problems with resistance to carboxamides in Ustilaginales have occurred in practice.

Organophosphates

Although mutants of *Pyricularia oryzae* resistant to edifenphos and 'Kitazin' were obtained easily in experiments in vitro, resistance has not been a problem in the field for about ten years. Development of resistance to 'Kitazin' has been reported only once, in an experimental field where it was used intensively (Uesugi, 1978). Resistance to pyrazophos in cucumber powdery mildew was found in greenhouses only after several years

of application; when the use of this fungicide was stopped in those greenhouses, the frequency and level of resistance dropped the following year. The resistant isolates were somewhat less pathogenic than the sensitive pathogen, and also less competitive (Dekker & Gielink, 1979b). Also pyrazophos-resistant isolates of *Pyricularia oryzae*, obtained in the laboratory by de Waard & van Nistelrooy (1980), showed a lower rate of growth and sporulation on agar. These observations indicate a somewhat reduced fitness of fungal isolates resistant to organic phosphates. In practice, however, resistance may develop under conditions of continuous severe selection pressure by the fungicide.

Antibiotics

Streptomycin resistance in bacteria has been known for a long time. After the introduction of streptomycin for control of a plant disease, namely fire blight in pears, caused by *Erwinia amylovora*, the occurrence of resistance caused problems. Schroth et al. (1979) showed that virulence among resistant and sensitive strains varied but that there was no consistent difference between the two groups. The resistant strains appeared to be relatively stable and were detected even six years after termination of streptomycin application.

Kasugamycin-resistant strains of *Pyricularia oryzae* have been easily obtained in the laboratory. Intensive use of this antibiotic against rice blast in Japan led to disease-control failure in some districts that was due to development of resistance. Comparison of resistant and sensitive isolates showed no differences in mycelium growth and sporulation in vitro, nor in the number and length of the lesions. In a mixture of resistant and sensitive isolates, the resistant ones still appeared less competitive; their incubation period was longer and their rate of appressorium formation appeared to be slower (Ito & Yamaguchi, 1979). Due to these phenomena and perhaps also to dilution of the resistant pathogen population by sensitive strains from neighbouring fields or areas, the population of the resistant strain decreased after the application of the antibiotic was stopped. Kasugamycin is now used again, but in a carefully planned way.

Resistance to polyoxins has been reported in *Alternaria kikuchiana* in Japanese pear orchards. Although the resistant strains appeared somewhat less competitive than the sensitive ones, they did not disappear entirely (Uesugi, 1978).

The level of resistance in fungi to polyene macrolide antibiotics, such as pimaricin and nystatin, appears to be inversely related with the degree of virulence (p.131-132). Therefore low virulence greatly hampers the build-up of a resistant pathogen population in vivo.

Inhibitors of ergosterol biosynthesis

It is known that various groups of fungicides act specifically on biosynthesis of ergosterol in the fungal cell, namely, triazoles, imidazoles, pyrimidines, morpholines, triforine and buthiobate. Although triforine-resistant colonies have been obtained in the laboratory, no problems with triforine resistance have arisen during a number of years of use in practical agriculture. All resistant strains have a lower fitness in vitro as well as in vivo, which suggests that this might be linked to development of resistance. There are indications that this might also be true for many of the other fungicides, that act on sterol biosynthesis. For a more detailed treatment of sterol biosynthesis see p.71-100.

Acylalanines

Metalaxyl and furalaxyl, exclusively active against fungi belonging to the Oomycetes, have been introduced only recently. In laboratory studies, strains of *Phytophthora nicotianae* (Dekker, unpublished data) and *Phytophthora megasperma* f.sp. *glycinea* (Davidse, 1980) have been obtained with decreased sensitivity to metalaxyl. Studies with the latter strains on alfalfa in growth room experiments did not reveal a reduced fitness. Soon after introduction in practice, resistance to 'Ridomil', a commercial product containing metalaxyl as an active ingredient, appeared in downy mildew on cucumber in Israel and Greece (Georgopoulos & Gregoriu, 1981), and in *Phytophthora infestans* on potatoes in the Netherlands (Davidse et al., 1981), causing considerable crop losses.

Dicarboximides

Vinclozolin, iprodione and dicyclidine are particularly active against *Botrytis*, *Monilinia* and *Sclerotinia*. Fungicide-resistant strains of these fungi have been readily obtained in laboratory experiments (Leroux et al., 1977; Hisada et al., 1979). Recently, various cases of development of resistance in the field have been reported, e.g. in *Botrytis* on strawberries (Davis & Dennis, 1979) and *Botrytis* on grapes (Spengler et al., 1979). Some authors report from laboratory studies that fitness of the resistant strains was less than that of wild strains (Spengler et al., 1979; Hisada et al., 1979), but others found also strains with equal fitness (Sztejnberg & Jones, 1978; Pappas et al., 1979). Therefore it cannot be concluded that resistance is linked to a decreased pathogenicity. Judicious application is necessary to avoid or delay resistance problems in practice. Resistance to dicarboximides is treated in more detail on p.101-117.

Organic tin compounds

Resistance to the organic tin compounds has been reported in Greece in *Cercospora beticola* on beets. The resistant strains tested were as virulent as the sensitive strains but appeared to be less competitive (Giannopolitis & Chrysayi-Tokousbalides, 1980).

Conclusions

From laboratory experiments circumstantial evidence has been obtained that fungi are able to develop resistance to all fungicides - whether systemic or not - that act as specific-site inhibitors. The frequency of mutation for resistance may vary, but this does not seem to be of great importance given the large number of propagules produced by fungi. Lack of emergence of resistance might theoretically be possible if mutation towards resistance is lethal for the mutating cells. This does not seem to have occurred for the fungicides tested so far, although a decrease of fitness of resistant strains has often been observed.

Emergence of resistance in vitro does not necessarily imply that a resistant pathogen population will build up in the field. This will depend on a number of factors, among them the type of pathogen, the nature of the disease, the mode of fungicide use and the fitness of resistant strains compared to sensitive strains. Strains that are resistant in vitro may be sensitive in vivo, e.g. when the fungicide is converted to another fungitoxic compound or induces resistance in the plant tissue to the pathogen. What then is the use and predictive value of laboratory experiments?

First, when it appears impossible to obtain resistance in vitro, even using mutagens, development of resistance in the field is unlikely. Second, laboratory and greenhouse experiments may provide information about at least a number of parameters of fitness, e.g. spore germination and growth of mycelium in vitro, infection rate and speed of colonization and sporulation on the host plant. If differences in fitness are difficult to detect, competition experiments in the greenhouse, using inoculation mixtures of spores from resistant and sensitive strains, may provide valuable information. As fitness and pathogenicity may vary widely among resistant as well as sensitive strains, only strains that show optimal fitness should be tested. Results from experiments with only a few resistant isolates, picked at random, should not be generalized without great care.

In spite of all these efforts, it will usually not be possible to predict, on the basis of laboratory and greenhouse experiments, that resistance to a particular fungicide will never occur in the field. First, even competitive experiments in the greenhouse may not provide all information that is important in field situations. Second, even if all resistant

strains tested were less fit than the sensitive pathogen, often it will be not justified to exclude the possibility that rare resistant strains with high fitness will emerge. Third, resistance problems may only arise after many years of use in practical agriculture; resistant strains that in the beginning do not cause problems, due to a low level of resistance may under constant selection pressure of the fungicide evolve with higher resistance. Fourth, the age of resistance may play a role. Wild-type strains have been subjected to selection for fitness for a very long time, but resistant strains are very recent and, hence, many of them have not been substantially selected for fitness.

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Dynamics of the pathogen population in relation to fungicide resistance*

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Abstract

The emergence of fungicide resistance depends on the nature of the fungicide, its effectiveness in the plant and the scale of its use. Detection of variation for resistance before a fungicide is introduced, or shortly afterwards, is limited by the technical difficulty of obtaining and recognizing rare phenotypes. Evaluation of such phenotypes must take account of likely changes in their pathogenicity following selection on untreated and treated host plants. The pathogen population may be subjected simultaneously to stabilizing, directional and disruptive selection for increased resistance. A simple model for estimating the relative fitness and size of sensitive and resistant populations of the pathogen indicates the relative importance of the difference in pathogenicity between such populations on untreated crops, and of the total crop area that receives the fungicide. Strategies of fungicide use that exploit population dynamics to limit the development of resistance are briefly described. Fungicide mixtures may be homogeneous (all components applied to the same plants), or heterogeneous (all components applied to different plants). Different fungicides may also be applied in an alternating sequence. It cannot be stated generally which of these strategies is the more suitable for particular compounds, but the most effective strategy is likely to be integrated control, for example by using limited, or heterogeneously mixed, fungicide applications on mixtures of host-plant varieties that differ in their resistance.

Keywords: fungicide resistance, population dynamics, selection model, *Erysiphe graminis*, ethirimol, tridemorph, triadimefon, fungicide strategies, integrated control.

* Editors' note: in this contribution - and the rest of the book - we conform with the FAO recommendation to denote the lack of or decrease in sensitivity to a fungicide by the term resistance. Dr Wolfe advocates the use of the term insensitivity, but he has agreed to the replacement of this term by that of resistance to maintain consistant terminology throughout the book.

Occurrence of fungicide resistance

It is a truism that the population of a target pathogen is sensitive to a newly introduced and successful fungicide, although the range of sensitivity within the population at the time of introduction is usually unknown. It is possible that the organism may be unable to cope with the fungicide: the system required to do so is beyond its metabolic capability. On the other hand, there may be mutants that are resistant to a lesser or greater degree; the potential ceiling for resistance may be moderate or high.

Although it is also true that the occurrence and potential range of resistance of the pathogen will be determined by the nature of the fungicide, it is usually impossible to distinguish a single character of the fungicide, such as site specificity, as the most important in this respect. Both the nature and the effectiveness of the fungicide are important in determining the pathogen's response to it. For example, resistance to a site-specific compound may involve one or more pathogen genes, whose ease of modification is unpredictable. Site-specific compounds are often systemic and persistent, and it is these characters that make them highly effective, but it also ensures a high degree of selection for resistance by maximizing exposure of the fungicide to the pathogen. Resistance to multi-site compounds may also involve one or more pathogen genes; again, the ease of modification of each gene is unpredictable. Multi-site compounds are often non-systemic and lack persistence, so that they have low effectiveness, which does not give such intense selection for resistance.

Initially, resistant forms are probably at a selective disadvantage and survive at only low frequencies in the population; they have a low fitness prior to the introduction of the fungicide. It is therefore necessary to monitor the population for this character from the earliest possible stage. Unfortunately, monitoring suffers from two defects. First, the majority of methods used are capable of detecting only relatively common mutants; they are often less effective than trial plots in detecting the more important, but less common, forms. This problem is illustrated in Table 1, which shows the sample sizes required to obtain with 95 % probability at least one of those mutants that occur at low frequencies. The figures were obtained from the binomial expression sample size = $\lg (1-P)/\lg (1-y)$ where P is the required probability and y is the frequency.

Table 1 also indicates the efficiency of cereal fields in trapping mutant spores of powdery mildew *(Erysiphe graminis)* (derived from suggestions of Wolfe & Schwarzbach, 1978), given a spore concentration in the atmosphere of only 1 per 1 000 m^3 and a spore deposition velocity of 1.5 cm/s. Only a small crop area is required to be certain of trapping a

Table 1. Sample size and sampling area required to obtain with 95 % probability an unusual mutant occurring at low frequencies in a pathogen population; sampling area required refers to the area per day of a cereal crop receiving spores of Erysiphe graminis.

Mutant frequency	Sample size required	Sample area required (ha/d)
10^{-3}	3×10^{3}	0.2
10^{-5}	3×10^{5}	23.1
10^{-6}	3×10^{6}	230.8
10^{-8}	3×10^{8}	23 077.0

relatively rare mutant. Of course, survival of a single spore after deposition is affected by many other factors.

The second problem in monitoring concerns the condition of the first-found resistant mutants. Tests often indicate that they do not grow well on treated host plants, and that they are less fit on untreated plants than the common sensitive strains. Unfortunately, once observed, these characteristics are often regarded as static. They may remain so, but if selection for resistance is maintained or increased, then genotypes with improved performance on both treated and untreated host plants may emerge. Because of the difficulties of adequate monitoring, the development of such changes may remain undetected for a considerable time.

Because of the problems of scale and dynamism in monitoring, many laboratory studies on the occurrence and performace of resistant strains of pathogens are inadequate and misleading. Typically, a genetically limited sample of the pathogen is challenged with a fungicide following the use of artificial mutagens. The results obtained are open to doubt: lack of response is unlikely to indicate the full potential of the pathogen. But even if a response does occur, it may not involve the strains that occur most commonly in the field (p.207-218).

Measurement of the pathogenicity of such artificially induced mutants on untreated media or host material in the laboratory may also be misleading because:

- it does not reflect the total dynamic potential of the field population
- it may be affected by mutations occurring at loci not involved in resistance
- it makes no allowance for the dynamics of fungicide use and the dynamics of the sensitive and resistant pathogen populations.

Laboratory studies on pathogen dynamics are generally of greatest value in testing the comparative performance of pathogen isolates from the field.

Dynamics of selection towards resistance

Given that the fungicide-resistant mutants do occur in pathogen populations not yet subjected to fungicide treatments, we need to understand the processes that cause an increase in frequency of these mutants when the fungicide is used. Mather (1953) defined three classes of selection acting on populations of organisms, namely, stabilizing, directional and disruptive.

Stabilizing selection is the process that maintains a high frequency of optimal phenotypes, i.e. those that best fit the individual to the usual range of circumstances that the population encounters. Because there are environmental variations, and because similar phenotypes may be produced by different genotypes, there will be genetic variability even in a population subject to strong stabilizing selection.

Directional selection occurs if the average phenotype in the population is not close to the optimum: then selection will favour those phenotypes, and their associated genotypes, that are so; selection will be away from the existing means, towards the new optimum. Stabilizing selection occurs simultaneously, to stabilize the population around the new optimum. Directional selection is a positive response to changes in the environment. It is of special importance in the context of fungicide resistance.

Disruptive selection occurs when two different environments that each favour distinct optimal characteristics are created. Selection for the two distinct optima may cause a break in the continuity of population variation. Stabilizing selection will again occur, to maintain the selected phenotypes and their respective optima.

All three types of selection may occur simultaneously and to varying degrees in response to the environmental change caused by introduction of a fungicide to control the pathogen. The relative importance of each will depend on the degree of use of the fungicide and the extent to which different mechanisms of resistance are available to the organism. One critical factor is the relation between the length of the reproductive cycle of the organism and the time during which the fungicide is active. If an organism has a long cycle relative to the duration of fungicidal activity, then the populations will change slowly, if at all. Conversely, if the pathogen has a short cycle relative to the duration of fungicide activity, there will tend to be a more rapid population response, and the population will tend to follow any changes in fungicide use.

The sequence of events

Prior to the introduction of a fungicide, the sensitive pathogen population can be regarded as being in equilibrium and subject to stabilizing selection for the character fungicide resistance. In Figure 1 the optimal

phenotype for resistance before introduction of the fungicide is at x_1 Stabilizing selection operates in the direction of the arrows, i.e. towards x_1; genotypes with some degree of resistance exist, but their frequency is low. Introduction of the fungicide, and its increasing use, represent a change in the environment, and the optimal phenotype may then be at x_2 or x_n, depending on the relationship between the fungicide and the metabolism of the pathogen, and the way in which the fungicide is used. If the fungicidal activity can be easily matched by the pathogen, and the fungicide is not widely used, then a shift from x_1 to x_2 by directional selection in each treated crop may be adequate for the pathogen to attain optimal fitness. Disruptive selection will also act on the population as a whole during the growing season, since untreated and treated crop areas will be different niches for randomly dispersed spores.

If the fungicide presents a more severe metabolic problem for the pathogen, or if it is very widely used, the new optimal phenotype may be at x_n. If it is metabolically possible, x_n may be attained by either of two routes. First, rare mutants may occur in the population represented by x_1 that are able to grow at or close to the optimum, x_n. If they infect the treated crop, they may be able to increase even if their fitness is low (w_1, Figure 1). Second, the process may be stepwise, from x_1 to x_2, from x_2 to x_3, and so on to x_n. Depending on the appropriate fitness values, a discontinuity between the sensitive and resistant fractions of the popu-

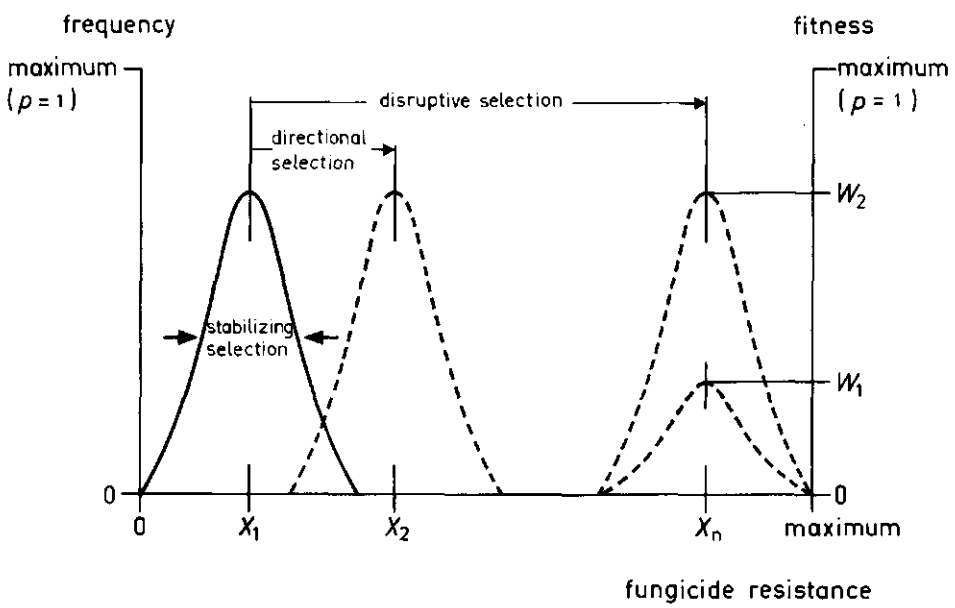

Figure 1. Stabilizing, directional and disruptive selection for the character fungicide resistance. $\underline{x}_1$, $\underline{x}_2$ and $\underline{x}_n$ represent different optimal phenotypes each depending on the type of fungicide and how it is used.

lation may develop and the intermediate stages may be more or less rapidly lost. Selection for resistance may also proceed simultaneously along both routes, but it is impossible to predict which may lead most rapidly to x_n. If of resistant genotypes are found it may be difficult to determine whether they occurred as the result of a single major change, or series of steps.

A model of disruptive selection

If two separate populations are produced as a result of disruptive selection, their progress relative to each other is dependent principally on their ability to reproduce on the part of the crop to which they are adapted, but to a lesser degree also on the part to which they are not adapted. This can be considered formally using a derivation of the discrete generation model for pathogen reproduction devised by Barrett (1978, 1980). The continuous generation model produces similar results.

The simplest model can be represented by a two-way table, given in Table 2. Nsu_0 represents the number of sensitive genotypes on area g of the untreated crop at the start of the epidemic, and wsu represents the fitness of the sensitive fraction of the pathogen population on the untreated crop. Let the basic multiplication rate of the pathogen, $m = 1 + \alpha$, where 1 represents the parent colony, and α the number of daughter colonies produced. After n generations there will be:

- on the untreated crop

$Nsu_n = Nsu_0.g(wsu.m)^n$ sensitive genotypes.

But if wsu is set = 1

then $Nsu_n = Nsu_0.g(m)^n$ sensitive genotypes.

Similarly there are

$Nru_n = Nru_0.g(wru.m)^n$ resistant genotypes.

- on the treated crop

$Nst_n = Nst_0.h(wst.m)^n$ sensitive genotypes.

But if wst = 0

then $Nst_n = 0$.

Table 2. Reference symbols for sensitive and resistant fractions of the pathogen population on untreated and treated crop areas.

	Untreated crop (u)	Treated crop (t)
Sensitive pathogens (s)	su	st
Resistant pathogens (r)	ru	rt
Proportion of total crop area[a]	g	h

a. g + h = 1.

$Nrt_n = Nrt_0.h(wrt.m)^n$ resistant genotypes.
Therefore
$\Sigma\ Ns_n = Nsu_n + Nst_n = Nsu_0.g(m)^n$,
and $\Sigma\ Nr_n = Nru_n + Nrt_n = m^n\ \{Nru_0.g(wru)^n + Nrt_0.h(wrt)^n\}$.
Therefore
$Nr_n/Ns_n = \{Nru_0.g(wru)^n + Nrt_0.h(wrt)^n\}/Nsu_0.g$.
Note: to follow the changes in populations size (and therefore epidemic size) m must be retained.
If the ratio $Nr_n:Ns_n$ exceeds $Nr_0:Ns_0$ then the resistant genotypes will become prevalent; if the reverse is true, the sensitive genotypes will remain prevalent. Stable equilibria are unlikely because the conditions required for stability are very restrictive (Barrett, 1978).

This model shows that the outcome is dependent on the initial frequencies of the two kinds of genotype, their fitness on the untreated and treated fractions of the host, the relative size of those two fractions, and the number of pathogen generations. If h is large and $wst = 0$, note that low values of wru and wrt may lead to prevalence of the resistant fraction; this prevalence would be accelerated if there were any increase in the values of wru or wrt. Conversely, if h is low, the sensitive fraction may remain prevalent even if wru and/or wrt increase relative to wsu and wst.

An example: the use of ethirimol in the UK

The declining effectiveness of ethirimol seed-dressing in controlling powdery mildew of barley (*Erysiphe graminis* f. sp. *hordei*) in the UK (Wolfe & Dinoor, 1973), indicates that some adjustment of the basic model may be necessary when considering a particular problem in practice:
- The relative crop area treated with ethirimol, h, though never large, varied from season to season.
- Since the fungicide was usually applied to the seed only, it was effective early in the epidemic but gradually became ineffective later in the season because of leaching and katabolism. Thus h decreased, although the originally treated crop area remained constant, so that the fitness of sensitive relative to resistant pathogen genotypes increased during the season.
- During the 1970s ethirimol was used only on spring barley, so that in the overwintering phase of the pathogen on winter barley $h = 0$.
- During the period of use, the fungicide was applied to different varieties covering a wide spectrum of host resistance genes. Different combinations of fungicide and host resistance may well have delayed the emergence of pathogenic recombinants, i.e. depressing wru and wrt relative to wsu. An exception was the pathogenicity for 'Sultan' and related varieties, which was positively associated with ethirimol resistance (Wolfe &

Dinoor, 1973; Wolfe & Slater, 1980).

These four features together suggest that resistance to ethirimol was not of overriding importance for the survival of the pathogen. Indeed, Bent (1978) showed that although highly sensitive and highly resistant forms of the pathogen occurred in the UK following the introduction of ethirimol for disease control of powdery mildew of barley, the genotypes that became most commonly established in the late 1970s had only an intermediate level of resistance, suggesting that directional selection had been prominent. As a consequence, the effectiveness of the fungicide declined, but not dramatically; under the right conditions, it still has some value in practical agriculture.

The circumstances described above in the first, third and fourth points also applied to the use of tridemorph for control of barley powdery mildew during the same period. However, this fungicide was only used as a foliar spray, for treating established populations of the pathogen, so that it probably had less effect than ethirimol in changing the direction of population development. Consequently, although Walmsley-Woodward et al. (1979) were able to detect resistance to tridemorph in the field, the effectiveness of this fungicide appears not to have changed during the period of use. However, it is evident that a different - more intensive - history of use of ethirimol and tridemorph involving a large increase in h could have led to a more rapid and obvious decrease in the effectiveness of both fungicides. For this reason the current large value of h for the use of ergosterol-biosynthesis inhibitors on cereals, and the finding of resistant forms of the barley-mildew pathogen in the field in 1980 and 1981 gives considerable cause for concern.

Strategies for fungicide use

The major problem in fungicide use is to minimize the exposure of a particular fungicide to the pathogen population while maintaining an acceptable level of disease control. The simplest strategy is to limit unnecessary use of the fungicide by not controlling disease that is not damaging. Improvements in disease monitoring and forecasting, better use of disease-resistant varieties, and improved methods of fungicide application, are important in this respect. Other strategies involve diversification, either between fungicides, or between fungicides and resistant varieties.

Homogeneous and heterogeneous mixtures

The principle behind this strategy (Wolfe, 1981) is simply that a multiple problem in terms of different fungicides is more difficult for a pathogen to overcome than a single problem. Field experience now suggests

that mixing fungicides does tend to decrease the rate of emergence of resistance (p.195-206). However, most experience has been gained with homogeneous mixtures, i.e. fungicides that are first mixed, and then applied as a homogeneous mixture to the crop. This may have the disadvantage that if one component is markedly more persistent than the others, it will be exposed uniformly to the pathogen when the dose levels of the less persistent components have declined.

A more effective strategy might be to use heterogeneous mixtures, in which the components are applied simultaneously to the crop, but to separate parts. In this system heterogeneity is preserved longer since the plants treated with the most persistent component will be mixed with, effectively, untreated plants after the dose levels of the other components have declined. Any resistant isolates will then face competition with sensitive isolates.

In practice, heterogeneous mixtures are obtained by mixing seed lots treated with different materials. Similar mixtures of foliar sprays require the development of appropriate machinery, the most promising of which is the electrodyn sprayer, which could easily be adapted for this purpose.

Alternating fungicides

The principle behind this strategy is to use one compound to reduce the absolute population size of forms of the pathogen resistant to the alternate compound. The alternative compound can then be safely introduced to minimize the population size of pathogen forms resistant to the first compound, and so on. It is not possible to state generally that alternating of fungicides is more or less effective than mixing for delaying the development of resistance. However, with alternating fungicide use, if one of the components is much less effective than the others, the cost of disease control while it is being used may be high, because of the more frequent applications necessary.

Integrated control

In an analogous development of strategies for delaying increased pathogenicity towards resistant host-plant varieties, Wolfe & Barrett (1980) proposed the use of mixtures of resistant plant varieties. They also suggested that fungicide use should be integrated with varietal mixtures, and methods for so doing have been described by Wolfe (1981). The most obvious method is to treat the seed of one or more components of the varietal mixture with different fungicides before mixing the seed. Alternatively, a small proportion of the already mixed seed can be treated with fungicide and then mixed either with untreated seed, or with

seed treated with other fungicides. The within-crop diversity for disease control that results is considerable. A pathogen is unlikely to match such a system for a considerable time, particularly if the mixture composition of host and fungicide is regularly changed.

If reasonable estimates of the appropriate parameters are available, the model of disruptive selection described can be extended to compare the basic strategies suggested. However for a more realistic analysis it is necessary to include parameters of spore dispersal (Barrett, 1980). The simple model described assumes redistribution of all spores produced over all host components. This is obviously unrealistic.

In cereal production much reliance is currently placed on ergosterol-biosynthesis inhibitors, but insensitive forms of *E.graminis* f. sp. *hordei* have now been detected in the field in England in 1980 (J.T. Fletcher, personal communication) and 1981, and in West Germany, also in 1980 and 1981 (E. Limpert, personal communication). There is no indication yet that there is a practical problem of reduced effectiveness. However, rather than wait to see if a problem does develop, it would be much more rational to try to implement some suitable strategies along the lines described in this contribution to reduce at least the likelihood of a problem developing.

Acknowledgement

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Can we use models describing the population dynamics of fungicide-resistant strains?

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Abstract

Vanderplank's equivalence theorem leads to an epidemiologic evaluation of fungicides. With the concepts of 'fitness' and 'competition' from population genetics and those of 'carrying capacity' and 'intrinsic growth rate' from population dynamics, it becomes possible to design a population biology of fungicide-resistant strains of phytopathogenic fungi. A first step towards a quantitative biology of plant diseases due to fungicide-resistant strains is ventured and a few examples are given.

Keywords: fungicides, competition, fitness, epidemiology, resistance.

The equivalence theorem

Epidemiologists cherish the disease triangle, which is a symbol of the interactions between environment, host and pathogen (Figure 1). The sides of the triangle represent mutual effects between pairs of factors; nothing is said about the nature of the effects. An economically desirable effect is the reduction of disease. This reduction may be brought about by reducing the virulence of the pathogen, or by increasing the resistance of the host, or by changing the environment. The change of environment, in its turn, can be caused by a change in cultivation methods, a change in crop climate, and/or application of fungicides.

Epidemiologists measure the rate of progress of a disease. For this

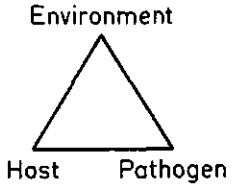

Figure 1. The disease triangle.

purpose they apply the logistic equation

$$dx/dt = r.x.(1 - x) \qquad (1)$$

in which x is the fraction disease ($0 \leq x \leq 1$), t is time, and r is the logistic infection rate. The value r is the 'speedometer' of the epidemic. Reduction of disease can be obtained by reducing the initial inoculum, x_0, and/or the speed of the epidemic, i.e. reducing r. Epidemiologically, resistance of the host, change of microclimate and application of fungicides can all lower r. All these very different actions have the same epidemiological effect, i.e. they are epidemiologically equivalent. This is the 'equivalence theorem' of Vanderplank (1963), which is illustrated by Figures 2 and 3. Note that in these figures the values plotted along the ordinates are not x, but logit x, where

$$\text{logit } x = \ln [x.(1-x)^{-1}] \qquad (2)$$

in order to obtain approximately straight lines, i.e. the logit lines.

The equivalence can be carried one step further. Among fungicides, protectants such as heavy metal compounds, sulphur, and some of the early carbamates, have an effect loosely equivalent to horizontal resistance, which is a host resistance approximately equal for all strains of the fun-

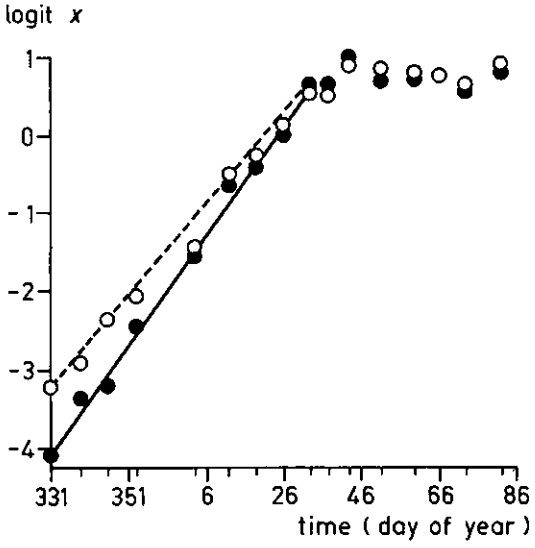

Figure 2. Development of epidemics of Cercospora apii on celery in Florida. ○= disease progress in unsprayed celery, wild type; ●= disease progress in celery receiving weekly sprays of benomyl, resistant strain. (Source: after Berger, 1973). The benomyl-resistant strain starts at a lower level than the wild type but overtakes it; the difference between the slopes of the two logit lines is statistically significant. (Source: Zadoks & Schein, 1979). Horizontal axis: t, time as day of the year from November to March. Vertical axis: logit x; x is disease severity.

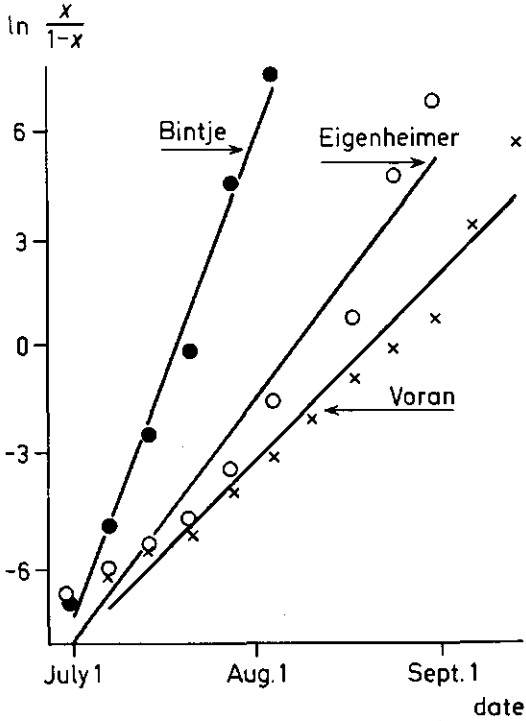

Figure 3. The progress of late blight in 117 fields in potatoes in sand areas of the Netherlands. Logit lines with different slopes represent different varietal resistances; x is the proportion of diseased foliage. (Source: Anonymous, 1954; Vanderplank, 1963).

gus. By analogy, these fungicides may be called 'horizontal' fungicides. They often affect many metabolic pathways without typical site-specific action. At the opposite end of the range of variation are the 'vertical' fungicides. These are the fungicides with a very specific metabolic target. They kill all strains of the fungus except the odd one that acquires resistance to the fungicide. Many of the systemic fungicides induce resistance. Their effect, then, is equivalent to 'vertical' or race-specific resistance. Resistant strains of a fungus are said to be equivalent to the classical physiologic races that have a gene-to-gene relation with their host plants.

Fitness

The word 'fitness' is borrowed from population genetics. The term is easily understood in a general way, but the concept of fitness is not so easy to handle in a scientific way. It is a comparative concept. One genotype is more or less fit than another genotype under given conditions; more specifically, one genotype can reproduce more, or less, successfully than another genotype.

The usual numerical approach to fitness is to choose one genotype as a reference and to assign it a fitness value of 1. The other genotype, to be compared to the reference, has the fitness value of $1 - s$. The symbol s stands for the difference in fitness between the two genotypes, with $0 \leq s \leq 1$. For our present purpose the non-resistant strain of the fungus, here indicated as a wild strain, will be the reference with fitness 1. The resistant strain will be indicated as mutant strain with fitness $1 - s$; for the time being we will assume it to be less fit than the wild strain.

Epidemiologists can be somewhat more precise than population geneticists. They have techniques to measure various components that contribute to fitness, such as latent period, p, infectious period, i, daily multiplication rate, R_c, and so on. Epidemiologists have also developed methods to compare genotypes by components and combinations of components. One method is the 'components analysis', which can be extended to 'life tables' and then 'survival tables'. Combinations of components can be used also to calculate relative growth rates of the same nature as the logistic infection rate, r, mentioned before (Zadoks & Schein, 1979).

The circle is now complete, beginning at the fitness reduction s that can be determined experimentally, passing on to a detailed analysis that leads to various r values, and, finally, coming to the differences between r values, which can be considered in terms of differences in fitness. Table 1 gives an example of how a 10 % reduction in one component leads to a change in relative growth rate. Note that a 10 % reduction in virulence by way of the basic infection rate, R, implies a decrease in R, but that a 10 % reduction of virulence by way of latent period, p, implies an increase in p. The effect of a 10 % reduction of virulence varies according to the component and the original level of virulence. Table 1 contains two warnings: it is difficult to predict the change in r from a known change in one component; and it is next to impossible to make conclusions about changes in components from a known change in r value.

The logic of population genetics would now compell us to the following argument. Under conditions of intensive and large-scale treatment with a

Table 1. The effect on r of a 10% reduction of fitness.

	Moderate r			High r		
Strain	wild	mutant	mutant	wild	mutant	mutant
p	10	11	10	10	11	10
R	1.48	1.48	1.33	21.84	21.84	19.66
pR	14.8	16.28	13.33	218.4	240.24	196.6
r	0.200	0.188	0.193	0.400	0.371	0.391
Reduction of r in %	-	6	4	-	7	2

Source: Zadoks & Schein (1979).

vertical fungicide the selection pressure for resistance is great. A mutant strain will appear, probably through a 'loss mutation' that is more successful than the wild type as long as the selection pressure continues, but less so as soon as the selection pressure ceases to exist. The implication is that the frequency of the resistant strain decreases as soon as the fungicide is no longer used. Though the reasoning is perfectly plausible, real life is not as simple as that. There are noteworthy exceptions. Figure 2 shows two epidemics of *Cercospora apii* on celery. The benomyl-resistant mutant strain has a higher r value than the wild type. It was reported that in some cases the resistance, the mutant character, persisted even when the selection pressure ceased to exist. Such anomalies can be explained in different ways. Either the general concepts of population genetics do not apply to resistance of fungicides in fungi, or the actual situation is more complex than the theory presented. There is some evidence for the latter view (Zadoks & Schein, 1979).

Competition

Population geneticists tend to think in terms of selection for strains with high fitness under given conditions. In the simplest case the fungal population is composed of two fractions, a fraction p, representing the weaker strain, and a fraction q, the stronger strain. The sum of the two fractions must equal unity:

$$p + q = 1. \qquad (3)$$

After one generation the fraction p_0 of the weaker strain has become p_1:

$$p_1 = (1 - s).p_0 \;/\; correction\ factor \qquad (4)$$

and

$$q_1 = q_0 \;/\; correction\ factor \qquad (5)$$

where the *correction factor* $= (1 - s).\ p_0 + q_0.$ (6)

Multiply Equation 4 by q_0 and substitute Equation 6 in Equation 4,

$$\begin{aligned} p_1 q_0 &= [(1 - s).\ p_0\ .\ q_0]/[(1 - s).\ p_0 + q_0] \\ &= (1 - s).\ p_0.\{\ q_0\ /[(1 - s).\ p_0 + q_0]\} \\ &= (1 - s).\ p_0\ .\ q_1. \end{aligned} \qquad (7)$$

Divide Equation 7 by $p_0\ q_0$.

$$p_1/p_0 = (1 - s).(q_1/q_0) \qquad (8)$$

Remember that s is the relative fitness reduction and $(1 - s)$ is the fitness of the weaker strain relative to the stronger one.
For the second generation the procedure can be repeated:

$$p_2/p_0 = (1 - s)^2.(q_2/q_0) \qquad (9)$$

Similarly after n generations:

$$p_n/p_0 = (1 - s)^n.(q_n/q_0) \qquad (10)$$

Equation 10 can be written as

$$\ln(1 - s) = (1/n).(\text{logit } p_n - \text{logit } p_0) \qquad (11)$$

Equation 11 can be compared to the integrated form of Equation 1, the logistic equation:

$$r = (t_n - t_0)^{-1}.(\text{logit } x_n - \text{logit } x_0) \qquad (12)$$

In these last two equations, x and p represent fractions ($0 \leq x \leq 1$, $0 \leq p \leq 1$). Time is represented differently: in Equation 12 time runs from day $t = 0$ to day $t = n$; In Equation 11 time is expressed in the number of generations, n.

At the left side of the equals sign, we see the logistic infection rate r in Equation 12 and $\ln (1 - s) = r_d$ in Equation 11. The 'speedometer' r is expressed in units per unit per day and has dimension T^{-1}. The value r_d has been called the relative rate of disappearance; when we choose to measure time not in seconds or days but in generation periods, taking the generation period as our unit of time, r_d also obtains the dimension of T^{-1}. So r_d becomes the 'speedometer' of disappearance by competition.

Note that the foregoing reasoning is applicable only in the early phases of the epidemic - the so-called lag or exponential phase - when there is no crowding or competition for space or nutrients; the present theory applies only to 'uncrowded' conditions.

Equation 12 shows that disappearance of a weaker strain due to the competition of a stronger strain is a logistic process. As in Figures 3 and 4, logistic processes can be represented by straight lines with time as the abscissa and the logit value of the fraction x or p as the ordinate of the coordinates. An example of such competition is given in Figure 5 for two strains of cucumber mildew (*Sphaerotheca fuliginea*), i.e. a wild type and a benomyl-resistant mutant strain. Plants were inoculated with a mixture of the two strains. After one generation (one time unit) spores were harvested and applied to fresh cucumber plants, and the

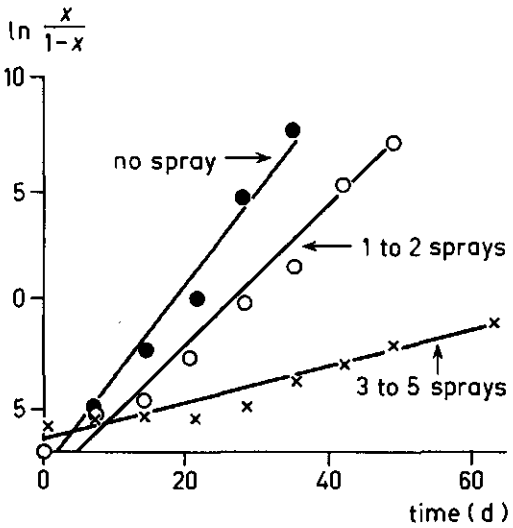

Figure 4. The progress of blight in 29 unsprayed and 31 sprayed fields of the susceptible potato cv. Logit lines with different slopes represent different treatments; x is the proportion of diseased foliage. Data from the Netherlands (Source: Anonymous, 1954; Vanderplank, 1963).

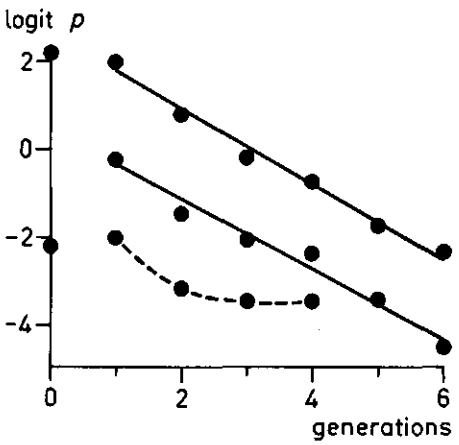

Figure 5. Competition between two strains of cucumber mildew (Sphaerotheca fuliginea) under fungicide-free conditions when mixed in 3 different proportions at n = 0. The wild benomyl-sensitive strain is the fittest. Curves show the decrease in the proportion, p, of the mutant fungicide-resistant strain. Except for the lowest line and the first reproduction cycle, the logit lines are reasonably straight and parallel. For the upper line 1 - s = 0.42. (Source: data by courtesy of Dr J. Dekker, Laboratory of Phytopathology, Agricultural University, Wageningen, unpublished; Zadoks & Schein, 1979). Horizontal axis: n, time in generations. Vertical axis: p, proportion of fungicide-resistant strain on logit scale.

cycle repeated, and so on. The mutant strain is less fit than the wild type when the selection pressure is removed; that means that s is positive ($0 \leq s \leq 1$). Calculation shows that $s = 0.58$ and $(1 - s) = 0.42$. The relative rate of disappearance is $r_d = -0.87$. Of course, r_d is negative as the weaker strain disappears gradually; the logit line representing the weaker strain runs downward. Figure 5 actually shows three lines or curves, representing three mixing proportions at $n = 0$, p being 0.99, 0.50, and 0.01, respectively. The line for $p = 0.01$ is an anomaly.

The competition between fungicide-resistant and fungicide-sensitive strains is not unlike that between different vertical races of a rust. Figure 6 shows the competition between two races, 15B-1 and 56, of black stem rust (*Puccinia graminis*) at two different temperatures. Figure 7 shows the same curves after logit transformation. At the start, the two races are present in equal proportions. Race 56 is the 'loser' at a low temperature. At a high temperature, however, it is the 'winner'. The example illustrates three important points:

- a relative fitness value is meaningful only under clearly specified conditions and pertains exclusively to those conditions.
- the equivalence theorem is valid also for situations of competition.
- it seems that the genetics of fitness is more complex than just single gene inheritance.

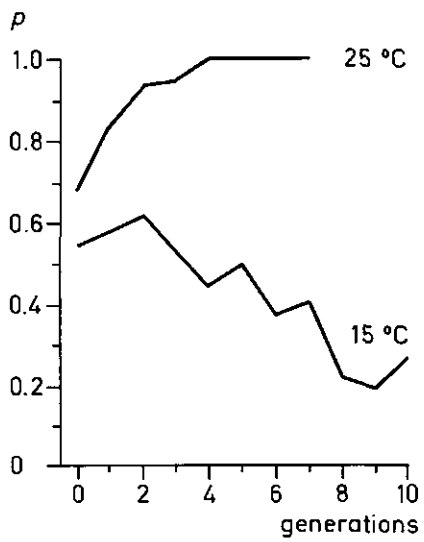

Figure 6. Competition between isolates of black stem rust (Puccinia graminis) on seedlings of wheat cv. Little Club. At generation 0 a 1 : 1 mixture of spores of one isolate of Race 15 B-1 and one isolate of Race 56 was applied to Little Club. The spores produced were collected, one part applied to fresh plants of Little Club (next generation), and one part used for frequency determination. The 0 generation yields approximately equal amounts of both races. At high temperature, the isolate of Race 56 outyields Race 15 B-1; at low temperature, the reverse is true. (Source: after Katsuya & Green, 1967; Zadoks & Schein, 1979). Horizontal axis: t, time in uredial generations. Vertical axis: p, relative frequency (or proportion) of Race 56 isolates.

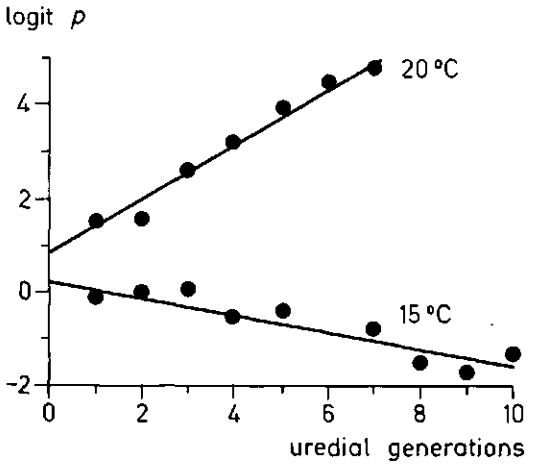

Figure 7. Competition between isolates of black stem rust (Puccinia graminis) on seedlings of wheat cv. Little Club. Data as used in Figure 6 are presented after logit transformation; the line for 20 °C is nearly the same as the line for 25 °C. (Source: Zadoks & Schein, 1979). Horizontal axis: t, time in uredial generations. Vertical axis: p, proportion of Race 56 isolates.

Recent studies on barley mildew (*Erysiphe graminis*) have shown that mutation for virulence is a prerequisite for establishment of the mildew on a new and vertically resistant cultivar, but that it is not enough (Wolfe & Barratt, 1979). Other genetic adaptations are needed for successful establishment. The same may be true for fungicide-resistant mutant strains.

Carrying capacity versus intrinsic growth rate

A desk study shows what can happen in the field. Imagine a disease of which the severity, x, can be expressed as the fraction of foliage infected. Assume that the initial inoculum x_0 of the wild strain is x_0 (wild) = 0.001 and that x_0 of the mutant strain is 10 000 times smaller. Assume that the logistic growth rate r(wild) = 0.20 and that r(mutant) = 0.15. In Figure 8A the heavy line represents the wild and the thin line the mutant strain. The vertical axis has a decimal logarithmic scale; leveling-off of curves represents crowding. At t = 0, the difference between the two strains is 4 $\log_{10}$ units, i.e. a 10 000-fold difference. At t = 20, it has become 5 $\log_{10}$ units, i.e. a 100 000-fold difference.

Without treatment the mutant strain is a 'loser'. With treatment, the mutant strain follows curve *c* (little crowding). The wild type follows curve *a* if the fungicide reduces the number of sites available to the fungus, or curve *b* if the fungicide reduces the logistic growth rate r by a factor of 2. The experiment is designed so that in both cases the dif-

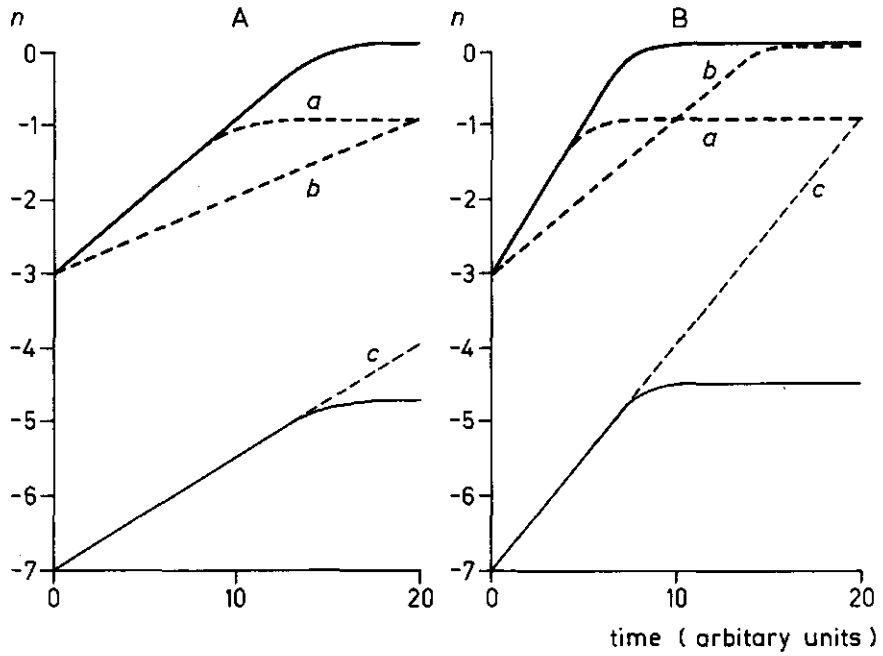

Figure 8. A desk study with a mixture of two fungal strains: a wild fungicide-sensitive strain (heavy lines) and a mutant fungicide-resistant strain (thin lines). Untreated controls (solid lines); fungicide application (broken lines). Note how the mutant strain increases in frequency relative to the wild type after fungicide application. In Figure 8B, the apparent infection rates are two times higher than those in Figure 8A, to represent severe epidemic conditions. For Curves a, the fungicidal effect is a reduction of the carrying capacity K; for Curves b, the fungicidal effect is a reduction of the infection rate r. Note how Curves c, representing the mutant strain when fungicides have been applied, approach Curves a and b, slower in Figure 8A and faster in Figure 8B. (Source: Zadoks & Schein, 1979). Horizontal axis: t, time in arbitrary units. Vertical axis: n, disease severity on log scale, to be read as $x = 10^n$.

ference between the two strains at $t = 20$ is 3 $\log_{10}$ units, i.e. a thousand-fold difference. In other words, the mutant strain wins.

Figure 8B shows what happens when the logistic growth rate of the wild and the mutant strains are doubled, the other things remaining unchanged. The replacement and competition process goes much faster, and the epidemiologic mode of action of the fungicide makes a difference. If the fungicide reduces the logistic growth rate by 2 (Curve *b*), the difference between the strains at $t = 20$ is one $\log_{10}$ unit, a 10-fold difference, but when the fungicide reduces the number of available sites by 10, the two strains end up on a par with each other.

This desk study illustrates a rule of population genetics: 'In an uncrowded environment the success of a population is determined mainly by its intrinsic rate of increase *r*; in a crowded environment the carrying capacity *K* may be more important' (Crow & Kimura, 1970). At what level of *x* does crowding begin? We assume this level to be $x = 0.05$ ($\ln x = 1.30$). When the fungicide is applied, crowding is not so relevant in the situa-

tion depicted by Curve *b*; relative growth rates determine the outcome of the process. For the situation depicted by *a* the carrying capacity for the mutant strain is $K_m = x_{max} = 1$, but the carrying capacity of the wild strain is $K_w = 0.1$, and the mutant rapidly gains an advantage over the wild strain.

Practical findings may corroborate the foregoing reasoning. In areas with regular epidemics of *Cercospora beticola* on sugar-beet, benomyl-resistant mutant strains are more prominent (Dovas et al., 1976). Resistance in *Venturia inaequalis* to dodine on apples appeared after a particularly wet season following years of good control (Szkolnik & Gilpatrick, 1969). The common denominator in both cases is an increase, locally or temporarily, of the logistic growth of the epidemic, *r*. The reasoning can be extrapolated to a higher level of generality: resistance to fungicides is to be expected especially after seasons conducive to severe epidemics i.e. epidemics with relatively high *r* values), and in localities specifically conducive to a particular disease.

Simple models for strategies of pesticide use have already been developed. Much depends on the nature of inheritance of resistance. Is there a gene dosage effect? Is there a regular sexual reproduction? What are the dominance relations of genes for tolerance? Two general conclusions seem to emerge:

- The use of a mixture of fungicides with different mechanisms of action, each applied at reduced dosages, is not recommended (Conway & Comins, 1979).
- Regular alternation of fungicides with different modes of actions applied at normal dosages has little advantage above the sequential use of these fungicides at normal dosages (i.e. using the first until it has become ineffective due to tolerance in the fungus, then use the second, etc., etc.) (B.R. Trenbath, personal communication, 1979).

However, for the time being conclusions based on models must be handled with caution, because these models are still primitive, incomplete and poorly corroborated by experimental evidence.

Conclusion

The title of this contribution asks 'Can we use models describing the population dynamics of fungicide-resistant strains?'. The answer is, definitely, yes. But this yes is pronounced under the following restrictions:

- The models must be time dependant (the duration of the persistance of the chemical and of the latent period *p* must be taken into account (see p.139-148)).
- The models must combine elements from population dynamics and population genetics.
- The models must contain stochastic elements.

- The models must be constructed specifically for the crop, pathogen and chemicals concerned.
- Such specific models must be validated by field and laboratory observations.

Such model studies could be part of the universities' contribution to the cooperative efforts toward a more sophisticated use of fungicides, in accordance with the plea by Schwinn elsewhere in this book (p.22-23).Many useful experiments can be performed with simple techniques that do not require sophisticated and expensive equipment, although they do require a considerable amount of time and labour. Such experiments will be useful for developing, first, the theory and, second, the strategy of control, with as the final objective finding a way of living with resistance to fungicides.

Acknowledgements

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Fungicide resistance and microbial balance

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Abstract

Plant pathogens are a minority within the microflora of the plant environment. In normal situations, a well-balanced ecosystem exists through numerous interactions between its components. Fungi are a major component, so the use of fungicides can affect the microbial balance. The responses of pathogens to a shift in this balance are discussed. Stimulation of the microbial antagonism to a pathogen leads to integrated or indirect control; inhibition of it can result in a change of the dominant pathogen or cause a 'boomerang' effect. Special attention is paid to responses of pathogens that are resistant to fungicides and to the role of resistant antagonists. When indirect effects are involved, closely related pathogens often differ in their responses to a fungicide. The same is also true for the same pathogen in different soils. It is suggested that these differences are caused by the specificity of microbial interactions.

Keywords: synergistic interactions, antagonistic interactions, competition for nutrients, amensalism, antibiosis, mycoparasitism, integrated control, change of dominant pathogen, boomerang effect, iatrogenic disease, benzimidazoles,chlorothalonil, dicarboximides, quintozene, *Botrytis, Fusarium, Microdochium, Penicillium, Pythium, Phytophthora, Rhizoctonia, Sclerotium, Trichoderma*.

Introduction

In the vast microbial world on the plant and in the soil, the plant pathogens are very much in the minority. Compared to their cohabitants of leaves and roots, most pathogens are weak competitors for the nutrients available. However, they escape from this competition by infecting host plants; the living tissue and its direct surroundings is their ecological niche. Nevertheless, during certain stages of their life cycle, the patho-

gens are susceptible to interactions with the saprophytic microflora. These stages include spore germination, infection of the host and, especially, survival in the absence of the crop.

The various components of the microflora in the plant environment are kept in a state of dynamic equilibrium through numerous interactions. As fungi constitute a major component, the use of fungicides may affect the microbial balance. Not only the populations of sensitive species will be affected, but also those of resistant micro-organisms interacting with the sensitive ones. In the natural environment, suppression of sensitive species will soon result in an increase of resistant species competing for the same substrate. Besides competition for nutrients, other mechanisms underlying the microbial balance, like production of antibiotics and mycoparasitism, may also be affected by fungicides.

In recent reviews on effects of fungicides on non-target organisms, Hislop (1976) discussed the effects on the microflora of aerial plant surfaces, Wainwright (1977) and Bollen (1979) those on the soil microflora and Papavizas & Lewis (1979) those on soil-borne pathogens. This contribution deals with responses of pathogens to fungicides brought about by the effects of those fungicides on the interactions between pathogens and microflora. Special attention is paid to the role of those fungi, that are naturally resistant to fungicides or have become resistant under selection pressure by a fungicide. The extent to which these fungi benefit from their resistance in competition with the microflora is discussed.

Types of microbial interactions

Interactions between species or populations may be synergistic or antagonistic (Table 1); it is hard to conceive a neutral relation between organisms present in one microhabitat. The effects of these interactions on the populations involved may be positive or negative (Table 2). In synergism, growth or reproduction of one or both of the associates is stimulated by the other. The term probiosis was coined by Sussman (1965) to describe an organism's production of stimulatory substances inducing spore germination and other specific processes in other organisms. The greater the dependence of one organism on the other, the greater the effect when one of the associates is suppressed by a fungicide. Synergistic interactions that are involved in the etiology of plant diseases are known for associations of various organisms, especially between nematodes and fungi. When a fungus predisposes a plant to infection by an associated pathogen, application of a fungicide may decrease disease incidence, even when the pathogen itself is resistant to the fungicide.

With effects of pesticides on antagonistic interactions it is often difficult to indicate the mechanism involved. It is especially difficult

Table 1. Microbial interactions.

Synergistic relations	Antagonistic relations
Provision of nutrients and growth factors by cross-feeding or commensalism	Competition for nutrients, oxygen and possibly space
Production of probiotic substances	Amensalism by production of antibiotics and simple metabolites, e.g. NH_3, nitrite, C_2H_2
Inactivation of substances including pesticides toxic to the associates	Parasitism and predation
Additionally for plant pathogens	
Predisposition of the host plant to infection by associated pathogens	Protection of the host plant from infection

to distinguish between effects on nutrient competition and those on amensalism. In amensalism, one species occupies the substrate by its ability to produce substances that are inhibitory to other organisms. In experiments where the occupation of the substrate is used as a parameter, it is impossible to indicate whether this has been achieved by reduced competition for nutrients or by keep other organisms from the substrate. There-

Table 2. Effects of microbial interactions on the populations involved.

Interaction	Population A	Population B
Positive		
mutualism, e.g. cross-feeding	+	+
commensalism	+	0
probiosis	+	0
detoxification	+	0
predisposition of a host plant by B to infection by A	+	0
mutual	+	+
Negative		
competition	-	-
amensalism	-	0
parasitism	-	+
predation	-	+
protection of a host plant by B from infection by A	-	0

Source: modified after Odum (1971).

0 = no effect; + = positive effect; - = negative effect.

fore preference should be given to the broad concept of competition of Clark (1965), in which the role of antibiotics in the occupation of the substrate and nutrient competition are included.

Reduced competition for the substrate is probably the main cause for an increase of tolerant pathogens after the use of selective fungistatic compounds. Examples are known for conventional and systemic fungicides: the increase of *Fusarium* and *Pythium* in quintozene-treated soils (Farley & Lockwood, 1969; Katan & Lockwood, 1970), and the increase of two cereal pathogens, *Cochliobolus sativus* (Fokkema et al., 1975) and *Rhizoctonia cerealis* (Reinecke & Fehrmann, 1979; van der Hoeven & Bollen, 1980) in benomyl-treated crops, respectively.

Fungicides of different categories, e.g. benzimidazoles and dicarboximides may affect the synthesis of antibiotics by fungi. Stimulation as well as suppression of the process have been reported (Bollen, 1979). The effect is highly specific and inhibition of growth by a fungicide does not imply that synthesis of antibiotics is also suppressed.

Under natural conditions pathogenic fungi are often parasitized by other fungi, for example parasitism on sclerotia of pathogens in soil and on mycelium and spores in pustules of rusts on leaves. In fungicide-treated fields, this parasitism will be influenced when the fungus host and its parasite differ in their sensitivity to the fungicides used. If the mycoparasite is less sensitive than the host fungus, application of a fungicide may result in a form of integrated control, e.g. the colonization of rhizomorphs of *Armillaria mellea* by *Trichoderma* spp. in soil treated with CS_2 and methylbromide (Bliss, 1951; Ohr et al., 1973). Recently, Davet (1979) suggested that the effective control of *Sclerotinia minor* in vegetables by dicarboximides may be due to the sensitivity of the pathogen as well as the resistance of its mycoparasites to these fungicides. In the reverse case - sensitive parasites and tolerant host fungi - aggravation of the disease may be expected. In spite of the occurrence of several pathosystems of a resistant host fungus and a sensitive mycoparasite, data on increase of disease incidence following treatment with fungicides are scarce. The high level of white-mold damage caused by *Sclerotium rolfsii* in peanuts sprayed with benomyl has been partly attributed to inhibition of *Trichoderma viride* (Backman et al., 1975). *Trichoderma* species are effective parasites of *S. rolfsii*.

Resistant pathogens and resistant antagonists

Two types of fungicide resistance can be distinguished. When the entire population of a species or a group at a higher taxonomic level is resistant to a fungicide this is called 'natural resistance'. Examples are the resistance of fusaria to quintozene, *Alternaria* spp. to benzimidazoles, and oomycetes to dicarboximides. This should be distinguished

from the other type of resistance, which under selection pressure by a fungicide develops in an originally sensitive population of a sensitive species; many examples of this kind of resistance are presented elsewhere in this book. In both types resistant pathogens will respond in the same way to a shift in the microbial antagonism caused by a fungicide.

Emergence of resistance to fungicides is not restricted to pathogens. Saprophytes, including antagonists of pathogens, may also become resistant. This has been observed for *Penicillium cyclopium* active as an antagonist of mercury-resistant strains of *Pyrenophora avenae* on oat seeds treated with an organo-mercury fungicide (Ward & Greenaway, 1976). Another example is the appearance of resistant strains of *Penicillium brevicompactum* in cyclamen crops treated with benzimidazoles. In a greenhouse where the crop was infested with a resistant strain of *Botrytis cinerea*, petioles and leaves of some benomyl-treated plants developed a green bloom because of abundant sporulation of *P. brevicompactum*. These plants seemed to be less affected by *Botrytis* rot than those without *Penicillium* spores. Antibiosis in vitro by this fungus to *B. cinerea* could be clearly demonstrated: in dual cultures on malt-agar, distinct inhibition zones were observed around the colonies of *P. brevicompactum*. In a greenhouse experiment, the effect of the antagonist on the development of the disease in a benomyl-treated crop was investigated. Although the non-inoculated plants did not remain free of *Botrytis* rot, probably because the plants were kept under extremely humid conditions, it was shown that inoculation with the resistant strain of *P. brevicompactum* partly restored the antagonism to the pathogen. The effect lasted one month (Figure 1).

In a study of the effect of benomyl on the fungus flora of the culm base of rye, *Microdochium bolleyi* (syn. *Aureobasidium bolleyi)* was among the species that first disappeared after the treatment of the crop with the fungicide (Platenkamp & Bollen, 1973; Reinecke, 1977). Therefore, *M. bolleyi* was used as an 'indicator species'. This role was easily explained later, when van Tuyl (1977) reported that the sensitivity of mycelial growth of *M. bolleyi* was the highest recorded for all fungi tested in his study. Nevertheless, in the 1975 crop the fungus was frequently isolated from rye plants in benomyl-treated plots of experimental fields at Wageningen. The fungus had lost its value as an indicator species, because part of the population had become less sensitive, which was confirmed in tests in vitro.

It is well-known that for pathogens the emergence of resistance occurs especially in certain species, e.g. *Botrytis cinerea*, whereas the populations of other species still remain sensitive despite intensive use of fungicides for long periods. The same seems to be true for the antagonistic mycoflora. Among the numerous isolates of *Trichoderma* spp. obtained from roots and bulbs from benomyl-treated crops, the author never found a resistant strain nor could any record of one be found in the literature.

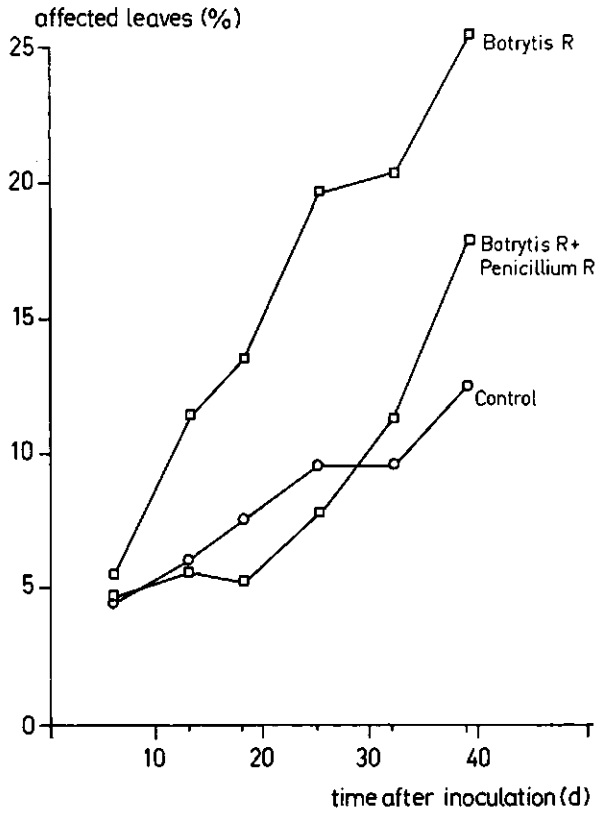

Figure 1. Antagonism between benomyl-resistant fungi on cyclamen sprayed with a suspension of benomyl (1 g/l). Three lots of 30 densely foliated plants were sprayed with benomyl. One day later they were sprayed with water (control), a spore suspension of a resistant strain of Botrytis cinerea, and a spore suspension of the same strain plus one of a resistant strain of Penicillium brevicompactum. The plants were kept at 100% relative humidity. For 32 days after inoculation the mean number of affected leaves per plant for plants inoculated with both resistant strains was significantly less ($P < 0.05$, Fisher test) than that for plants inoculated with B. cinerea alone.

However, resistant isolates of *Penicillium brevicompactum* were obtained frequently from the benomyl-treated crops.

Resistant antagonists may contribute to disease control if their population increases following a fungicide treatment. Then the ultimate disease control is the result of both direct inhibition of the pathogen and stimulation of its antagonists. Recently, successful attempts have been made to induce resistance in effective antagonists of pathogens. Papavizas (1980) obtained strains of *Trichoderma harzianum* that were resistant to captafol, chlorothalonil, iprodione and other fungicides. A number of these strains showed an increased ability for biocontrol of white rot of onion caused by *Sclerotium cepivorum*.

Responses of resistant pathogens to fungicides due to a shift in the microbial balance

The side-effects of fungicides on the population of synergists or antagonists of pathogens may be positive or negative for disease control. Selective stimulation of antagonism leads to integrated or indirect control; selective inhibition of antagonism can result in a change of the dominant pathogens or cause 'boomerang' effects (Figure 2). Diseases that have increased as a result of these phenomena belong to the iatrogenic diseases, which were defined by Horsfall (1979) as those 'that are induced or worsened by a plant pathologist's prescription for the crop'. Of the types of iatrogenic diseases distinguished by Griffiths & Berrie (1978), the examples mentioned in this chapter belong to the category in which the disease is induced through action of agrochemicals on the ecosystem. In view of the important role of fungi in microbial ecosystems, one would expect more records of the effects of selective fungicides on such ecosystems than there are.

Under normal circumstances, farmers will not use a pesticide when they know that the pathogen infesting their crop is resistant to it. Application of fungicides to crops infested with resistant pathogens occurs in two situations. First, when resistant strains are present among the population of a sensitive pathogen. Second, when the causal organism has been wrongly diagnosed, as happens occasionally for diseases that have similar symptoms but are caused by different pathogens. For example, the symptom

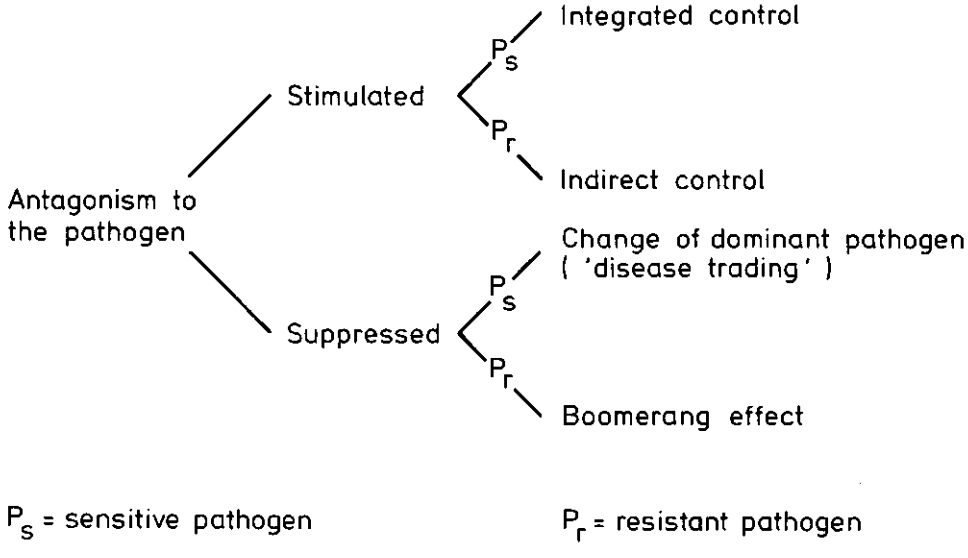

Figure 2. Phenomena observed in plant-disease control caused by differential sensitivity to fungicides of pathogens and their antagonists.

damping-off can be caused by a variety of pathogens, viz. *Pythium* and *Phytophthora* species, *Rhizoctonia solani*, *Botrytis cinerea* and *Fusarium* species.

The effects mentioned in Figure 2 need not be brought about only by changes in the microflora: fungicide applications may also influence the plant resistance mechanism or alter the micro-environment in the crop through an effect on plant canopy.

Integrated control

Disease control brought about by direct inhibition of the pathogen together with an enhanced microbial antagonism to the pathogen is a form of integrated control. A prerequisite for such control is that either the population of antagonists is less affected by the fungicide than the pathogen or that the antagonists are more successful than the pathogen in their colonization of plant surfaces or soil after treatment of the crop. Since the microflora plays an essential role in this form of integrated control, the ultimate result of fungicide application is highly dependent on the biotic environment. A well-known example is the effect of carbon disulphide and methyl bromide on the viability of rhizomorphs of *Armillaria mellea* (Bliss, 1951). When infected roots are treated with sublethal doses of the fumigants and buried in natural soil the pathogen dies, but when buried in sterile soil the pathogen can survive: the rhizomorphs are colonized by *Trichoderma* in natural soil, but not in sterile soils.Ohr & Munnecke (1974) showed that the treatment with methyl bromide inhibits the antibiotic production of the rhizomorphs so that they cannot ward off their antagonists, among which *Trichoderma* ranks first. In this context, Baker & Cook (1974) suggested an approach to chemical control in the sense of 'rather than kill the pathogen, it may only be necessary to weaken it and make it more vulnerable to antagonism of the associated microflora'.

An increase of microbial antagonism as a positive side-effect has been observed for fungicides of various categories, e.g. organo-mercury fungicides, dithiocarbamates, benzimidazoles, quintozene and, according to Langerak (1977), also the antibiotic fungicide pimaricin. It is not surprising that most of the examples concern soil-borne pathogens, since the role of the biotic environment is thought to be more important for pathogens in the soil than on the aerial parts of plants.

In integrated control a number of mechanisms may operate simultaneously. This has been shown for the effect of organo-mercury fungicides on common root rot of wheat (Chinn, 1971), caused by *Cochliobolus sativus*, and fusarium diseases of bulbs and roots of daffodils (Langerak, 1977). The ultimate disease control was achieved by four distinct effects: a direct effect of the fungicide on the pathogen; stimulation of production

of fungistatic substances by *Penicillium* spp.; a significant increase in the populations of antagonists (*Penicillium* spp. and *Trichoderma* spp.); and a 'cross protection' mechanism. Cross-protection was assumed since the subcrown internodes of wheat seedlings were more densily colonized by *Penicillium* spp. in treated plots. In daffodils, *Trichoderma viride* and *P. janthinellum* became more established on roots of bulbs treated with 2-methoxyethylmercury chloride ('Aretan'), thiram or a formulated product of pimaricin than on roots of untreated bulbs.

In most of the instances where the microflora has contributed to effective disease control by a fungicide, that supplementary role was recognized only afterwards. Occasionally, integrated control by fungicides and antagonists has been used intentionally. Carter & Price (1974) reported on the intentional use of integrated control for *Eutypa armeniacae*, an air-borne, vascular pathogen of apricot. Upon invading wounds after pruning, the pathogen is antagonized by *Fusarium lateritium*. Benomyl effectively controlled the disease. *F. lateritium* was less sensitive to the fungicide than the pathogen. When a conidial suspension of the antagonist was added to the fungicide it colonized the wounded tissue, resulting in a control supplementary to that by the fungicide. An example of synergistic action between an antagonist and a fungicide in controlling soil-borne pathogens has been reported by Chet et al. (1979). The control of *Sclerotium rolfsii* obtained by supplying a *Trichoderma* preparation to the soil was significantly improved when quintozene at sub-inhibitory doses was applied together with the preparation.

Indirect control

Examples of effective disease control due to effects on microbial interactions only are scarce. The most important examples of indirect control of fungal pathogens in the field are side-effects of nematicides on root-infecting fungi. This concerns the so-called complex diseases, where nematodes predispose roots to infection by *Fusarium* and other pathogens.

An interesting laboratory example of disease control of a tolerant pathogen is that of *Pythium debaryanum* on cucumber seedlings treated with 6-azauracil. Stankova & Dekker (1969) observed that treatment of seeds with this experimental fungicide at a low dose rate resulted in a significant increase of the number of bacteria in the rhizosphere. It was suggested that the enhanced bacterial population protected the roots from infection by *P. debaryanum*.

In field trials, reduced incidence of resistant pathogens in fungicide-treated crops is occasionally observed. Joyner & Couch (1976) reported reduced severity of *Pythium* blight and red leaf spot caused by *Helminthosporium erythrospilum* in *Agrostis palustris*, one of the grasses in turf treated with benzimidazole fungicides. No examples of disease control have

been found where the effects of fungicides on microbial antagonism were high enough for practical use in the field.

Changes of the dominant pathogen

Suppression of a target pathogen by control measures may be followed by the increase of a pathogen that was initially of minor importance. This phenomenon was first recognized in soil treatments. It was termed 'disease trading' by Kreutzer (1965), who defined it as 'a situation in which the dominant pathogen is controlled by soil treatment, and a minor pathogen is elevated to major importance, thus becoming the new pathogen'. Most examples are associated with the use of selective fungicides and concern an exchange of sensitive pathogens for pathogens tolerant to the fungicides. Among non-systemics, this side-effect is known for 'Dexon' and quintozene. Suppression of *Pythium* by 'Dexon' may be followed by an increase in *Rhizoctonia*. *Rhizoctonia*, in turn, may be exchanged for *Pythium* and *Fusarium* in soil treated with quintozene. A recent example is the shift in the pathogens causing fruit rot in strawberry following treatment with dicarboximide fungicides. An increase in yield obtained by the suppression of *Botrytis cinerea* was partly lost due to the incidence of *Phytophthora cactorum* (Hunter et al., 1979) or *Mucor* and *Rhizopus* spp. (Figure 3).

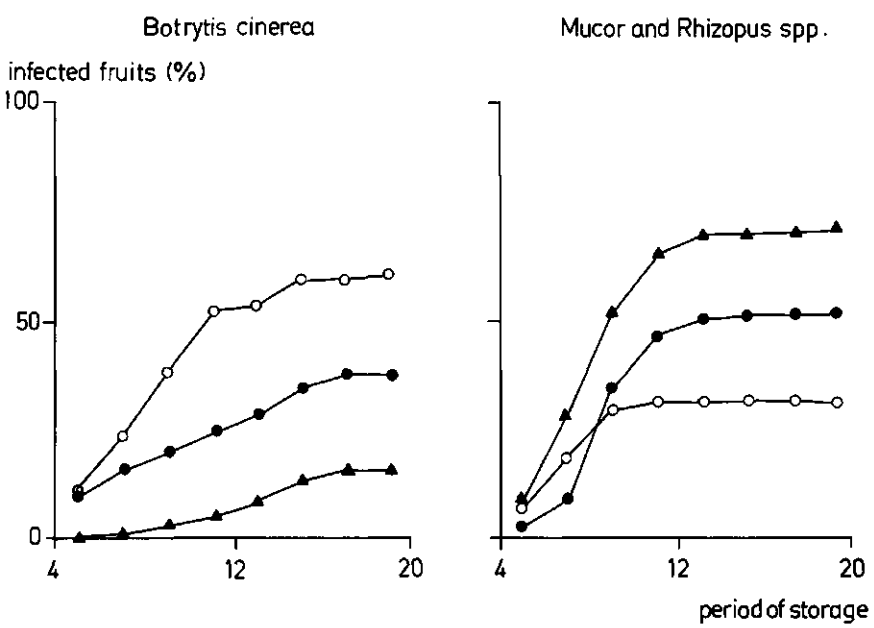

Figure 3. Effect of dicarboximides on incidence of spoilage fungi on stored strawberries: unsprayed (○), iprodione (●), vinclozolin (▲). The crop from which the fruits came was sprayed during the flowering period. The fruits were stored at 5 °C and relative humidities of 95-97%. Source: Davis & Dennis (1979).

Since the use of systemic fungicides with a selective antifungal spectrum became common, farmers and plant pathologists have been confronted with new examples. Most reports on the appearance of non-target pathogens in fungicide-treated crops concern the incidence of pythiaceous fungi and other benzimidazole-tolerant species after treatment with benzimidazole fungicides. Nevertheless, given the frequent use of the fungicides, the number of cases of an increased incidence of resistant pathogens is relatively low. Only few of these fungi seem to benefit by their resistance.

Appearance of non-target pathogens in a fungicide-treated crop cannot be attributed merely to suppression of microbial antagonism by the fungicide. Another cause may be a decrease of host resistance. Swinburne (1975) found that benomyl reduced enzyme activities in barley seedlings. He pointed out that this might result in inhibition of resistance mechanisms. Still another mechanism operates through a change of the microclimate in the crop, for instance, brought about by a denser canopy because of control of foliar pathogens. Backman et al. (1975) observed that the control of peanut leaf spot caused by *Cercospora arachidicola* and *Cercosporidium personatum* was associated with an enhanced damage caused by *Sclerotium rolfsii*. Control of leaf spot by spraying benzimidazole fungicides and chlorothalonil promoted a dense foliage. In the sub-canopy environment, a humid atmosphere is conducive to infection by the white mold fungus. Backman et al. pointed out that, in addition to this side-effect, the fungicide suppressed the population of *Trichoderma viride*, a natural antagonist of *S. rolfsii*. The effect was more pronounced in benomyl- than in chlorothalonil-treated plots, probably because *S. rolfsii* is highly resistant to benzimidazoles. Porter (1980) presumed change in microclimate also to account for the increase of severity of blight caused by *Sclerotinia minor* in peanuts treated with captafol and chlorothalonil. However, unlike *S. rolfsii*, this pathogen was controlled by a high rate of benomyl, which is to be expected, since *S. minor* is very sensitive in vitro to the fungicide. In *Sclerotinia*-infested fields, peanut yield was significantly lower in captafol and chlorothalonil-treated plots than in untreated and benomyl-treated plots. From the data given by Porter it can be estimated that the loss of yield and value due to this change of the dominant pathogen was 13-16 %.

In a few instances there is substantial evidence that a decreased microbial antagonism was a cause of enhanced incidence of pathogens. An example for aerial pathogens was well-documented by Fokkema et al. (1975). They showed that benomyl sprayings reduced the microflora of rye leaves, resulting in an increased susceptibility to *C. sativus*, a pathogen tolerant to the fungicide. The effect was only observed if the leaves were densely populated with saprophytes. Another example is decreased antagonism to *R. cerealis*, which causes sharp eyespot, in cereals treated with benzimidazoles. Spraying with these fungicides have resulted in an exchan-

ge of eyespot by *Pseudocercosporella herpotrichoides* for sharp eyespot or, when a high dose was used, *Fusarium* foot rot for sharp eyespot. The effect was attributed to differential sensitivity of the pathogens competing for host tissue on the culm base (Reinecke & Fehrman, 1979) and suppression of antagonism to *R. cerealis* by the soil microflora (van der Hoeven & Bollen, 1980).

Reduced antagonism due to fungicides can also lead to an increase in competitive saprophytism of pathogens in the soil, as has been shown for *Pythium* and *Fusarium* in quintozene-amended soil (Katan & Lockwood, 1970) and *Phytophthora cryptogea* in benomyl-amended soil (Bollen, 1979).

Boomerang effect

This effect is defined as an accentuation of the disease caused by the target pathogen itself after treatment to control the disease. The term was used originally for the reappearance of soil-borne pathogens in disinfested soil in quantities greater than before the treatment (Kreutzer, 1965).

During the last decade, farmers and plant pathologists have been confronted with a special category of boomerang effects: the accentuation of diseases in fungicide-treated crops caused by resistant strains appearing in the population of the pathogen. If resistance emerges in a treated crop the pathogen population may be either unaffected or increased by inhibition of the natural antagonists. Accentuation of disease caused by resistant strains in benzimidazole-treated crops has been reported for *Penicillium* rot in lily bulbs (Rattink & Beusenberg, 1972), dollar spot in turf grass caused by *Sclerotinia homeocarpa* Warren et al., 1974) and dry bubble of commercial mushroom caused by *Verticillium fungicola* (Figure 4).

Substantial evidence for suppressed microbial antagonism as a cause of the boomerang effect by resistant strains is available for only a limited number of examples. The possibility that resistant strains that are more virulent than a wild strain incidently appear should not be overlooked. In the experiment on the incidence of dry bubble in mushrooms (Figure 4), the yield of plots infested with a resistant strain (RO) was lower than of those infested with a wild strain (SO). These results suggest that the strain was more virulent than the wild strain.

Specificity of indirect effects

Records of indirect effects of pesticides on the microflora, including pathogens, are often inconsistent. Contradictory results are sometimes obtained for different crops or soils, especially when effects of pesticides with high selectivity to the microflora are involved. The specificity of effects is well illustrated by the study of Joyner & Couch (1976), in

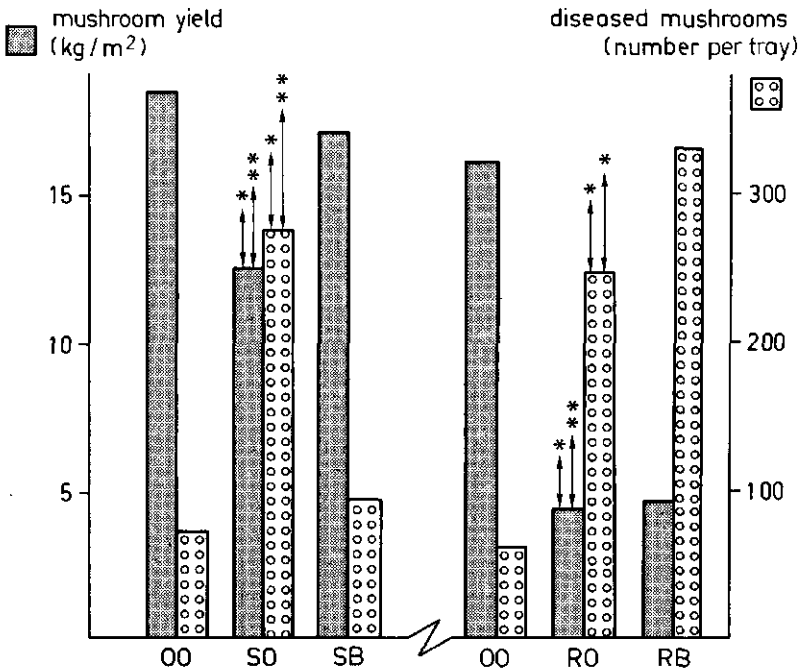

Figure 4. Incidence of dry bubble and mushroom yield of benomyl-treated plots infested with either a sensitive or a resistant isolate of Verticillium fungicola. Treatments: no fungicide and not inoculated (00), benomyl (0.75 g/m^{-2}) (B), inoculated with a sensitive strain (S), innoculated with a resistant strain (R). The yields and numbers of diseased mushrooms of Trial 1 (8 replicates) and Trial 2 (9 replicates) were processed by analysis of variance; * and ** are least significant differences for $P = 0.05$ and $P = 0.01$, respectively. Source: Bollen & van Zaayen (1975).

which the effect of four benzimidazole fungicides on non-target diseases of turf grasses was estimated. *Pythium* blight was reduced in *Agrostis palustris*, but unaffected in *Lolium perenne*. A similar specific response was found for the incidence of leaf-spot disease, which is caused by various *Helminthosporium* species.

In greenhouse experiments where the effects of benomyl on pythiaceous fungi were estimated, the author found that in the same soil *Phytophthora cryptogea* and *Pythium aphanidermatum* growth was enhanced, whereas growth of *Pythium irregulare* was unaffected. In vitro, the pathogens were equally resistant to the fungicide. Differential effects like these can be understood when it is realized that each pathogen has its specific spectrum of antagonists. It depends on the fungicide sensitivity of the dominant species within the population of antagonists whether an effect appears or not.

A similar specificity holds for the response of the antagonism to one and the same pathogen in different soils. The effect of benomyl on antagonism to *R. cerealis* was measured in five field soils cropped with cereals

(van der Hoeven & Bollen, 1980). Two were affected in their antagonistic activity, the other three were not. Again the response to the fungicide depends on the sensitivity of those species that predominate in the antagonist population. Because the microflora differs from soil to soil, the effects of selective inhibitors on microbial antagonism will be specific for the soil. To a certain extent this is also true for above-ground habitats.

Final comments

The application of fungicides to crops or soils can cause a shift in the microbial population in favour of resistant species, which may be pathogens or their antagonists. Therefore the effects may be either undesirable or favourable for disease control. Resistance of antagonists offers a possibility for integrated control by applying a combination of spores of antagonists and low dose rates of fungicides.

Most instances of accentuation of diseases after fungicide treatments involve selective fungicides, both the conventional non-systemics and the systemics. Use of selective toxicants demands a more accurate diagnosis of the causal pathogen than for broad-spectrum pesticides. When in a nursery damping-off of seedlings is caused by *Pythium*, but has erroneously been attributed to *Rhizoctonia*, the use of quintozene or benomyl may aggravate the disease.

During the last few years the number of reports of undesirable side-effects of selective fungicides is declining. This is probably due to a more careful use of the fungicides and, especially, to their use in combination or alternation with other fungicides. Boomerang effects caused by development of resistance may have declined, because the increased use of combinations of fungicides retards the build-up of a resistant population (Dekker, 1977). Changes of dominant pathogen are also less likely to occur than after the use of a single fungicide, since fewer pathogens will escape the broad-spectrum activity of the combinations.

The study of side-effects of pesticides on the microflora, including resistant pathogens, has its own side-effect - a positive one. In some cases, interactions between organisms have been noticed for the first time when one of the interacting populations was suppressed. A meticulous study of the side-effects on resistant pathogens can provide information on the kind of organisms that operate as their synergists or antagonists, on the plant or in the soil. Such studies can provide the tools for integrated control.

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Countermeasures for avoiding fungicide resistance

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Abstract

Attention is paid to factors that may influence the build-up of a resistant pathogen population, such as type of fungicide, type of disease and selection pressure exerted by the fungicide. Measures are discussed that may delay or avoid the build-up of resistance in practice.

Keywords: fungicide resistance, specific-site inhibitor, multi-site inhibitor, fitness, selection pressure, negatively correlated cross-resistance, alternating use, combined use.

Introduction

Unexpected failure of a fungicide to provide adequate disease control may be due to development of resistance in the pathogen to the chemical. This may cause serious crop losses to the farmer and endanger the financial returns for the company that developed invariably at high costs the fungicide. It is therefore important for a grower to know what to do to avoid or delay the development of fungicide resistance in his fields, and for a company that develops new agricultural fungicides to obtain information about the risk of development of resistance in the pathogen, so that it can take countermeasures and advise farmers about proper use of the new product. For such knowledge it is necessary to obtain insight in the factors, that influence the development of fungicide resistance in the field. In particular the type of fungicide, the nature and severity of the disease and the selection pressure exerted by the fungicide are of prime importance.

Type of fungicide

During a century of large-scale use of conventional fungicides, such as compounds based on copper, few problems with fungicide resistance have

been encountered. However since the introduction of systemic fungicides, some 15 years ago, disease-control failure due to fungicide resistance is reported with increasing frequency. It has been explained (Georgopoulos, 1977; Dekker, 1977) that genetic changes in a pathogen resulting in fungicide resistance occur much more readily with fungicides acting primarily at one particular site in the metabolism of the fungal cell than with fungicides that interfere at many sites with metabolic processes. All systemic fungicides appear to be single-site inhibitors, while the majority of conventional fungicides are classified as multi-site inhibitors.

As has been outlined on p.132-136, considerable differences also exist among single-site inhibitors as to the chances of resistance building up in practice. There are indications that, at least with some fungicides, resistance is linked to decreased fitness (Dekker & Gielink, 1979), which will hamper the build-up of a resistant pathogen population. Experience has shown that some types of systemic fungicides do encounter resistance problems more readily than others (Table 1).

Table 1. Risk of failure of disease control in practice due to development of resistance to specific-site fungicides; rough indication only, since risk not only depends on type of fungicide, but also on type of disease and use strategy for fungicide.

Site-specific fungicides	Risk of failure
Antibiotics	
kasugamycin, streptomycin	moderate to high
pimaricin	very low
Acylalanines	
metalaxyl, furalaxyl	high
Benzimidazoles	
benomyl, carbendazim, thiabendazole	high
Carboxamides	
carboxin, oxycarboxin	low to moderate
Dicarboximides	
iprodione, procymidone, vinclozolin	moderate
Hydroxypyrimidines	
ethirimol	low to moderate
dimethirimol	high
Imidazoles	
imazalil	low
Morpholines	
dodemorph, tridemorph, fenpropimorph	very low
Organic phosphorus compounds	
pyrazophos	low
edifenphos, kitazin	moderate
Piperazines	
triforine	very low
Pyrimidines	
fenarimol, nuarimol	low
Thiophanates	
thiophanate-methyl	high
Triazoles	
triadimefon, triadimenol, bitertanol	low

It would be wrong, however, to assume that resistance problems only occur with the use of systemic fungicides, and not with conventional fungicides. A few of these, although not systemic, are considered to be specific-site inhibitors, e.g. various aromatic-hydrocarbon compounds (Georgopoulos & Zaracovitis, 1967), dicarboximides (Pappas et al., 1979) and organic tin compounds (Giannopolitis & Chrysayi-Tokousbalides, 1980; these may encounter resistance problems similar to the systemic fungicides. Moreover, in a few cases resistance has developed to some multi-site inhibitors, e.g. organic mercury (Noble et al., 1966), possibly due to a change in permeability of the fungal membrane or by a detoxification mechanism.

The nature and the severity of the disease

Disease-control failure will only occur when a majority of the pathogen population has become resistant. Whether this happens, and how rapidly, depends op the type of disease, the nature of the pathogen and circumstances that promote or hinder disease development. For instance, development of resistance to benzimidazole fungicides has occurred rapidly with various powdery mildew diseases, but only after a few years of use with apple scab and up to now not at all with *Pseudocercosporella* foot rot in cereals (Horsten & Fehrmann, 1980).

Factors that may play a role are:

- Sporulation and spread of pathogens. It is easy to understand that fungicide resistance may build up more rapidly in a pathogen with abundant sporulation on aerial plant parts than in a pathogen that sporulates scarcely or produces spores that are not readily transported, as may be the case with several soil-borne root or foot diseases.
- Survival of sensitive forms. If some sensitive forms survive, these will, during periods of reduced selection pressure, compete with the resistant forms, so that the build-up of a resistant pathogen population will be counteracted. This may happen with pathogens in plant parts that are not easily reached by fungicides applied as sprays, not even systemic fungicides. Examples are vascular wilt and root pathogens. There may also be survival of pathogens present in plant parts from which the fungicide is rapidly removed by apoplastic transport, e.g. eyespot on wheat culms.
- Infection threshold. With diseases that have a high infection threshold, the appearance of single mutant cells will rarely result in infection, so that the risk of development of fungicide resistance in the population is low.
- Multinucleate cells. The rare presence of genes for resistance in a multi-nucleate fungus may under selection pressure by the fungicide lead to a rapid shift towards resistance in the pathogen population. Build-up of resistance may also occur through heterokaryosis in fungi.

- Severity of epidemic. In several cases fungicides, despite a number of years of satisfactory performance, have failed to control disease in seasons with very severe epidemics.

The absence of fungicide resistance problems in the control of eyespot in cereals by benzimidazoles may, according to Horsten & Fehrmann (1980a, 1980b), be attributed to:
- limited sporulation and slow spread of the pathogen
- escape of sensitive forms on crop debris in the soil or in the culm, from where, after spraying, fungicides disappear by apoplastic transport
- high infection threshold
- low selection pressure, because sprays are applied only once or twice per season.

During three years of fungicide treatment they found only very little increase in the frequency of resistant forms (Table 2). In view of this they expect that the use of benzimidazole fungicides against *Pseudocercosporella herpotrichoides* at current rates will not lead to resistance problems.

Selection pressure by the fungicide.

Strains resistant to a particular fungicide may arise in a number of ways. When the fungicide concerned is used it exerts a selective action upon the pathogen population in favour of the cells that have become resistant. The selection pressure may be high, intermediate or low; and it may be continuous or intermittent. When strains emerge with a high level of resistance, a high selection pressure will strongly favour the build-up of a highly resistant pathogen population by elimination of the sensitive pathogen and forms with low resistance. The opposite, low selection

Table 2. Influence of one carbendazim application per season on the frequency of fungicide-resistant spores of Pseudocercosporella herpotrichoides in winter barley, c.v. Vogelsanger Gold.

Fungicide treatment	Number of isolates	Number of conidia (x 10^4)	Number of resistant conidia	Frequency of resistant conidia
1975				
unsprayed	40	865	11	1 : 78 x 10^7
carbendazim	40	794	10	1 : 79 x 10^7
1976				
unsprayed	34	1 617	23	1 : 70 x 10^7
carbendazim	46	2 346	40	1 : 58 x 10^7
1977				
unsprayed	19	509	20	1 : 25 x 10^7
carbendazim	18	471	39	1 : 12 x 10^7

Source: after Horsten (1979).

pressure, will not kill forms with low resistance, thus saving a larger quantity of resistant forms. If only strains with a low level of resistance are expected to emerge, high selection pressure may be favourable since it may kill all resistant strains. Absence of highly resistant strains may indeed occur when a link exists between resistance and decreased fitness, as has been suggested for pimaricin (Dekker & Gielink, 1979) and triforine (Fuchs & Drandarevski, 1976). However the possibility can not be excluded that under constant selection pressure by the fungicide, an increase in fitness of these resistant strains may take place. Moreover, accumulation of mutations, each of a low level of resistance, may eventually lead to forms with higher resistance.

The persistence or continuity of the selection pressure may also play a role. Interruption of selective pressure may give the sensitive cells a chance to compete with resistant forms, many of which often have a lower pathogenicity. This will tend to counteract the build-up of a resistant pathogen population. In general, high and continuous selection pressure favours most the development of resistance.

Introduction of new chemicals

Before a new fungicide is introduced into practical agriculture, knowledge about the risks of fungicide resistance developing is very desirable if growers are to be given sound recommendations on its use. Information about the potential of pathogens to become resistant to such a fungicide is of prime importance. If a non-obligate parasite is concerned, resistance can be studied in laboratory experiments on artificial media, containing the fungicide, with or without use of ultra-violet (UV) irradiation or mutagenic chemicals, as described on p.129.

It must be realized, however, that classification of a pathogen in the low risk category does not guarantee that resistance problems will never arise. It cannot be excluded that the pathogenicity of resistant strains will increase over the course of years. Resistance problems may then occur only after a long period of practical use, as happened with dodine resistance in *Venturia inaequalis* (Gilpatrick & Blowers, 1974).

It has been suggested that fungicides with indirect action might be less liable to meet resistance. But such a general statement is not possible: it will depend on the mechanism of action of the compound whether the pathogen will be able to break the artificially induced resistance of the plant. Whether this type of resistance can be compared with the naturally occuring 'horizontal' or 'vertical' resistance of the host plant may be relevant here.

It is important that the search for systemic fungicides with low risk for development of fungicide resistance be continued.

Fungicide management

General

If it is possible to choose different types of chemicals for the control of a particular disease, those with the lower risk for development of fungicide resistance should be preferred. This is especially important when the type of disease is conducive to development of fungicide resistance. If for control of such a disease only vulnerable fungicides are available, careful pesticide management will be necessary. Then special attention should be paid to measures that influence selection pressure in such a way that the chance of a build-up of a resistant pathogen population are reduced, without decreasing the impact of the fungicide. The doses applied, the frequency of application, the mode of application, the efficiency of the treatment, the extension of the area treated with one chemical and alternating or combined use of different fungicides are important here. Early detection of the build-up of resistance is also important, so that timely, adequate measures can be taken.

Dose, frequency and method of application

If it is advisable to avoid unduly high selection pressure, the amount of fungicide sprayed on the crop should not exceed the minimum necessary for adequate disease control. This demands careful consideration of the doses applied and the frequency with which they are administered. If the risk of development of fungicide resistance is great, it may be better to restrict application to critical periods in the development of the disease. For example, if a vulnerable fungicide is used for post-harvest disease control, this or related fungicides should not be applied in the field to prevent early build-up of resistance.

The mode of application may also be important. A systemic fungicide applied to the soil or seed allows uninterrupted uptake, which favours continuous selection pressure for resistant pathogens. Moreover, thorough treatment of the crop will provide little opportunity for sensitive pathogens to survive, so that resistant forms will meet little competition.

The extent of the area treated with a particular fungicide may contribute to the selection pressure too. When a large area is treated with a single chemical, or with related compounds, few sensitive forms can enter the crop from outside, which limits competition between sensitive and resistant forms during intervals of low selection pressure. Growth of a crop in a more or less isolated environment, for instance in a greenhouse, may also result in limited competition.

Alternating or combined use of fungicides

To delay or prevent the development of fungicide resistance, the use - alternately or combined - of fungicides with different mechanisms of action has been advocated. With a combination of two specific-site inhibitors the possibility exists that the pathogen will aquire resistance to both compounds. This is much less likely if a combination of a systemic fungicide and a multi-site inhibitor is used. This is now becoming a common practice.

The use of different fungicides in mixtures or in an alternating strategy has been studied by Kable & Jeffery (1980), who developed a mathematical model for such use. The model has three variables: the efficacy of the fungicide to which resistance has developed against the sensitive and the resistant sub-population; the efficacy of the second fungicide, assumed to be equal for the two sub-populations; and the degree of spray coverage, i.e. the proportion of the fungal population not coming into contact with the fungicide. The variable that has the largest effect on the rate of selection appears to be spray coverage. With complete coverage, the use of a mixture does not slow down the build-up of a resistant pathogen population; two fungicides used alternately is then the better strategy. However complete coverage will seldom occur when the crop is sprayed: the use of mixtures appears to be increasingly effective as the coverage decreases. These relations are illustrated by the data presented in Table 3, which were calculated on the basis of assumed model values for the above mentioned parameters. Dekker & Teng (unpublished), using the same parameters as for the model of Kable & Jeffery, calculated that alternating a mixture of the two fungicides with a conventional fungicide may delay the development of resistance even more efficiently (Table 4).

Table 3. The effects of combined or alternating use of two fungicides and spray coverage [1] on build-up of a resistant pathogen population, assuming specific model values for efficacies of the fungicides [2] and initial resistance level [3]; based on data from simulation model by Kable & Jeffery (1980).

Fungicide application	Number of sprays before the populations become 90 % resistant for various values of E					
	0	1	5	10	30	50
S repeated	10	11	13	15	25	42
(S + C) repeated	10	13	20	29	75	157
S alternating with C	20	22	26	30	50	84

[1] E (escape) = proportion of the pathogen population escaping fungicide contact
[2] S = vulnerable fungicide, e.g. systemic fungicide; efficacy against sensitive and resistant sub-populations set at 95 % and 10 %, respectively. C = conventional fungicide; efficacy against both S-sensitive and S-resistant sub-populations set at 80 %
[3] Initial resistance frequency = 10^{-9}

Table 4. The effects of combined or alternating use of two fungicides on build-up of a resistant pathogen population, assuming specific model values for efficacies [1] spray coverage [2] and initial resistance level [3]. Simulation model by J. Dekker & P.S. Teng (unpublished).

Fungicide application	Proportion of the population resistant after various numbers of sprayings				
	5	10	20	30	40
S repeated	0.000	0.826	1.000	1.000	1.000
(S + C) repeated	0.000	0.000	0.996	1.000	1.000
S alternating with C	0.000	0.000	0.826	1.000	1.000
(S + C) alternating with C	0.000	0.000	0.000	0.261	0.996

[1] E (escape) = proportion of the pathogen population escaping fungicide contact = 5%
[2] S = vulnerable fungicide, e.g. systemic fungicide; efficacy against sensitive and resistant sub-populations set at 95 % and 10 %, respectively. C = conventional fungicide; efficacy against both S-sensitive and S-resistant sub-populations set at 80 %
[3] Initial resistance frequency = 10^{-9}

Kable & Jeffery state that their model is a simple one and that various parameters that may play a role in practice have not been included, for example, the reproduction of the target organism. They also state that reproduction is not incompatible with the model provided the resistant and sensitive sub-populations reproduce at equal rates. However it appears that the fungicide resistant sub-population may in the absence of the fungicide concerned have a lower reproduction rate, which can partly be ascribed to the mechanism of resistance (Dekker & Gielink, 1979; Fuchs & Drandarevski, 1976). In such a case, sequential fungicide use will favour directional selection towards the sensitive form when the application of the vulnerable fungicide is interrupted, thus allowing recovery of the sensitive pathogen. Introduction of parameters for reproduction during the selection pressure by the fungicide and recovery during absence of selection pressure may give a model that simulates reality more closely (J. Dekker & P.S. Teng, unpublished).

Negatively correlated cross-resistance

The build-up of fungicide-resistant populations might be prevented by using a mixture of fungicides with negatively correlated cross-resistance. This means that resistance to fungicide A is linked to sensitivity to fungidice B, and vice versa. Studies of negatively correlated cross-resistance have been made for phosphoramidate and phosphorothiolate fungicides, and for isoprothiolane and phosphoramidate (Uesugi, 1978), but no such combinations are yet available for practical use.

Conclusions

Among the fungicides considered to be specific site inhibitors, the probability that resistance will develop varies greatly. Therefore it is important to continue the search for new fungicides to which resistance will not develop readily, e.g. compounds where resistance is linked to decreased fitness, or a combination of compounds with negatively correlated cross-resistance. When vulnerable fungicides are used, strategies to cope with resistance problems, should they arise, become necessary. Reviewing the problems of resistance in insects, Brown (1976) concluded that 'the best policy is to continue using the insecticide recommended for the job until the control effect becomes inadequate, and then replace it with the most insecticidal substitute'. This strategy is very costly, especially in cases of a very rapid build-up of resistance, as has occurred with some fungicides. Moreover, it seems to be possible to prolong the useful life of fungicides by strategies of fungicide treatment that delay or avoid the development of resistance. The most important aspect of such strategies is to avoid a continuous high selection pressure, by restricting application to critical periods, reducing the amounts applied and limiting the area treated with one particular fungicide. Also the type of disease and the method of application should be considered. Development of resistance may be counteracted by the use of a second fungicide, preferably a multi-site inhibitor, either in combination with the vulnerable fungicide or in sequence with it.

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Case study 1: Cercospora beticola of sugar-beets

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Abstract

Fungicide resistance in the sugar-beet leaf spot pathogen, *Cercospora beticola*, is discussed, drawing mainly on experiences in Greece. Resistance to benzimidazoles developed after 2 years of intensive and almost exclusive use. Benzimidazole-resistant strains are highly adaptable and, even in areas where benzimidazoles have not been used for 6-8 years, they represent a very high percentage of the population of the fungus, rendering the fungicides ineffective. The first signs of poor performance of fentin derivatives were reported only after several years of use. Resistance to fentin does not appear to be equally stable under field conditions, but satisfactory disease control is seldom achieved. Some fungicide treatments that give good results against *C. beticola* of sugar-beets that are resistant to both benzimidazoles and fentin are identified.

Keywords: sugar-beets, leaf spot, benzimidazoles, fentin, stability of resistance, selection pressure, maneb, bitertanol, nuarimol, daconil.

Introduction

The leaf spot of sugar-beet, due to *Cercospora beticola*, is known to all of the sugar-beet-growing countries but it is only in areas with warm summers that it becomes a major disease. The pathogen requires high humidity, but not free moisture, for infection. In irrigated fields, humidity is practically always high inside the thick foliage. Sprinkler irrigation is particularly favourable for the development of leaf spot because it provides moisture directly to the foliage and washes off fungicide deposits. Under such circumstances, temperature is the most decisive factor for the devolopment of an epidemic, because the incubation period becomes shorter as the mean daily temperature increases.

Conditions particularly favourable for *Cercospora* leaf-spot epidemics prevail in most of the sugar-beet-growing areas of Greece.

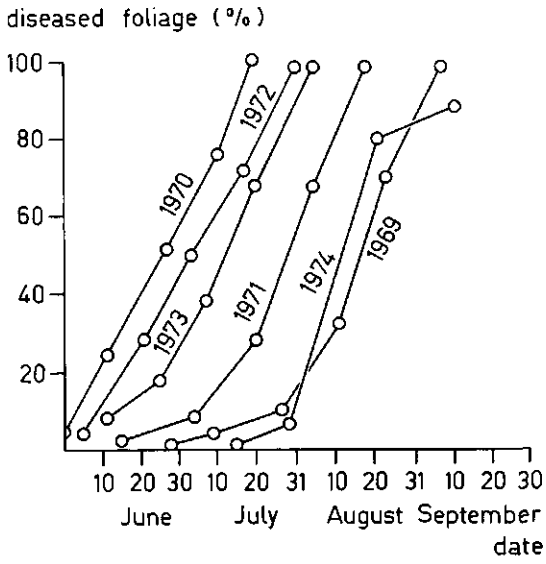

Figure 1. Six years data on the progress of sugar-beet leaf spot during summer in untreated sprinkler-irrigated plots in northern Greece (Source: after Dovas, 1975).

Figure 1, which summarizes data collected by Dovas (1975), shows that the destruction of the foliage of sprinkler-irrigated sugar-beets in Greece is complete between the middle of July and the end of August. The chemical control of leaf spot is therefore indispensable, and many difficulties have been created by the development of fungicide resistance in *C. beticola*.

Under conditions of high disease pressure most protective fungicides fail to give satisfactory disease control. Until recently the most successful of the protectants were the triphenyltin derivatives, namely, triphenyltin hydroxide (fentin hydroxide) and triphentyltin acetate (fentin acetate). These compounds were almost exclusively used against leaf spot in Greece from 1964 to 1970. The Hellenic Sugar Industry, S.A., which carries out most of the sugar-beet disease control for the growers, sprayed practically every field in most areas with organotin fungicides, usually from the end of May until the middle of September, at about 15-day intervals. In most cases the results were good but Hellenic Sugar was always looking for replacements for the organotins, which are rather toxic to mammals and lack strong post-infection activity.

Field testing of the first systemic fungicides that became available for *C. beticola* control began in Greece as early as 1967. The results obtained with benzimidazole derivatives were excellent. Data from trials performed in 1970 show that benomyl was practically twice as effective as fentin acetate in protecting the foliage throughout the growing season (Table 1). On the basis of field tests the use of organotins was gradual-

Table 1. Comparison of benomyl and fentin acetate for the control of sugar-beet leaf spot in Greece during the 1970 growing season.

Fungicide treatment	Spraying interval (days)	Proportion of diseased foliage (%)			
		21 July	2 August	21 August	4 September
Benomyl 300 g/ha	12	1.5	3.6	5.9	13.8
	17	4.4	4.3	8.8	25.5
	22	4.5	4.6	37.5	44.0
Fentin acetate 500 g/ha	12	4.4	4.5	19.3	35.5
	17	5.5	7.2	39.2	57.0
	22	8.5	20.0	83.0	95.0
Control		100.0			

Source: after Dovas (1975).

ly reduced, and in 1972 more than 3000 ha were sprayed exclusively with benomyl.

Resistance to the benzimidazoles

The results of the commercial applications of benomyl against sugar-beet leaf spot in Greece were excellent in the years 1970 and 1971. In the summer of 1972 no signs of inadequate control were observed until the middle of July. Suddenly, the disease incidence increased very rapidly near the end of the month, and within 20 days the proportion of spotted foliage increased from 5-10 % to 80-100 %, in spite of regular benomyl sprayings. The development of the disease was not affected even by shortened spraying intervals or increased benomyl doses. At first conditions unusually favourable for the disease were thought to be responsible for the control failures. However, in demonstration plots the performance of the organotins continued to be satisfactory during the summer of 1972. The data in Table 2, collected only two years after those of Table 1, from the same area, show the distinct superiority of fentin hydroxide over benomyl and thiophanate methyl after the end of July 1972.

The possibility of resistance to the benzimidazoles in *C. beticola* was considered only towards the end of the 1972 growing season; specimens were then brought to the laboratory. Resistance to this group of fungicides is very easy to detect, so it took only a few days to recognise that most conidial isolates obtained from fields where benomyl did not control leaf spot were highly resistant to that fungicide (Georgopoulos & Dovas, 1973). The data even suggested a correlation between the number of benomyl sprayings during 1971 and 1972 and the proportion of resistant isolates in various fields. The same study showed that all of 250 isolates of *C. beticola* obtained from garden beets in areas where no sugar-beets were grown

Table 2. Comparison of effectiveness of fungicides for control of sugarbeet leaf spot in 48 demonstration plots in Greece during the 1972 growing season.

Fungicide treatment	Spraying interval (days)	Proportion of diseased foliage (%)		
		16 July	30 July	15 August
Benomyl 300 g/ha	15	6.5	29.0	85.9
Thiophanate methyl 500 g/ha	15	8.0	32.0	90.0
Fentin hydroxide 500 g/ha	15	11.0	28.0	39.6
Control		70.0	100	

Source: after Dovas (1975).

and no benomyl had been used were sensitive to benomyl. That benomyl-resistant strains of the pathogen were responsible for the failures in the control of leaf spot in 1972 was shown in growth-room experiments conducted during the winter months: resistant and sensitive strains produced comparable amounts of disease on artificially-inoculated, untreated sugarbeet plants. However on benomyl-treated plants sensitive strains failed to produce leaf spot, but resistant strains produced the same amount of disease as on untreated plants (Georgopoulos & Dovas, 1973).

The high selection pressure of the benzimidazole treatments on the population of *C. beticola* was demonstrated in a large field experiment conducted in the summer of 1973 (Dovas et al., 1976). In experimental plots in which only 3.5 % of leaf spot was caused by resistant strains at the end of June, the proportion increased to 91.5 % by the beginning of August. This was due to two benomyl applications, one on 30 June and one on 13 July. As expected, in plots with a high proportion of benomyl-resistant *C. beticola* at the beginning of the season the benomyl treatments had very little effect, but fentin hydroxide continued to give good control. The reduced effectiveness of benomyl was always correlated with the increase in the proportion of the resistant conidia. The composition of the population of the pathogen with respect to sensivity to the benzimidazoles has been monitored in several areas. This monitoring has shown that, as a whole, resistant strains are not less fit than sensitive strains in the absence of benzimidazoles. The proportion of conidia that are resistant to concentrations of benomyl of 1 µg/ml remains constant for years, without the use of any related fungicide (Dovas et al., 1976). A similar stability of benzimidazole resistance has been found in other fungi, e.g. *Botrytis cinerea* (Miller & Jeves, 1979).

In Greece, the benomyl sensitivity of *C. beticola* populations is determined as follows. Preferably young leaves with lesions are thoroughly washed with tap water to remove fungicide residues and old conidia. They are incubated overnight in a moist chamber. A single conidium is then picked up with a fine glass needle. Since conidia from the same lesion

usually behave similarly, single spore isolation is not necessary. Conidia from each lesion are transferred to a premarked position on a water agar plate. Of each small colony produced, one transfer is made to a potato dextrose agar slant (if maintainance of the isolate is desired) and another transfer to a premarked position of a plate of complete medium containing a concentration of benomyl of 1 µg/ml. If examination of the transfers under the microscope is feasible, the distinction between resistant and sensitive isolates can be made the following day: transferred hyphae of sensitive isolates develop swellings and other distortions, and often hyphal tips burst; normal appearance and growth of hyphae is observed for benzimidazole-resistant isolates (Georgopoulos & Dovas, 1973). If early determination is not required the plates are incubated for a few days and then examined macroscopically. The distinction with the concentration of benomyl of 1 µg/ml is very clear cut because, even after prolonged incubation, the sensitive isolates show no growth and the transferred water-agar blocks remain transparent, but resistant isolates form normal colonies.

When a large number of lesions have to be tested, it saves time to transfer conidia directly to a fungicide-containing medium (Yoder, 1979). Benomyl can be added even to the water-agar medium at the concentration of 0.1 µg/ml and scoring can be done macroscopically four days later. Sensitive isolates do not survive on the treated medium, however, and establishment on fungicide-free medium is necessary if sensitive as well as resistant isolates need to be maintained for future studies.

For the measurement of resistance dosage-response curves are obtained and compared. The effect can be measured by the inhibition of dry weight increase in liquid medium or, less accurately, but more easily, by the inhibition of radial growth on agar plates. When adjusting the concentration the water solubility of benomyl, which does not exceed 27 µg/ml, should be taken into account.

Following the first report from Greece, resistance to benzimidazole fungicides was noticed also in other countries (D'Ambra et al., 1974, 1980; Ruppel & Scott, 1974; Ruppel et al., 1980; Uesugi, 1978). Resistant strains in those countries have been found to behave similarly to the ones studied in Greece and to be responsible for failures in disease control.

Resistance to organotins

From 1973 to 1977 the control of sugar-beet leaf spot in Greece depended again almost entirely on fentin hydroxide and fentin acetate. During this period poor performance of these fungicides was occasionally observed. Perhaps due to the increased awareness of the problem of resistance - following the experience with the benzimidazoles - these occurrences

were carefully studied. It has now been established that the use of organotins can also lead to selection of moderately and highly resistant strains of *C. beticola* and to failures in disease control (Giannopolitis, 1978). It was first found that conidia collected from areas with and without a history of organotin usage differ considerably in their ability to germinate in solutions of fentin hydroxide and fentin acetate. Further, the growth rate on agar media containing these fungicides differs, and according to that criterion *C. beticola* isolates can be classified as sensitive, moderately resistant, and resistant to organotins. The abundance of resistant and moderately-resistant strains in a given location is proportional to the number of years triphenyltin fungicides have been used.

Recent studies (Giannopolitis & Chrysayi-Tokousbalides, 1980) have shown that resistance of *C. beticola* to organotins is stable in culture and does not affect colony morphology and growth. There is no cross-resistance between organotins and benzimidazoles, but many field isolates are resistant to both groups of fungicides, apparently because of two independent mutations. On untreated sugar-beets, both fentin-acetate sensitive and resistant strains are virulent, but on plants treated with fentin acetate (300 g/ha) only resistant strains can cause an appreciable amount of leaf spot. From untreated plants inoculated with a 1:1 mixture of sensitive and resistant conidia, sensitive isolates are obtained at higher frequency, indicating reduced competitive ability of the resistant strains. Although no extens ive monitoring for organotin resistance has been carried out, there are also indications that the resistant populations in the field tend to decline in the absence of the selective agent.

The distinction between organotin-resistant and organotin-sensitive strains of *C. beticola* is not as clear-cut as with benzimidazoles. Microscopic examination of the water agar blocks the day following transfer does not reveal any distinct differences. A difference in the amount of growth becomes recognizable after 2-3 days, although the water agar block is covered with mycelium, even in the case of sensitive isolates. Safe distinction can be made 6 days after the blocks are transferred: the sensitive and the moderately-resistant isolates do not grow out of the agar block into a medium containing 0.25 µg/ml and 1.00 µg/ml fentin acetate, respectively, but colonies of resistant isolates on a medium containing 1.00 µg/ml of the fungicide are about half the size of those on a control medium (Giannopolitis & Chrysayi-Tokousbalides, 1980).

Failures in *C. beticola* control due to resistance to triphenyltin fungicides have not been reported from countries other than Greece. In the recent publication of D'Ambra et al. (1980) it is stated that although resistant strains were present in an Italian population of *C. beticola* that was studied, reduced effectiveness of organotins has not been observed in Italy.

Chemical control of Cercospora beticola today

There are large areas in Greece where since 1978 most of the *C. beticola* population has been resistant to both the benzimidazoles and the organotins, so that satisfactory control of leaf spot cannot be achieved with fungicides of either group. Giannopolitis (1978) observed that in areas where organotins perform poorly, a mixture of carbendazim and maneb was superior to fentin acetate. It was first thought that partial replacement of organotins with such a mixture might solve the problem. However the data of Table 3 show that for an organotin-resistant pathogen population even maneb alone is preferable. And as the population has long been resistant to the benzimidazoles, addition of a member of this group has no effect (Table 3).

Extensive experimentation carried out by the Hellenic Sugar Industry S.A., in recent years has provided very useful information. The non-systemic bitertanol has given excellent results so far and the systemic nuarimol appears equally good. But at the doses required both of these fungicides are too expensive to be generally adopted. They can be recommended for use alone only once a year and then only at the most critical time for disease control. Organotins are used only once or twice at the beginning of the season, when disease pressure is low. For the remaining sprayings alternation of mixtures containing maneb (plus 'Daconil', fentin

Table 3. Comparison of fungicides and fungicide combinations for the control of sugar-beet leaf spot in the summer of 1980 in an area of northern Greece where most of the Cercospora beticola population is resistant to both the benzimidazole and the organotin fungicides.

Fungicide	Treatment[a] (g/ha)	Proportion of diseased foliage (%)		
		9 August	25 August	8 September
Fentin acetate	300	11.0	43.0	75.0
Maneb	2 000	6.0	31.0	52.0
Maneb + carbendazim	1 850 + 150	3.0	32.0	52.0
Maneb + 'Daconil'	2 000 + 750	1.0	21.0	27.0
Maneb + fentin acetate	2 000 + 300	3.0	22.0	35.0
Mancozeb	2 000	5.0	29.0	46.0
Propineb	1 750	9.0	38.0	69.0
Difolatan	1 500	10.0	32.0	46.0
Dithianon	750	17.0	75.0	100.0
Triadimefon	100	14.0	51.0	90.0
Bitertanol	450	1.0	17.0	23.0
Nuarimol	250	1.0	11.0	17.0
Bitertanol + maneb	300 + 2 000	3.0	16.0	20.0
Bitertanol + fentin acetate	300 + 300	2.0	25.0	34.0
Nuarimol + maneb	55 + 2 000	5.0	20.0	30.0
Control		46.0	95.0	100.0

Source: unpublished data kindly provided by the Hellenic Sugar Industry S.A.

a. Spraying interval 15 days.

acetate, bitertanol or nuarimol, the last two at reduced concentrations) have been adopted with good results so far.

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Case study 2: Venturia of pome fruits and Monilinia of stone fruits

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Abstract

Dodine resistance in the apple scab fungus, *Venturia inaequalis*, is now common in eastern Canada and northeastern U.S.A. Resistant populations that developed after 9-10 years of intensive, and often exclusive, use of dodine have been detected at least 10 years after spraying with this fungicide was discontinued. Resistant strains are only two to four times less sensitive to dodine than wild-type strains. Benzimidazole resistance in *V. inaequalis* is now reported from most areas of the world where apples are grown, and in the pear-scab fungi, *V. pirinia*, in Israel and France, and in *V. nashicola*, in Japan. Resistance to benzimidazoles in *Monilinia* spp., the brown-rot fungi, also has been reported world-wide. Benzimidazole resistance has developed after about three to four seasons of intensive use of these fungicides. The level of resistance to benzimidazoles in *Venturia* and *Monilinia* spp. is a factor of at least five times normal sensitivity, but is often much higher. Prevention of resistance to fungicides on these crops may be achieved by strategies that reduce the selective pressure. These approaches may include the use of mixtures or alternative fungicides and pest management techniques.

Keywords: dodine, benzimidazole, pest management, *Venturia inaequalis*, *Venturia pirinia*, *Monilinia* spp., selective pressure.

Introduction

Scab of apple and pear and brown rot of stone fruits caused by *Venturia* and *Monilinia* spp., respectively, are economically important fungal diseases wherever these fruits are grown. To prevent severe crop losses these diseases are controlled by numerous applications of fungicide each growing season. Under such intensive regimes, fungicide resistance problems have occurred world-wide on pome and stone fruits and they have severely limited the use of some of the most effective fungicides.

From experience with resistance on these crops there is much information available that should be useful in dealing with this problem in the future.

The diseases and their control with fungicides

Scab of apple and pear

Apple scab caused by *Venturia inaequalis* (Cke.) Wint. is a great problem in cool, moist areas of the world. The fungus produces one sexual overwintering generation per year in which ascospores develop in perithecia in fallen leaves on the ground. These spores are forcibly ejected in the spring and cause primary infections on foliage and fruit until about four weeks after bloom. Many secondary cycles of the asexual stage occur during the growing season and each scab lesion is capable of producing thousands of conidia. Thus the potential for disease incidence in a favourable environment is extremely high. To grow commercially acceptable crops in most apple growing areas, 8-20 fungicide applications are required each growing season, starting at the green-tip growth stage and continuing until the fruit and leaves become resistant to infection. Several fungicides are available for the control of apple scab (Table 1). These vary in efficacy, control mechanism, biochemical modes of action and apparent proneness to resistance. In recent times, captan, ethylene-

Table 1. Fungicides available for apple-scab control in the U.S.A. and their properties.

Fungicides	Scab performance	Control mechanism	Biochemical sites	Resistance reported
Captan	good	protectant	many	no
Benzimidazoles	good	protectant antisporulant curative systemic	one	yes
Mancozeb	good	protectant	many	no
Dodine	good	protectant curative antisporulant	few	yes
Other carbamates	moderate	protectant	many	no
Sulfur	fair	protectant	many	no
Dichlone	good	curative	many	no
Captafol [a]	good	protectant	many	no

a. Limited to single application at green tip.

bisdithiocarbamates, dodine, and benzimidazoles have been used most by apple growers against scab, but resistance has greatly restricted the use of dodine and benzimidazoles. The ergosterol-synthesis-inhibiting (ESI) fungicides are promising new products for apple-disease control.

Pear scabs caused by *V. pirina* Aderh. and *V. nashicola* Tanaka & Yamamoto are economically important diseases with life-cycles similar to *V. inaequalis*. In some areas *V. pirina* may overwinter in lesions on twigs and produce conidia in the spring, as well as ascospores in perithecia in fallen leaves, so that the disease pressure may be extremely intense. Control methods are similar to those for apple scab, using the same fungicides and spraying schedules, depending on the pear cultivar and geographic area. Resistance to benzimidazoles has occurred on pears; the ESI fungicides are the most promising replacements.

Brown rot of stone fruits

Brown rot is caused by three species of *Monilinia*, including *M. fructicola* (Wint.) Honey, *M. fructigena* (Aderh. & Ruhl) Honey, and *M. laxa* (Aderh. & Ruhl.) Honey. The disease caused by *M. fructicola* has two periods of major activity: the bloom and ripe-fruit stages, which are separated by an interval of several weeks when the green fruit is not highly susceptible unless injured. The brown-rot fungi overwinter on infected dried fruit (mummies) or other plant parts. The following year conidia are produced in the tree or ascospores in apothecia from mummies on the ground. Both spore types infect flowers and young fruit, causing rots with profuse conidial sporulation, thus increasing inoculum pressure to an extremely high level by harvest time. Extensive loss of the fruit crop can occur on the tree and post-harvest rot is extremely destructive, especially for stored or shipped fruit.

Control of brown rot is highly dependent on the application of fungicide sprays at bloom and shortly before harvest. In rainy areas or when fruit injury occurs, it also may be necessary to spray during the immature-fruit stage. Stored or shipped fruit must be treated after harvest. Before the introduction of the benzimidazoles, brown-rot control was often unsatisfactory, requiring numerous applications of such fungicides as captan, copper, dichlone, dichloran, maneb and sodium pentachlorophenate. After the benzimidazoles became available, early in the 1970s, they were the primary fungicides until resistance to them developed. The benzimidazoles are now used mostly in combinations with broad-spectrum fungicides such as captan. Dicarboximide and ESI fungicides are gradually being approved and used for brown-rot control on stone fruits throughout the world, and they are expected to replace the benzimidazoles within the next few years because of problems of resistance.

Resistance to dodine

Emergence of resistance

Dodine was introduced as a fungicide for apple-scab control in the late 1950s. Because of its low cost and excellent protectant, curative, and antisporulant properties, it was quickly accepted and used extensively in many apple growing areas of the world. After about 10 years of use in the western fruit growing areas of New York State, U.S.A., widespread and unprecedented scab-control failures occurred in 1969. These failures were quickly demonstrated to be due to resistance of *V. inaequalis* to dodine (Szkolnik & Gilpatrick, 1969). Subsequently, workers have documented this phenomenon in other areas of the northeastern U.S.A. and eastern Canada (Jones & Walker, 1976; McKay & Mac Neill, 1979; Ross & Newbery, 1977). Meanwhile, the volume of dodine used on apples has declined from a high level in the 1960s to a very low level today, at great economic loss to the manufacturer of the product. In addition, the loss of dodine stimulated the extensive use of benzimidazoles as replacement fungicides and, no doubt, contributed to the early development of resistance in *V. inaequalis* to this new class of compounds.

Detection and measurement

A disease-control failure must be carefully studied before fungicide resistance can be confirmed as the cause. Several factors that can lead to poor disease control with fungicides are listed in Table 2. After all other factors, such as faulty application or unusual weather, have been eliminated, resistance may be suspected. Unfortunately, attempts to detect resistance prior to its appearance as a control failure are not often successful with *Venturia* and resistance is usually proven only after the failure occurs.

Table 2. Factors leading to poor control of plant disease with fungicides.

Improper fungicide or formulation
Inadequate or faulty equipment
Improper dosage, velocity, volume, or timing
Incompatability with other pesticides
Severe epidemic conditions: high inoculum level, heavy rains, etc.
Human error
Resistance

Proof of resistance to dodine in *V. inaequalis* has been carried out in in vitro and in vivo tests. For tests in vitro, agar plate or glass-slide drop-dilution methods are used. The sensitivity of non-resistant strains to the fungicide is first established using previously unexposed wild types or standard dodine-sensitive laboratory strains. In germination tests, spores of dodine-sensitive *V. inaequalis* exhibit LD_{50} values of 0.25-0.5 µg/ml; resistant isolates are two to four times less sensitive (Gilpatrick & Blowers, 1974). The values are determined from dosage-response curves (log_{10} probit) developed within the range of 0.2-2.0 µg/ml of dodine. Inhibition of the fungus is expressed as non-germination of the spore. Because *V. inaequalis* grows very slowly and sporulates poorly on agar media, colony-growth tests are not as rapid as spore germination tests, but they have been used by some workers with success (Jones & Walker, 1976; Mac Neill & Schooley, 1973; McKay & Mac Neill, 1979; Ross & Newbery, 1977).

Tests in vivo may include the spraying of trees in the orchard or greenhouse with a dosage series of dodine and comparing its performance to another standard, unrelated fungicide (Szkolnik & Gilpatrick, 1969).

Build-up of a resistant population

In western New York State orchards, where apple scab is the only primary disease requiring chemical control, dodine was often the only fungicide applied in the 9-10 years before resistance occurred. During that time, it is estimated that dodine was applied about 80 times in orchards before resistance was demonstrated. Similar use patterns were common in other areas of the U.S.A. where resistance has subsequently developed (Gilpatrick & Blowers, 1974; McKay & Mac Neill, 1979; Ross & Newbery, 1977). To date, dodine resistance has not been reported in areas where the compound has had only limited use or has been used in mixed programmes with other fungicides (Gilpatrick & Blowers, 1974). Such is the case in the Hudson Valley of New York State, where, in addition to scab, it is necessary to control cedar-apple rust and summer diseases, against which dodine is ineffective. Carbamate fungicides are applied three or four times each year during bloom and early-fruiting periods to control rust; other fungicides, such as captan, are applied later for summer-fruit rots. Both carbamates and captan also provide adequate scab control. Thus in the Hudson Valley of New York State, dodine is used for early scab control by some growers, usually for two or three sprayings, followed by benomyl or other fungicides in combination with a carbamate during bloom, and captan only during later sprayings. Under this regime, resistance in the scab fungus to dodine has not been found after more than 20 years of use. These experiences clearly indicate that in the Hudson Valley resistance has been avoided by alternating and mixing fungicides and that in

western New York State and other areas of the U.S.A. exclusive use of dodine has resulted in resistance.

Laboratory and field studies have shown that the sensitivity to dodine of individuals in a population of *V. inaequalis* follows a normal distribution for concentrations of about 0.1-1.0 µg/ml. Mac Neill & Schooley (1973) found a few conidia in a population of *V. inaequalis* that were resistant in vitro and retained this characteristic in repeated subculturing. In the field repeated exposure of the scab fungus to dodine causes the mean sensitivity of populations to this fungicide to decrease by selection of the less sensitive individuals. Eventually, a level of resistance is reached at which dodine no longer controls scab satisfactorily (Gilpatrick & Blowers, 1974; Jones & Walker, 1976; McKay & Mac Neill, 1979; Ross & Newbery, 1977). The change in sensitivity is usually only by a factor of two to four and there is often an overlapping of the distribution of dodine sensitivity between resistant and non-resistant strains. Thus in small sample populations it often is difficult to clearly separate these two types (Gilpatrick & Blowers, 1974).

The evidence therefore supports the belief that *V. inaequalis* must be exposed to dodine for a considerable time and that dodine must be used intensively as the principal, if not exclusive, fungicide if resistance is to develop to a point where apple scab no longer can be controlled satisfactorily with this fungicide.

Resistance to benzimidazoles

Venturia spp.

Resistance in *V. inaequalis* to benomyl was first reported in South Australia in 1974 (Wicks, 1974). Subsequently, resistance to benzimidazoles in this fungus has occurred in many apple-growing areas of the world (Carreno & Pinto de Torres, 1979; Jones & Walker, 1976; Kiebacher & Hoffman, 1976; Novacka et al., 1977; Olivier, 1979; Tate & Samuels, 1976). Benzimidazole resistance in pear scab caused by *V. pirina* has been reported in Israel and France and by *V. nashicola* in Japan (Ishii & Yamaguchi, 1977; Olivier, 1979; Shabi & Ben-Yephet, 1976).

Proof of resistance to benzimidazoles in *Venturia* spp. is similar to that described for dodine resistance in *V. inaequalis*. Spore-germination tests on agar plates using either conidia or ascospores are most useful for field monitoring (Jones & Ehret, 1976; Umemoto & Nagai, 1979; Yoder, 1978). Sensitive spores germinate but exhibit marked germ tube abnormalities at concentrations of 0.1 µg/ml of benomyl in agar. Resistant isolates grow normally at this level. Some isolates may be resistant to much higher levels of benomyl in the agar, even 1 000 times that tolerated by sensitive strains.

Resistance has occurred primarily in apple and pear orchards where benzimidazoles have been used intensively for three or four years (Ishii & Yamaguchi, 1977; Jones & Walker, 1976; Kiebacher & Hoffman, 1976; Olivier, 1979; Sawamura et al, 1976; Shabi & Ben-Yephet, 1976; Tate & Samuels, 1976; Wicks, 1974). However, in the Hudson Valley of New York State and in certain other areas of the northeastern U.S.A. where cedar-apple-rust control with other fungicides is required, as described under dodine resistance, no case of benzimidazole resistance in *V. inaequalis* has yet been recorded. However, in North Carolina this is not so. In orchards surveyed in that state, benomyl was most commonly used in three or four pre-blooms prays on apples, and other fungicides unrelated to benomyl were used for the remainder of the season (Sutton, 1978). After three to four years, benomyl-resistant strains developed in about one-third of the orchards surveyed, but not in the others. The reason for this rapid build-up of resistance in North Carolina has not yet been explained (Sutton, personal communication, 1981).

The rapid development of resistance in the scab fungi to benzimidazoles may be related not only to the selection of resistance through ascosporic and conidial inoculum by spraying during the scab season, but also to a further selective pressure during the overwintering period. Seasonal spraying of benomyl is known to inhibit to a high degree perithecial formation of sensitive strains of *V. inaequalis* in fallen infected apple leaves, but resistant strains are not so inhibited (Gilpatrick, unpublished data). Thus, in orchards sprayed with benomyl, development of resistance may occur very rapidly from one growing season to the next by elimination of sensitive strains within the overwintering fungus population.

Monilinia spp.

Because of the superior value of the benzimidazoles in controlling diseases caused by *Monilinia* spp., these fungicides were used extensively on stone fruits throughout the world following their introduction in about 1970. Within three or four years many regions reported crop failures resulting from resistance to these fungicides (Jones & Ehret, 1976; Penrose & Koffman, 1977; Szkolnik & Gilpatrick, 1977; Whan, 1976). In regions where use was less intense, resistance was delayed (Tate et al., 1974). Detection methods and sensitivities of resistant strains closely parallel those for *Venturia* spp. (Yoder, 1978).

Because available alternatives to the benzimidazoles often have given less than adequate control compared to benomyl, stone-fruit growers tend to use benomyl despite resistance, until it no longer controls brown rot. Many growers have taken to mixing benomyl and captan themselves to try to avoid severe crop losses.

Fitness of resistant strains

Observations in New York State indicate that fungicide-resistant strains of *Venturia* and *Monilinia* are as pathogenic as sensitive strains (Szkolnik & Gilpatrick, 1969, 1977), and that once resistance occurs in a field population of these fungi, it continues for a long time. Dodine-resistant strains of *Venturia* have been isolated from New York State orchards 10 years after the use of this fungicide was stopped, and benomyl-resistant strains have been isolated after 3 years. A similar situation exists with benomyl resistance in *M. fructicola*. Thus, re-use of these fungicides after several years of abstinence does not seem advisable (McKay & Mac Neill, 1979; Wicks, 1979; Gilpatrick, unpublished data).

Strategies to avoid resistance

Apples

In New York State, growers have been provided with as much information on fungicide resistance as is available. Once resistance is suspected or proven, the growers are advised to change to an alternate fungicide immediately. For scab, a list of suitable alternate materials for benzimidazoles or dodine is available (Table 1). Apple growers have largely refrained from using dodine and benomyl alone in areas where resistance has appeared, but they often use benomyl in combination with multi-site inhibitors such as captan.

Resistance probably can be best prevented by using certain treatment regimes from the first use of a new fungicide, rather than taking measures after it occurs. Based on experiences in New York State, several treatment strategies may be proposed for apple-scab control (Table 3). The exclusive and intensive use of a resistance-prone fungicide (e.g. benomyl → benomyl → benomyl) for scab control is discouraged. The use of single and multi-site fungicides in mixtures or alternations that seem successful in preventing resistance in *V. inaequalis* in the Hudson Valley appears promising (e.g. dodine → (benomyl + macozeb) → captan). Resistance also appears to have been delayed in the scab fungus when captafol was used in the single-application technique (SAT) at bud burst, followed by benomyl later in the season (e.g. captafol SAT → benomyl). In this case, about one-half of the ascospore inoculum is eliminated each year from the selection pressure of benomyl by the captafol, which may account for the delay of resistance to benomyl.

Several other strategies are suggested to prevent resistance in *V. inaequalis*. One is to alternate a multi-site inhibitor, such as captan, with a limited-site inhibitor, such as benomyl, in the spraying programme (e.g. captan → benomyl → captan → benomyl → etc.). Another is to use half dosa-

Table 3. Suggested spraying strategies to prevent fungicide resistance in the apple-scab fungus in New York State.

Strategy number	Spraying sequence during season [a]										Post-harvest foliar application	Probability of resistance to benomyl or ESI
	1	2	3	4	5	6	7	8	9	10		
1	B	B	B	B	B	B	B	B	B	B	-	very high
2	Dod	Dod	Dod	$\frac{M+B}{2}$ [b]	$\frac{M+B}{2}$	$\frac{M+B}{2}$	$\frac{M+B}{2}$	C	C	C	-	low
3	$\frac{C+B}{2}$ [b]	$\frac{C+B}{2}$	$\frac{C+B}{2}$	$\frac{C+B}{2}$	$\frac{C+B}{2}$	$\frac{C+B}{2}$	$\frac{C+B}{2}$	$\frac{C+B}{2}$	$\frac{C+B}{2}$	$\frac{C+B}{2}$	-	unknown
4	C	B	C	B	C	B	C	B	C	B	-	delay
5	Dif	-	-	-	B	B	B	B	B	V	-	medium or delay
6	Dif	-	-	-	S	-	-	S	-	-	B	low
7	$\frac{B+S}{2}$ [b]	$\frac{B+S}{2}$	$\frac{B+S}{2}$	$\frac{B+S}{2}$	$\frac{B+S}{2}$	$\frac{B+S}{2}$	$\frac{B+S}{2}$	$\frac{B+S}{2}$	$\frac{B+S}{2}$	$\frac{B+S}{2}$	-	unknown

a. B = benomyl; Dod = dodine; M = mancozeb; C = captan; Dif = captafol single application, high dosage; S = ESI.
b. Half dosage of that normally recommended for each fungicide.

ges of two dissimilar fungicides at each spraying (e.g. captan + benomyl)/ 2 → etc.). Similarly, use of two single-site inhibitors of different biochemical action in combination at reduced dosages has been suggested (e.g. (benomyl + ESI)/2 → etc.). The latter three programmes involving alternating and mixing of fungicides have not yet been proven to be useful in practice. Such approaches also present certain tactical problems of optimum timing for each fungicide, adequate dosages, residue dynamics, and economics.

In the long run, the pest-management approach may be the best solution to prevent fungicide resistance in apple. Under Integrated Pest Management (IPM), control tactics will be based on absolute need as determined by computerized spray-warning devices and knowledge of the dynamics and mode of action of fungicides. Furthermore, use of resistance-prone chemicals can be selectively managed, so that the selection pressure on the fungus can be minimized. A scheme of scab control might involve a SAT application of captafol at bud burst followed by two or three sprayings of a curative fungicide as needed, and a post-harvest spray of benomyl or another suitable fungicide to prevent perithecial formation during the winter e.g. captafol SAT → ESI(curative) → benomyl(post-harvest).

Stone fruits

Similar strategies to prevent resistance may be proposed for control of brownrot in stone fruits, e.g. triforine at bloom → captan in cover sprayings → vinclozolin (pre-harvest) → benomyl(post-harvest). Resistance is unlikely to develop to any of these fungicides under this regime because each has a different biochemical mode of action and the selection pressure would be low for each product. This example illustrates the need for a variety of fungicides to be available to combat resistance. In addition, research is needed to develop IPM strategies for stone fruits that lead to reduced dependence on or better timing of fungicide application for the control of brown rot. Such strategies may reduce the selection pressure for resistance and delay or prevent resistance becoming a practical problem.

Conclusions

The history of resistance to fungicides by *Venturia* and *Monilinia* spp. on pome fruits illustrates how resistance arises and how this problem may be prevented for new fungicides. The case of dodine and *V. inaequalis* shows that resistance may be at a very low level and require a long time for development. Finally, however, complete breakdown of control occurs and the use of the fungicide in disease-management strategies must be discontinued, causing the manufacturer and often the grower to suffer extensive economic losses. Thus the detection of a low level of resistance to a fungicide in a fungus or a slow loss of population sensitivity should not be regarded as an indication that resistance will not be a problem in practice, but should be considered as a warning that with extended use of the substance its effectiveness may decline. In practical agriculture it should be possible to delay or even avoid resistance of this type by good management practices.

Benzimidazoles resistance on pome- and stone-fruit diseases illustrates that extensive and exlusive use of a resistance-prone fungicide can quickly lead to a high level of resistance, crop losses, and greatly limited use of the product. However resistance may be avoided if the product is mixed or alternated with other fungicides from its first use. After the discovery of resistance, mixtures of benzimidazoles and multisite inhibitors have been widely used, but the impact of this approach on the problem of resistance is not yet clear. The future status of resistance under the mixture regime should clarify the value of this strategy.

New fungicides are now available for use on pome and stone fruits, the most notable of which are the ergosterol-synthesis inhibitors for both scab and brown rot, and the dicarboximides for brown rot. Both classes of compounds appear to have limited-site-inhibition characteristics. Thus,

the potential for resistance in these new fungicides exists. The scab and brown-rot diseases are both multicycle diseases requiring multiple applications of fungicides each year. With dodine and the benzimidazoles this has led to resistance problems. New fungicides used on stone and pome fruits will be subjected to similar pressures and the development of resistance in the future seems probable. Thus, steps taken early in the use of new fungicides to avoid resistance would be appropriate. From past history with dodine and the benzimidazoles on these crops, it appears that strategies that reduce selective pressure on the pathogens by the fungicide offer a hopeful approach. As more and more pest management strategies are introduced that will lead to more direct control of grower practices, our ability to manage resistance should improve. Though much more research is needed before strategies are available that can be relied upon to avoid fungicide resistance in *Venturia* and *Monilinia* on pome and stone fruits, these crop-disease scenarios are convenient model systems for studies of fungicide resistance in both practice and theory. The continuing economic production of these crops may depend on the outcome of these studies.

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Case study 3: Pyricularia oryzae of rice

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Abstract

Fungicide-resistant strains of *Pyricularia oryzae* have been obtained in vitro. Resistance to the antibiotic kasugamycin and to organophosphorus fungicides has also been found in the field. Resistance to kasugamycin in field isolates is controlled by a major gene. Three loci for resistance were identified in mutants obtained in vitro. In these mutants resistance appeared to be due to modification of the ribosomes at the site of action of the antibiotic. Kasugamycin-resistant isolates from the field are somewhat inferior to normal sensitive strains. Strains resistant to organophosphorus fungicides have been obtained in vitro and, only recently, in the field, although these fungicides have been used for 15 years. The resistant mutants obtained in vitro were cross-resistant to isoprothiolane, whose molecule does not contain phosphorus atoms, and showed negatively-correlated cross-resistance to some phosphoramidate compounds. In these respects they clearly differ from most resistant field isolates, which lack negatively-correlated cross-resistance to phosphoramidates and have a lower level of resistance to organophosphorus fungicides. The resistance mechanism in the moderately resistant field isolates appeared to be increased detoxification, but in laboratory-derived mutants the mechanism was different. The availability of a variety of fungicides with different modes of action may be a great help in coping with the resistance problem.

Keywords: rice blast, cross-resistance, negatively-correlated cross-resistance, detoxification, kasugamycin, organophosphorus fungicides, phosphoramidate, isoprothiolane.

Introduction

Rice blast, caused by *Pyricularia oryzae*, is the most important disease of rice in Japan. In other rice-growing countries where rice blast is less serious, its occurrence may increase because of changes in cultural

methods, such as increased use of nitrogen fertilizers, which accelerate the development of the disease.

In principle, three methods of control are available, namely, breeding for resistance, cultural methods and chemical control. The first method does not, however, provide lasting results, as new physiologic races of the pathogen readily emerge. Moreover, the most appreciated, tasty rice varieties are often susceptible to the disease. The possibility of preventing blast by cultural methods is often very limited. Early season cultivation, for example, may help to avoid damage caused by typhoons, which may strike Japan just before the harvest, but it may result in the development of the disease at a younger stage of the plant, due to the lower temperatures then prevailing. In view of these facts, chemical control is indispensable, and it probably will remain the most important measure for coping with blast.

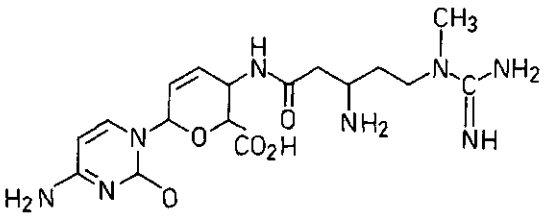
In the past, Bordeaux mixture has been used to control blast, but the effect was unsatisfactory. Food shortages after the Second World War led to the dusting of the rice crop with phenylmercury compounds, which until that time had only been used for seed disinfection. From 1952 to about 1967 these compounds were the prime agents for control of rice blast.

A search for antibiotics effective against blast started in 1953. It led to the discovery of blasticidin-S, which was introduced commercially in 1961. This promoted the development of other non-mercurial fungicides, among them the antibiotic kasugamycin, organophosphorus fungicides, such as IBP and edifenphos, and other types of compounds, such as tetrachlorophthalide, probenazole and isoprothiolane (Figure 1). Most of these non-mercurial fungicides act specifically on rice blast. However some of these have encountered problems with fungicide resistance in the field. Fortunately, different types of fungicides are now available, so that control of blast remains possible by using a variety of types of chemicals.

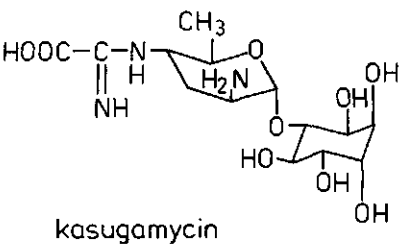
Resistance to kasugamycin

Emergence of resistant strains

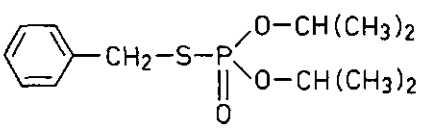
Mutants of *P. oryzae* resistant to kasugamycin were readily obtained by plating conidia on agar containing a discriminating concentration of the fungicide, without the use of mutagenic agents (Ohmori, 1967; Uesugi et al., 1969; Katagiri & Uesugi, 1978; Taga et al., 1978). The frequency of emergence of resistant mutants in these experiments was usually about 10^{-5}; it varied with the parent strains tested, but, within a certain range, not with the concentration of the antibiotic. This suggests that it is unlikely that the antibiotic exerts mutagenic activity (Miura et al., 1976). Resistant mutants were also obtained from fast-growing sec-



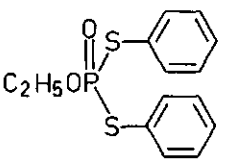
blasticidin-S



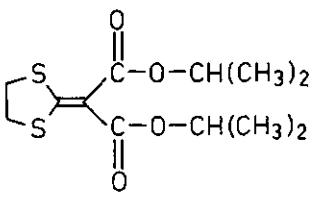
kasugamycin



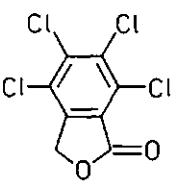
IBP 'Kitazin P'



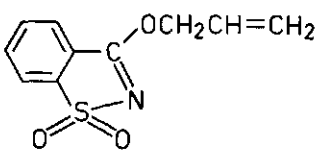
edifenphos (EDDP, 'Hinosan')



isoprothiolane



tetrachlorophthalide ('Rabcide')



probenazole ('Oryzemate')

Figure 1. Structural formulae of fungicides used to combat Pyricularia oryzae on rice.

tors of mycelium on a medium containing the antibiotic (Taga et al., 1979), and from subcultures successively transferred to media containing increasing concentrations of the antibiotic (Uesugi et al., 1969).

Resistance of *P. oryzae* to kasugamycin in the field was found in 1971 in the Shōnai district of the Yamagata Prefecture, an important rice-growing area in Japan, where the antibiotic had been used intensively and exclusively for several years (Miura et al., 1975). As some resistant mutants obtained in vitro appeared virulent and able to sporulate, it is plausible that the resistant strains found in the field also develop by mutation and that survival in the field is possible, at least for a limited period of time.

Monitoring and detection of resistant strains

As kasugamycin does not inhibit germination of conidia of *P. oryzae*, assessment of resistance was carried out by observing inhibition by the antibiotic of mycelium growth on agar and calculating the minimum growth-inhibiting concentration (MIC). Although these MIC values for sensitive strains appeared to vary widely (Uesugi et al., 1969), resistance in the field exceeded this broad distribution of sensitivity (Sakurai et al., 1977; Sakurai & Naito, 1976). A good method to measure resistance is the agar-diffusion technique, in which inhibition zones are formed by diffusion of an antibiotic solution in an agar medium uniformly seeded with conidia of the strain to be tested. However this method is less convenient than measurement of radial mycelium growth (Katagiri & Uesugi, 1974).

Genetics

The perfect stage of the rice-blast pathogen *(Magnaporthe grisea)* has recently been found in cultures isolated from rice and various other graminaceous plants. Strains were isolated from finger millet that could be crossed with other strains from this crop plant and with strains from rice plants, so that genetic analysis was possible. Resistance to kasugamycin in field isolates was found to be controlled by a major gene (Taga et al., 1978). In resistant mutants originally isolated from finger millet, so far three loci for kasugamycin resistance have been detected. Of these, one appears to be responsible also for resistance to blasticidin-S (Taga et al., 1979).

Mechanism of resistance

The mode of action of kasugamycin is reported to involve protein biosynthesis, by affecting the ribosomes in fungal cells. The resistance me-

chanism in a mutant selected in vitro for resistance to kasugamycin was investigated using reconstituted reaction systems of ribosomes and tRNA extracted from the resistant mutant and the sensitive parent strain. Incorporation of labelled aminoacyl tRNA into the ribosome fraction was always inhibited by the antibiotic when ribosomes from the sensitive strain were used, but not with ribosomes from the resistant mutant, regardless of the source of the tRNA. This indicates that resistance is due to decreased affinity of the ribosomes to the antibiotic (Misato & Ko, 1975).

Population dynamics of resistant strains in the field

When the resistant strains were found in the Shōnai district in 1971, most of the isolates from the area appeared to be resistant. A striking decrease in the population of resistant strains was observed after application of the antibiotic was stopped in 1972; the preportion of resistant strains in the isolates in 1975 decreased to 2-20 % (Miura & Takahashi, 1976). Resistant isolates were compared with sensitive ones on mycelial growth, sporulation and virulence, but no significant differences were found (Miura et al., 1976). When rice plants were inoculated with a 1:1 mixture of conidia of sensitive and resistant isolates, the number of lesions formed by the sensitive strain was, in most cases, far greater than for the resistant strain, suggesting a superior competitive ability of the former over the latter. This seemed to be due to the higher rate of infection of the sensitive strain (Ito & Yamaguchi, 1979).

Resistance to organophosphorus fungicides and isoprothiolane

Emergence of resistant strains

Mutants of *P. oryzae* resistant to organophosphorus fungicides, such as IBP and edifenphos, are also readily obtained by selection in vitro from a large number of conidia (Uesugi et al., 1969). However the frequency of emergence of resistant mutants seems a little less than for kasugamycin (Katagiri & Uesugi, 1978). Note that these mutants resistant to organophosphorus fungicides are cross-resistant to another new fungicide, isoprothiolane, which has no phosphorus atom in its molecule. Mutants selected in the presence of isoprothiolane are also cross-resistant to organophosphorus fungicides, and the frequency of their emergence was nearly the same as for organosphosphorus fungicides (Katagiri & Uesugi, 1977, 1978). Contrary to what one may expect, some phosphoramidate compounds are specifically fungicidal to these resistant mutants, but not to normal wild-type strains (Uesugi et al, 1974).

Organophosphorus fungicides have been in practical use since 1965,

but field emergence of resistant strains of *P. oryzae* was not reported until 1976. One unreported and exceptional case was observed in 1973, in an experimental greenhouse where IBP had been intensively used as a standard for evaluating fungicides. The preventive effect of IBP against blast disease on rice seedlings was remarkably diminished, but a phosphoramidate was quite effective, suggesting the occurrence of resistant strains similar to laboratory-derived resistant mutants.

In 1976, eight isolates resistant to IBP at 0.1 mM - a concentration to which normal wild-type strains used to be sensitive - were found among 173 isolates from Toyama Prefecture, where large quantities of IBP granules had been applied to the paddy soil. Monitoring was continued in 1977: the sensitivity of isolates was compared with that of stock strains isolated before 1964, when no organophosphorus fungicides had been applied in practical agriculture. The results shown in Table 1 indicate development of resistance, though the level was rather low.

Among the resistant field isolates, two levels of resistance were found. One isolate exhibited a phosphoramidate sensitivity equal to that of resistant mutants obtained in vitro. Most of the resistant field isolates, however, showed a moderate level of resistance, and were different from resistant mutants obtained in the laboratory; the former did not show negatively-correlated cross-resistance to phosphoramidate (Katagiri & Uesugi, 1980).

Monitoring and detection of resistant strains

The measurements of MIC in Table 1 show an increase in the level of resistance. It was difficult, however, to distinguish resistant strains from originally sensitive ones. In fact, the 28 isolates identified in 1977, which had MIC values of 0.1 mM, seemed to consist of resistant and sensitive strains.

Some phosphoramidates are specifically toxic to in-vitro-obtained mutants resistant to IBP, edifenphos and isoprothiolane, but not to normal

Table 1. Development of resistance to IBP of field isolates of Pyricularia oryzae.

MIC (mM)	Number of isolates resistant [a]			
	before 1964	1974	1976	1977
≧ 0.2	0	0	8	4
0.1	3	165	165	28
≦ 0.05	27			46

a. Source of isolates: stock strains isolated before 1964, when organophosphorus fungicides were not used in practice; in 1974 isolates from Ibaraki and Yamagata Prefectures; and in 1976 and 1977 isolates from Toyama Prefecture.

wild-type strains. Combination of the phosphoramidates with IBP, edifenphos or isoprothiolane gives, moreover, a synergistic fungicidal effect on normal wild-type strains (Uesugi et al., 1974; Katagiri & Uesugi, 1977). The synergism, evaluated by the crossed-paper technique, is not pronounced in laboratory and field isolates resistant to IBP. The difference in extent of synergism makes it possible to distinguish resistant isolates from sensitive ones. Inhibition of radial growth of mycelium on agar also makes it possible to recognize resistant isolates (Katagiri & Uesugi, 1980).

Genetics

Conidia of a mutant obtained by selection in vitro in the presence of an organophosphorus thiolate were selected again on a medium containing a phosphoramidate. A second mutant was thus obtained. In this test the first mutant was resistant to IBP and sensitive to phosphoramidate; the second mutant was resistant to phosphoramidate and sensitive to IBP just like the original wild-type strain. Therefore the second mutation seems to be a back-mutation, and resistance to IBP was accompanied by sensitivity to phosphoramidate (Uesugi & Katagiri, 1977).

Genetic analyses of resistance in laboratory-derived mutants of isolates from finger millet are being conducted; a major gene for resistance to IBP and isoprothiolane has already been identified (Taga et al., 1980). Resistance found in the field is different from that obtained in the laboratory, but genetic analyses of resistant field isolates have not been reported.

Mechanism of resistance

A field isolate moderately resistant to organophosphorus fungicides detoxified IBP by metabolic cleavage of the S-C linkage in the IBP molecule (Uesugi et al., 1978). This detoxification may account for the resistance. However, for the resistant mutants obtained in vitro the rate of detoxification was even lower than in sensitive wild-type strains, so that resistance may not be due to increased detoxification but to other factors, such as site modification or decreased permeability of the fungal cell membrane to the fungicide. Since IBP and isoprothiolane are inhibitors of the oxidative metabolism of phosphoramidate, there is a possibility that the target site of these fungicides is the oxidative enzymes: modification of the enzymes, as suggested by decreased phosphoramidate metabolism, might be the actual resistance mechanism (Uesugi & Sisler, 1978). But, inhibition of phospholid biosynthesis was recently proposed as the possible mode of action of IBP (Kodama et al., 1979); thus both mode of action and resistance mechanism have yet to be ex-

plained.

Field isolates moderately resistant to organophosphorus thiolate fungicides and normal wild-type strains are resistant to phosphoramidate, but laboratory-derived mutants resistant to organophosphorus thiolate fungicide are sensitive to phosphoramidate. The rate of fungal metabolism of a phosphoramidate through hydroxylation or N demethylation was high in field isolates with moderate resistance to IBP and in normal wild-type strains, but low in laboratory-derived mutants resistant to IBP. Fungal metoblism of phosphoramidate thus coincides with sensitivity to phosphoramidate in all the strains tested.

Population dynamics of resistant strains in the field

Since emergence of resistance in the field has been observed only recently, little is known about the population dynamics of resistant strains. Also, it has not yet been explained, why resistant strains in the field only emerged after the fungicides had been used for more than ten years, and why most of the resistant strains emerging in vivo were different from those obtained by selection in vitro.

Although the level of resistance in the field isolates is mostly only half that of the isolates obtained in the laboratory, still it is thought that it diminishes the effect of the fungicides, especially when they are applied as granules to submerged paddy fields; then the fungicide is translocated within the plant. Its concentration in the plant remains at a moderate level for a long time, so that presumably sensitive strains, but not moderately resistant ones, are controlled. This may stimulate further selection of strains with moderate resistance.

Resistance to other fungicides

Blasticidin-S

There have been many reports of emergence of mutants of *P. oryzae* resistant to blasticidin-S under laboratory conditions (Suzuki, 1962; Nakamura & Sakurai, 1968; Uesugi et al., 1969, Hwang & Chung, 1977). Although the application of this antibiotic is rather limited in Japan, and therefore there is little chance of resistance occurring in practice, cross-resistance in field isolates resistant to kasugamycin has been reported (Sakurai & Naito, 1976). However, there are also other reports of no cross-resistance being found (Miura et al., 1975; Ito & Yamaguchi, 1976). Maybe there are two or more types of kasugamycin-resistant strains with various cross-resistance to blasticidin-S. On the other hand, since the distribution of MIC values of the antibiotic is rather broad among sensitive strains (Uesugi et al., 1969; Hwang & Chung, 1977), it might be dif-

ficult to detect resistance to blasticidin-S. The mechanism of resistance in a mutant has been investigated. It was attributed to decreased penetration by the antibiotic into the fungal cell (Huang et al., 1964).

Benomyl

Although this fungicide is not used for control of rice blast in Japan, it is fairly fungicidal to *P. oryzae*. In experiments with a large number of conidia, the frequency of emergence of resistant mutants was about 10^{-7}. This is lower than the frequency of resistance to kasugamycin and organophosphorus fungicides (Katagiri & Uesugi, 1978). The rather low frequency of benomyl resistance is interesting in view of its many resistance problems with other plant pathogens.

Disease-control agents having little or no fungicidal activity

Tetrachlorophtalide and probenazole have little or no fungicidal action on *P. oryzae*, yet they prevent blast disease when applied to rice plants. The question remains whether development of resistance to these agents is probable or not. In an experiment of 20 successive inoculations of fungicide-treated rice plants with *P. oryzae*, no development of resistance was observed with tetrachlorophthalide, although resistance to compounds with direct fungicidal activity has been observed so often (Aoki & Yamada, 1979).

General discussion

Under laboratory conditions *P. oryzae* can develop resistance to antibiotics, organophosphorus fungicides and isoprothiolane. With kasugamycin, resistance was due to spontaneous mutation. Selection on media containing IBP, edifenphos or isoprothiolane resulted in resistant strains at nearly the same frequency, with cross-resistance among the three fungicides. This suggests spontaneous mutation for resistance (Katagiri & Uesugi, 1978). These resistant mutants, or at least some of them, are virulent to the host plant and seem quite similar to the wild-type parent strain in many respects, except for fungicide resistance. Hence they are assumed to be able to occur in the field and survive under natural conditions.

Nevertheless, the following is also conceivable. Since the resistant mutants emerge by spontaneous mutation, they emerged and existed a long time before the fungicides were developed, but their populations were limited as there was not selection pressure of the fungicide. Thus the resistant mutants must be inferior and unable to increase their population in a natural environment free of the fungicide.

In laboratory studies, kasugamycin-resistant field isolates were not found to be significantly inferior to sensitive isolates. In the field, however, the fitness of resistant strains must be somewhat lower than that of the sensitive strains, since the population of the former declined after application of the antibiotic was stopped. At least two types of IBP-resistant field isolates have been found. One is similar to mutants obtained in the laboratory; the other is clearly different. The former is rarely found in the field, but the latter occurs more frequently. It seems that most of the (moderately) resistant strains occurring in the field are mutants that emerge far less frequently but are fitter. Mutants that occur very readily under laboratory conditions, however, appear to be less fit for survival under field conditions.

The resistance mechanism in the field isolates is presumably an increased detoxification, as distinct from the mechanism in laboratory mutants, for which activity of the enzymes involved in detoxification was even lower. In this case changes in the physiology of the fungus may cause its lower fitness in the environment. Note that increased detoxification is the most common resistance mechanism to medical drugs of clinical isolates of bacteria, but this is seldom so in bacterial mutants obtained on artificial medium in the laboratory (Benveniste & Davis, 1973; Davis & Smith, 1978).

Selection pressure under field conditions is another important factor determining the occurrence and survival of resistant strains. Time and frequency of application, dose and persistence of the fungicide may all be important. If the level of resistance occurring in the field is low, as is for organophosphorus fungicides, the concentration of fungicide on or in the plant may be critical. A moderate concentration may discriminate between resistant and sensitive forms, in favour of the former.

Recently a novel type of fungicide that has no direct fungicidal action on *P. oryzae*, yet is effective against the disease, has been introduced. The mode of action of this type has not yet been elucidated, but in principle one of the following may be possible:

- It increases the resistance of the host plant.
- It does not inhibit growth of the pathogen, but rather changes its metabolism, resulting in a decrease of virulence.
- It increases both the resistance of the plant and decreases the virulence of the fungus.

Strains of pathogens that are insensitive to this type of compound have not yet been reported.

Many new and very effective fungicides to combat rice blast have been introduced during the last two decades. But there have also been problems, of which development of resistance to some of these new fungicides is the most important and urgent one. Variation, by using fungicides with different mechanism of action, has helped greatly to cope with these pro-

blems. A study of the mode of action and the mechanism of resistance will deepen our insight greatly, which is necessary if we are to maintain adequate control of rice blast.

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Case study 4: Powdery mildews of barley and cucumber

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Abstract

The occurrence of acquired resistance to fungicides in powdery mildews, and the methods of investigating such phenomena in field populations are discussed briefly. Responses to the 2-amino-pyrimidines, dimethirimol and ethirimol, are considered in greater detail. A comparison of surveys of sensitivity of cucumber powdery mildews to dimethirimol in glasshouses in the Netherlands and of barley powdery mildews to ethirimol in the U.K. indicates widely divergent behaviour patterns, which probably reflect different methods of use and agricultural situations. A widespread and rapid loss of effectiveness of dimethirimol against cucumber mildew was experienced in 1970. This was associated with the incidence of resistant populations of the fungi. The almost universal adoption of dimethirimol in Dutch glasshouses and its use at highly effective doses over long periods of the year probably created very favourable conditions for resistance. In the case of ethirimol, which has been studied more extensively, field populations of barley mildew varied considerably in sensitivity. Disease control by ethirimol treatment was associated with slightly reduced sensitivity in the surviving populations. However, the most resistant forms did not spread or become predominant over a five-year period of surveys (1973-1977). The continued effectiveness of ethirimol during this period probably depended on limited use (one application per year) and the re-invasion of treated areas by more sensitive forms when selection pressures were low or absent. An interesting shift in the spectrum of response was discerned: over the period of monitoring, both the most sensitive and the most resistant populations decreased markedly in number, and isolates of intermediate sensitivity became predominant.

Keywords: fungicide, resistance, powdery mildew, barley, cucumber, ethirimol, dimethirimol.

Introduction

The powdery mildews form a well-defined group of plant pathogens that cause serious damage in many types of crops throughout the world. They are controlled mainly by chemical treatment, and a wide range of chemicals is available for this purpose (Bent, 1978). Some of these fungicides (e.g. the dinitrophenols and 2-aminopyrimidines) act only against powdery mildews. Others (e.g. the benzimidazoles, triazoles and pyrimidine carbinols) affect a greater range of fungal pathogens, but are known to have specific mechanisms of biochemical action within the cells of susceptible fungi. Given the marked selectivity of action against one group of fungi or at particular biochemical sites, and given the well-known ability of powdery mildews to adapt to 'resistant' varieties of crop plants, it is not surprising that fungicide resistance should have arisen. Indeed, the first reported case of acquired resistance to the benzimidazole fungicides - the forerunner to many other instances - was the occurrence in field plots in New York State of isolates of cucumber powdery mildew (*Sphaerotheca fuliginea*) that were resistant to benomyl (Schroeder & Provvidenti, 1969). Subsequently, resistance to benzimidazole fungicides in cucurbit powdery mildews, coupled with obvious failure in disease control, has been found in many countries, both in glasshouse and field crops (Netzer & Dishon, 1970; Kooistra et al., 1972; Burth, 1973; Peterson, 1973; Iida, 1975).

Other documented instances of fungicide resistance in powdery mildews concern the response of cucumber powdery mildew to dimethirimol (Bent et al., 1971) and triforine (Gilpatrick & Provvidenti, 1973), and barley powdery mildew to ethirimol (Wolfe & Dinoor, 1973; Shephard et al., 1975; Hollomon, 1975a; Smith et al., 1977; Walmesley-Woodward et al., 1979a,b). Resistance to dimethirimol and ethirimol, which has been examined in extensive surveys, will receive particular attention in this contribution. There are very few reports of fungicide resistance amongst powdery mildews other than those of cucurbits and barley, and none, to my knowledge, of fungicide resistance among the powdery mildews of grapevine (*Uncinula necator*), apple (*Podosphaera leucotricha*) or rose (*Sphaerotheca pannosa*). Yet it seems very unlikely that these important and widespread pathogens differ from the cucurbit and barley mildews in their response to fungicide use. The repeated observations of variation in sensitivity to fungicides in cucurbit and barley powdery mildews probably result from the very obvious and discrete lesions caused by the cucurbit mildew, which show up strikingly any loss of disease control, and the extent to which barley powdery mildew in Europe has been the subject of special investigations designed to disclose differences in sensitivity. Furthermore, the cucurbit and barley powdery mildews are for various technical reasons easier to study in laboratory or glasshouse experiments on leaf pieces

than other major powdery mildews.

Methods of assessment of fungicide resistance in powdery mildews

Most studies on fungicide resistance have been based on the degree of inhibition of growth of isolates of the fungus on agar or in liquid media containing known concentrations of fungicides. However, powdery mildews are obligate parasites, and hence other techniques, which are more difficult and less precise, have to be used. If the conditions and procedures are carefully controlled, the conidia of powdery mildews will germinate to produce germ tubes and appressoria when placed on artificial substrates such as glass slides or cellulose-acetate membranes, or when floated on water. Assessment of the germination of cucumber powdery-mildew spores on cellulose-acetate membranes impregnated with dimethirimol (Bent et al., 1971) revealed relationships between the responses of different isolates that were broadly similar to those revealed by other methods. Hollomon (1975b) observed germination and appressoria formation with spores of isolates of barley powdery mildew on the surface of ethirimol-treated barley leaves. However these microscopic techniques are tedious to use with a large number of samples, and they require stringent precautions to make them accurate and reproducible. Also, they indicate the effect of the fungicide at only one or two stages of the development of the pathogen, whereas in practice most powdery-mildew fungicides exert their action at various phases of infection, for example on haustorial formation, on hyphal growth and on sporulation, and some (e.g. benomyl) do not affect the initial formation of germ tubes or appressoria (Schlüter & Weltzien, 1971).

The amount of visible growth of powdery mildew on detached leaves or on leaf pieces inoculated with spores and treated with fungicide at differing concentrations is the common basis of the assessment methods of several workers. Schroeder & Provvidenti (1969) used detached cucumber leaves, some of which were taken from plants grown in soil drenched with standard amounts of benomyl solution. Bent et al. (1971) used a similar method, in which cucumber-leaf discs were floated on dimethirimol solutions at six concentrations (0.052-5.0 µg/ml) in petri dishes, inoculated with conidia and incubated for 7-10 days. Visible mildew on each disc was assessed on a simple 0-4 scale according to the proportion of its area covered by mildew.

Floating leaf pieces can also be used to examine isolates of barley powdery mildew, although growth of this mildew is less even and harder to assess than that of cucumber mildew, there is more variation in infection from piece to piece, and curling and senescence of the leaf pieces can cause difficulties. A more convenient technique, adopted by Wolfe & Dinoor (1973), and used in modified form by Shephard et al. (1975), is to treat barley seeds with different amounts of chemical

(ethirimol), grow the seedlings under controlled environmental conditions, cut replicate leaf pieces from the seedlings, embed the leaf pieces at one end in tap-water agar in a petri dish, and apply mildew spores. The amount of visible mildew on each leaf-piece is recorded after six days incubation at 19 °C. In the study of Shephard et al. (1975) tests with ^{14}C-labelled ethirimol indicated some variation in the ethirimol content of replicate leaf pieces, proximal pieces tending to contain less fungicide than distal pieces. Nevertheless an adequate degree of statistical significance between the differing results can be obtained, particularly if the tests are repeated two or three times. Precise standardisation of inoculum would further increase the accuracy of the tests, but this is very difficult to achieve with powdery mildews. Some degree of uniformity can be achieved by subculturing field samples on fresh leaf tissue under controlled conditions, blowing off old spores and allowing a fresh crop to develop, and then applying the spores to the test material by blowing them with a standardized airblast into a settling tower for a given period.

Another approach used by several workers is to treat whole plants with fungicides at different concentrations, and to apply conidia of a particular isolate to replicates of treated plants. Such procedures can provide useful additional tests to complement leaf-piece methods, or they can be used as primary tests on pathogens for which leaf-piece methods are not practicable (e.g. apple powdery mildew), although they tend to be less convenient and less precise than leaf-piece methods. If pre-test culture on plants is required, to build up inocula for the tests, then special precautions, such as the use of screening or individual air-flows, must be taken to contain the individual isolates on their 'correct' host plants and to avoid cross-contamination.

All the techniques described can be used successfully to reveal relative differences in sensitivity between powdery-mildew isolates. Extensive comparisons of the methods have not been made, but the available evidence indicates that differences in sensitivity among particular isolates shown by the different types of procedures are broadly similar. However, it is very important to avoid labelling the behaviour of an isolate in absolute terms, for example designating an isolate as 'resistant' or 'sensitive' or even as 'resistant to a dosage of *x* mg/l fungicide', without defining the terms used carefully. One worker's 'resistant' form may be another worker's 'sensitive' or 'intermediate' form, according to the base-line adopted. Also the concentration of fungicide used may bear a greatly different relationship to fungitoxicity according to how or where the fungicide is applied. For example, a seed treatment with ethirimol at 8 000 µg/g seed gives mean concentrations of 1.9-8.3 µg/g fresh weight in leaf pieces (Shephard et al., 1975), whilst the amounts actually reaching or penetrating spores at the leaf surface are unknown and virtually im-

possible to define in terms of concentrations.

Yet another difficulty is deciding whether to compare the responses of different mildew samples at a single, discriminating, fungicide concentration or over a range of concentrations. In practical agriculture isolates are likely to encounter a wide range of concentrations, and for this reason as well as for increased accuracy it is probably best to use a range of concentrations for testing. One can quote as a response score the highest concentration that allowed some degree of mildew development, the lowest concentration at which complete control was obtained, estimated ED_{50} or ED_{95} values, or summations of the degrees of control given at each concentration tested. Since each method of scoring will give different numerical values, it is advantageous to define and stick to a particular system throughout a particular study or series of related studies. The merits and limitations of the different scoring systems will vary according to the resources, conditions and objectives of each investigation, to the assessment method adopted, and to the degree of variation between the dose-response curves of different isolates. It would be unwise, therefore to lay down standard procedures for studying fungicide resistance in powdery mildews.

Great care must be taken when interpreting the results of glasshouse or laboratory tests of fungicidal activity in terms of predicted field performance. It is a common occurrence that fungicides giving complete disease control when sprayed to run-off at concentrations of 1-10 µg/ml in routine glasshouse tests on small plants need to be sprayed at concentrations of 50-500 µg/ml to give effective control of the same disease in the field. Seed treatments applied at a particular dosage appear to be more closely related in their glasshouse and field effects, but the possible influence of, for example, soil type, weather, time of infection and fitness of the pathogen under field conditions on the relation between the effects must be carefully considered.

Methods of sampling powdery mildews for resistance testing

The selection of suitable procedures for obtaining representative samples of fungi from field populations forms a difficult but very important part of the planning of all investigations into fungicide resistance. The method chosen will depend on the objectives of the research and the effort available. If the aim is to monitor the fungicide for continued effectiveness over large regions, it is necessary to take samples from many sites or trial plots where the fungicide has been used, together with samples from untreated sites or plots that are closely situated and as comparable as possible. It is important that estimates of the degree of disease control, or at least the level of disease, should be made at the time of sampling, and both sampling and disease assess-

ment should be repeated several times during the season, whenever possible.

The simplest method, and the one adopted by ICI workers in surveys of sensitivity to ethirimol and dimethirimol, is to take a single representative sample of a large mildew population in each field or commercial glasshouse in the survey by mixing together spores taken from a number of pustules at different points in each crop area. Such a sample should elicit an 'average' response typical of the site concerned. Since the sample will be heterogeneous, it is possible that its composition and its response will alter during incubation or sub-culturing in the laboratory, either in the presence or absence of fungicides. Hence any incubation or sub-culturing between sampling and testing, which is sometimes necessary to obtain sufficient viable inoculum, should be as brief as possible. Repetitive testing should give some indication of any gradual shifts in response. However, the problems of possible loss of resistance during the pre-test period may not be too serious, because the more transient the resistance and the greater its dependence on continued fungicide selection pressure, the less likely it is to build up and cause problems in the field. In practice, bulk samples of cucumber and barley mildew tend to retain their particular responses to pyrimidine fungicide through several sub-cultures in the absence of the fungicide; only minor fluctuations occur between tests (Shephard et al., 1975).

One way of avoiding any shifts in sensitivity during incubation in the laboratory, growth room or glasshouse, and also of obtaining a direct sample of the field inoculum at the time of sampling, is to place fungicide-treated test plants (or plant material) at points within or near the crop so that they are exposed to the natural spore inoculum for a certain length of time, say 4-24 hours, and then to transfer them to the controlled conditions of the growth room or glasshouse for incubation and assessment (Wolfe & Minchin, 1976). Some possible drawbacks of this method for larger surveys include a relatively high labour requirement, a logistical problem of providing uniform test material over wide areas, and dependence upon the weather and the inoculum level in the crop for obtaining satisfactory spore depositions and infection.

It can be rewarding to take multiple samples of mildews from one or a few sites, to study in detail the incidence and the spatial distribution of forms with different degrees of sensitivity. Mildew from single pustules or even single spore chains may be transferred to host material in individual containers and maintained as separate cultivars. The samples obtained from one site may vary considerably in their responses to fungicides (Wolfe & Dinoor, 1974; Shephard et al., 1975), and also in the stability of response during laboratory culture (Hollomon, 1975). Indeed, uniform mildew populations may be rare or non-existent.

Resistance of cucumber mildew to dimethirimol

Dimethirimol is a systemic fungicide with a highly specific action against powdery mildews. It was introduced in the Netherlands in 1968 as a soil drench to control cucumber powdery mildew in glasshouses. In 1969 dimethirimol treatment was adopted very widely, because one application provided excellent protection for several weeks and there were no effects on predators used for biological control. However by spring 1970 a number of failures had occurred. Leaf-disc tests on mildew samples taken from many glasshouses revealed a close correlation between lower sensitivity to dimethirimol and lack of disease control (Table 1). Some resistant mildew populations were also detected in glasshouses in the U.K. and West Germany: the more resistant isolates appeared to be at least as vigorous in growth and sporulation on leaf disc and small cucumber plants as sensitive isolates.

The product was promptly withdrawn from use in the Netherlands. The incidence of resistance remained high the next year, 1971; subsequent surveys suggest a gradual decline (J.A.W. Turner & A.M. Cole, personal communication), although samples still vary greatly, and it is difficult to make valid year-to-year comparisons because of unavoidable variations in sampling sites, experimental conditions and research personnel. Since 1977, in response to requests from growers and advisors, a limited amount of dimethirimol is being sold again in the Netherlands. It is recommended that only one application per year - at the most necessary time - should be made, to minimize the risk of reselection of resistant forms. Unrelated fungicides are used at other times. Used in this way, dimethirimol worked well in 1977 and 1978 in the Netherlands, but there were some instances of inadequate control in 1979, and little of it was used in 1980 (B. Anema, personal communication). Resistance to

Table 1. Sensitivity to dimethirimol of cucumber powdery mildew from Dutch glasshouses.

Tolerance level [a] (µg/ml)	Number of isolates	
	from sites of poor control	from sites of good control
5.0 or > 5.0	8	-
2.0	9	-
0.80	10	-
0.32	2	1
0.13	-	-
0.05 or < 0.05	2	5

Source: Bent et al.(1971).

a. Highest dimethirimol concentration that allows any visible growth of mildew on floating leaf discs.

dimethirimol in powdery mildew of cucurbits has not been reported during the roughly 10 years of use in Spain and the Middle East. Glasshouse and outdoor applications have been made, by soil drench and by spraying, but the intensity and duration of treatments have been much less than in the Netherlands.

Resistance of barley mildew to ethirimol

Ethirimol is chemically closely related to dimethirimol, and probably has the same mode of action against powdery mildews. It has been in widespread use since 1971 in the U.K. as a barley seed treatment. In annual trials it has shown very good control of powdery mildew during the critical phase of infection and this has resulted in substantial yield increases. Surveys showed little overall change in sensitivity during the period 1973-1977 (Table 2). However, in each survey mildew samples from treated fields were generally less sensitive than samples from nearby untreated fields. Mildew populations were heterogeneous in their response to ethirimol (single-pustule isolates obtained within single fields gave varied responses), and the less sensitive forms survived the ethirimol treatment.

There was no clear-cut correlation between the ethirimol sensitivity of mildew isolates and the levels of mildew in their fields of origin at the time of sampling. When all data from the 1975 survey were amalgamated there was a low-significance, positive correlation coefficient ($P = 0.10$) between sensitivity and amount of field mildew (M. Woolner & K.J. Brent, unpublished data). This confirmed indications obtained by Shephard et al.

Table 2. Sensitivity [a] to ethirimol of powdery mildew samples [b] from barley fields treated and not treated with ethirimol.

Period	Scotland		East Anglia	
	untreated	treated	untreated	treated
1973 summer	17.1	15.4	11.9	10.5
1974 spring	14.8	10.5	12.3	9.3
1974 summer	13.8	10.7	14.2	10.0
1975 spring	12.6	11.3	13.0	10.6
1975 summer	12.7	10.9	12.8	11.3
1977 spring	13.6	12.8	14.3	12.8

Source: Bent(1978).

a. Mean scores: 0, insensitive; 20, very sensitive.
b. Mildew samples taken from 26 to 80 fields per region were applied to leaf segments grown from seed treated at five concentrations of ethirimol (100-8000 µg/g) under controlled conditions (see p.221-223). Data to 1974 from Shephard et al. (1975), in which further details are given. Later data from M. Woolner, D.R.E. Mills & A.M. Skidmore (personal communication).

(1975) in the 1974 survey that reduction in sensitivity of field populations tends to be associated with the survival of the less sensitive elements of a mixed population during and after a period of good disease control, rather than with failure of control. There was an early decrease in mean sensitivity in Scotland between 1973 and 1974; it is not known whether a similar early effect occurred in East Anglia, where widespread treatment started a year earlier than in Scotland. The mean sensitivity of the mildew populations of individual crops in East Anglia and Scotland was found to fluctuate in either direction as selection pressures changed. Sensitivity tended to be lowest at the earlier growth stages, when uptake of ethirimol and disease control were greatest.

Table 3 shows the range of responses found in East Anglia and Scotland in successive surveys from 1973 to 1977. Initially isolates from East Anglia differed widely in their responses. However, the range has since narrowed. Isolates of intermediate sensitivity have become predominant, and both the more resistant and the highly sensitive isolates seem to have disappeared. This effect is reminiscent of the action of stabilizing selection for pathogen virulence, which can lead to the predominance of strains with intermediate degrees of virulence (Van der Plank, 1968; Crill, 1977). One might speculate that the extreme forms are less competitive in the field; that the most sensitive isolates were eliminated by usage of the fungicide; and that the most resistant forms, which may have increased initially, declined when, in 1973, ethirimol was no longer recommended for use on winter barley. Wolfe & Dinoor (1973) also observed differences in sensitivity to ethirimol amongst barley mildew isolates, and advised that ethirimol should no longer be applied to winter barley,

Table 3. Distribution of sensitivity to ethirimol in powdery mildew samples from barley fields.

Survey	Distribution [a] of sensitivity scores					
	<6	6-9	9-12	12-15	15-17	>17
East Anglia						
1973	5	14	41	21	12	7
1974	1	16	60	13	7	2
1975	0	3	55	37	5	0
1977	0	0	19	76	4	1
Scotland						
1973	0	4	9	12	54	21
1974	0	26	21	30	12	12
1975	0	6	50	38	6	0
1977	0	0	14	80	6	0

Source: Bent(1978).

a. Values are percentages of samples (from treated and untreated fields) in each category. For explanation of sensitivity score and sources of data see Table 2.

to avoid selection of resistance in the periods between successive spring barley crops. This policy was followed as far as possible, between 1973 and 1978. The survey results show a similar reduction in Scotland since 1973 in the range of sensitivities, particularly of the most sensitive isolates; very resistant forms have never been detected in Scotland, where little winter barley is grown.

The more resistant forms appear to grow and sporulate normally under growth-room conditions, but a lack of competitiveness with more sensitive forms has been indicated in field experiments (Hollomon, 1978). Smith et al. (1977) reported that of two mildew strains differing in resistance to ethirimol, the more resistant strain persisted in the field longer than the more sensitive one. However, since comparisons with other strains were not included, it seems possible that an intermediate form was being compared with a very sensitive one. Variation in the response of barley mildew isolates to tridemorph has also been detected (Walmsley-Woodward, 1979a,b), but the resistant forms appear not to cause practical problems. Genetic studies on strains of barley mildew differing in response to ethirimol have been difficult to interpret, but suggest a complex, non-Mendelian system of genetic control (Hollomon, 1978). An association in barley mildew isolates between the frequency of ethirimol resistance and virulence to certain barley varieties has been noted by Wolfe & Dinoor (1973), and possible relationships between fungicide resistance and the growing of different cereal varieties deserve further study.

Discussion

It is interesting to contrast the rapid onset of failures of dimethirimol to control cucumber powdery mildew in Dutch glasshouses with the absence of problems arising from its use in Spain and the Middle East, and with the continued good performance of the closely-related ethirimol on U.K. barley crops. Wolfe (1975) pointed out that variations in environmental conditions and in the population dynamics of pathogens largely determine whether resistant forms become dominant. Experience now confirms that the following conditions favour increase of resistance:

- When mutants of the pathogen arise that not only are little affected by the fungicide applications, but also approximate ordinary forms in their ability to infect and multiply.
- When the fungicide exerts a potent selective action on fungal populations.
- When contact between the fungicide and the pathogen is prolonged, either by repeated application or because of persistence; when this contact extends to the whole population, without the ingress of untreated populations or of those treated with unrelated fungicides.

The year-round, almost universal use of dimethirimol in Dutch glass-

houses, coupled with near-perfect levels of control in the isolated glasshouse environment, may be seen with hindsight as a recipe for misfortune. The risk of control failure with single, annual applications of ethirimol in a proportion of open barley crops is clearly lower. Note here that the resistance problems of the benzimidazole fungicides also have been observed commonly in glasshouses or under other intensive horticultural practices, but rarely in cereal crops.

Autumn usage of ethirimol was re-introduced in the U.K. in 1979, in response to increased farmer interest in growing winter barley and maximizing its yields. A wider range of mildew fungicides is now in use in both winter and spring barley in the U.K. Also, the survey data suggest that less sensitive forms of mildew may not persist in the spring, when the activity of the autumn-applied ethirimol has declined. Nevertheless, with applications being made in the autumn- and spring-sown crops, it is important that the performance of this fungicide be kept under review.

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Case study 5: Penicillium decay of citrus fruits

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Abstract

Sodium *o*-phenylphenate (SOPP), *sec*-butylamine, thiabendazole, benomyl and diphenyl are applied to harvested citrus fruits to prevent infection by *Penicillim digitatum* and *P. italicum* during storage and marketing. Spontaneous mutations give strains of *Penicillium* that are resistant to one or more of these fungicides. These resistant mutants persist at low frequencies in the natural population, but under selection pressure created by the post-harvest fungicide treatments, they may become the dominant component of the population in packing houses. A single conidium of a resistant strain could produce under favorable conditions a progeny of about 10^8 spores in one disease cycle in the absence of the competing wild strain. SOPP-resistant strains of *Penicillium* are resistant also to diphenyl; benzimidazole-resistant strains are not controlled by thiabendazole, benomyl or thiophanate-methyl. Treatment of fruit with SOPP or benomyl before storage may cause an increase in the frequency of mutant strains that cannot be controlled by post-storage treatment with biphenyl or thiabendazole, respectively. If a strain resistant to the benzimidazole fungicides and SOPP or *sec*-butylamine is present in the population, a pre-storage treatment of SOPP or *sec*-butylamine may cause an increase in benzimidazole resistance of the *Penicillium* population during the storage period. Benomyl-resistant strains usually are less competitive than wild strains in untreated fruits that have been inoculated with a mixture of both strains. This interaction may explain the low frequency of benzimidazole-resistant strains in citrus groves that are not treated with these fungicides. Appropriate strategies for delaying the emergence of resistance are sanitation, fungicide programmes that do not favor the proliferation of resistant strains, and fungicidal compounds that the pathogens cannot escape readily by mutation.

Keywords: benomyl; *sec*-butylamine; carbendazim; diphenyl; green mould; imazalil; lemons; oranges; *Penicillium digitatum*; *Penicillium italicum*;

thiabendazole; thiophanate-methyl, sodium o-phenylphenate; sodium carbonate; sodium tetraborate.

Disease aetiology and biology of the pathogens

Green and blue mold of citrus fruits are incited by the ubiquitous fungi, *Penicillium digitatum* Sacc. and *P. italicum* Wehm., respectively. Green mold is the most important cause of post-harvest decay of citrus fruits produced in areas with scant rainfall during the period of fruit development. Blue mold is of lesser over-all importance, but it may become the major problem under environmental conditions or fungicide treatments that selectively suppress the development of green mold (Eckert, 1978). Penicillium molds are important also in humid production areas, but they tend to be overshadowed there by the stem-end rots, *Diplodia natalensis* P. Evans and *Phomopsis citri* Fawc. In nature, *P. digitatum* completes its life cycle only on citrus fruits, whereas *P. italicum* can infect an array of different fruits and vegetables. Spores (conidia) of *P. digitatum* and *P. italicum* are produced on the surface of diseased fruit lying on the ground in citrus groves or in packing houses. These spores can survive for months under dry conditions. They are transported by air currents to healthy fruits, and the surface of virtually every citrus fruit is contaminated with these spores at harvest time, but germination and infection occurs only at injured sites. Potentially, a single spore of *P. digitatum* inoculated into a susceptible fruit may give rise to progeny of about 10^8 spores in ten days under optimum environmental conditions. These spores serve as inoculum for infection of other citrus fruits and they may visibly contaminate adjacent fruit in the same container, giving rise to the problem 'soilage', which seriously reduces the market value of the fruit.

The hyphal cells of *Penicillium digitatum* and *P. italicum* are multinucleate, but each conidium contains only one nucleus. The nuclei usually are haploid, like other species of the class Deuteromycotina (Fungi Imperfecti). No true sexual stage has been reported for either *P. digitatum* or *P. italicum*. Some isolates of *P. italicum* may undergo a 'parasexual cycle' in which the hyphae of two compatible haploid isolates anastamose to form a heterocaryon. Some of the haploid nuclei fuse to form diploid mycelium. The diploids revert spontaneously to haploid segregants, accompanied by a recombination of the chromosomes among the progeny (Strømnaes et al., 1964; Beraha & Garber, 1980). This parasexual cycle has never been observed in *P. digitatum*, despite repeated attempts using techniques that have been successful with other *Penicillium* spp.

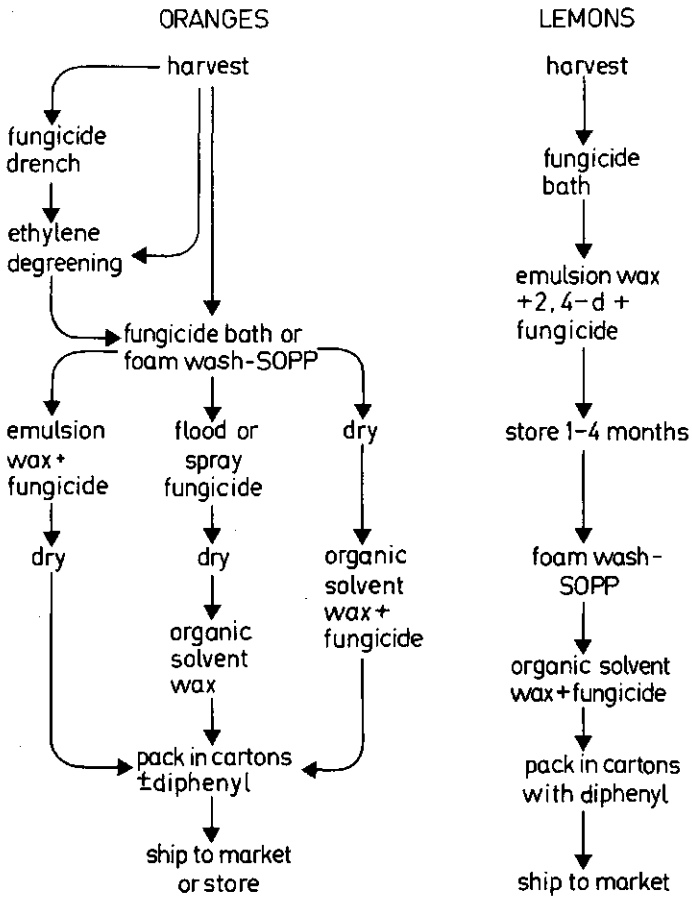

Figure 1. Flow diagram of handling of oranges and lemons after harvest showing fungicides applied and other treatments.

Conventional decay-control practices

Figure 1 shows the sequence of fungicide treatments on Californian citrus fruits after harvest to control *Penicillium* decay (Dawson & Eckert, 1977). The structural formulae of the recommended organic fungicides (diphenyl, *o*-phenylphenol, thiabendazole, benomyl, *sec*-butylamine and imazalil) are shown in Figure 2.

Early and late-season oranges often require degreening with ethylene gas to attain the uniform orange color that is preferred by most consumers. The harvested fruits are held for several days (before cleaning) in an atmosphere containing ethylene gas at concentrations of 5-10 µl/l at 20-25 °C and 90 % relative humidity. Since these environmental conditions are optimum for infection of the fruit by *Penicillium* spp., the fruit may be drenched with a solution of *sec*-butylamine (phosphate salt) or either thiabendazole or benomyl before degreening. Next, the fruit are cleaned

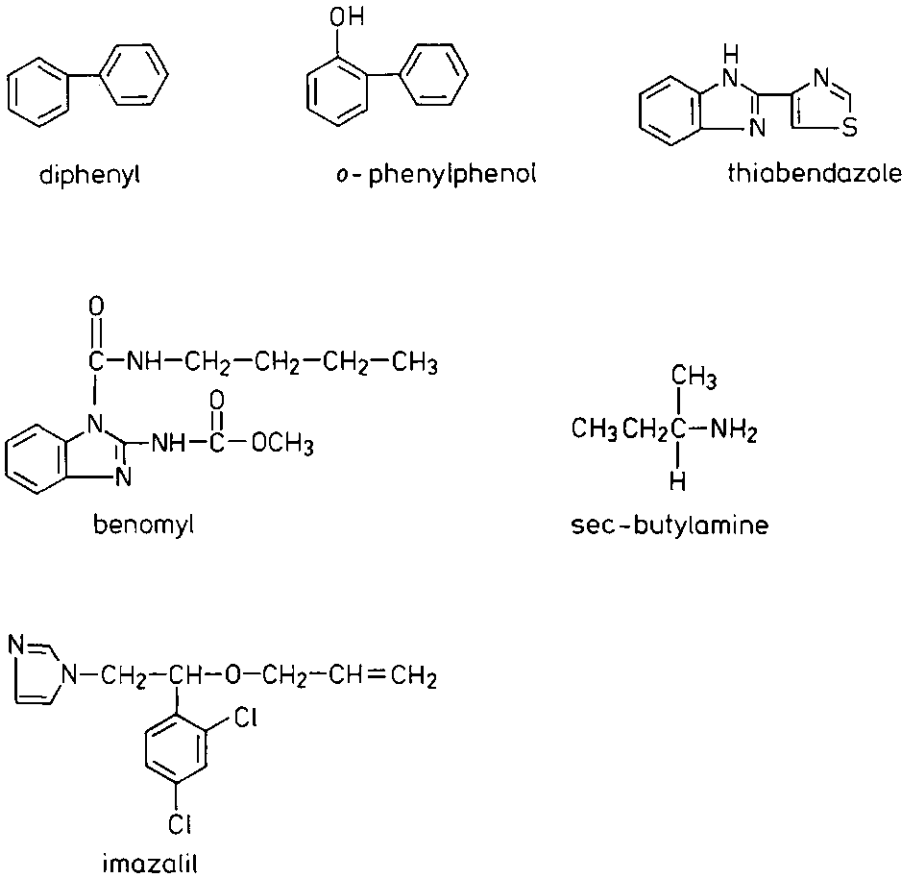

Figure 2. Structures of fungicides that are effective for control of post-harvest decay of citrus fruits.

and disinfested with one of the broad-spectrum fungicides - sodium carbonate, sodium tetraborate or sodium o-phenylphenate (Eckert, 1977, 1978). The fruit are rinsed with fresh water and covered with a hydrophobic coating ('wax') containing a fungicide, e.g. thiabendazole (2-4 g/l), benomyl (1-2 g/l) or *sec*-butylamine (10-20 g/l). The 'wax'-fungicide treatment retards water loss and prevents *Penicillium* decay during storage and transport of the fruit to market. Finally, the fruit are placed in a fibreboard box lined with a paper sheet impregnated with the volatile fungistat, diphenyl.

Several variations of this sequence in commercial practice can have a major influence upon the development of fungicide-resistant strains of *P. digitatum* and *P. italicum*. Lemons produced in the coastal districts of California usually are stored for 1-4 months after harvest to improve fruit quality and to regulate supply and demand. Oranges and lemons may be stored for days or weeks after packaging for shipment, depending upon

the market demand for certain fruit sizes and grades. Since the residues of all of these fungicides are stable on fruit, the practice of storing fungicide-treated fruit creates intense selection pressure for the fungicide-resistance genes in the populations of *P. digitatum* and *P. italicum* in citrus packing houses.

Thiabendazole, benomyl or carbendazim, suspended in water or a wax formulation, are applied to citrus fruits harvested in all production areas except Japan. In Japan, benomyl or thiophanate-methyl are applied to satsuma mandarin fruit as a single spray before harvest; none of these fungicides are registered in Japan for post-harvest application. Pre-harvest sprays of benomyl have been used effectively in Florida also (Brown, 1977), but this has been discontinued since it could select for benzimidazole-resistant fungi that would reduce the effectiveness of the post-harvest benzimidazole treatment, which is necessary for successful marketing of the crop.

Thiabendazole is stable on citrus fruits, whereas thiophanate-methyl and benomyl undergo transformation to carbendazim (Figure 3), which is the stable residue on these fruit. Carbendazim appears to be the principal fungitoxicant present in plant tissue and in fungi that have been treated with benomyl or thiophanate-methyl (Vonk & Kaars Sijpesteijn, 1971; Hammerschlag & Sisler, 1973). Benomyl and carbendazim are roughly equally fungistatic on an equimolar basis, whereas thiabendazole is significantly less inhibitory to most fungi (Eckert & Rahm, 1979). Benomyl is more lipophilic than thiabendazole or carbendazim and, therefore, penetrates hydrophobic barriers (wax and cuticle) on the plant surface more readily than thiabendazole or carbendazim (Eckert et al., 1979). For this reason, benomyl is more effective than the other benzimidazole fungicides in situations that require penetration of the fungicide into the plant tissue, e.g. eradication of latent infections, internal protective action and sporulation inhibition.

Benzimidazole fungicides have been highly effective against *Penicillium* decay of citrus fruits, as well as the stem end rots incited by *Di-*

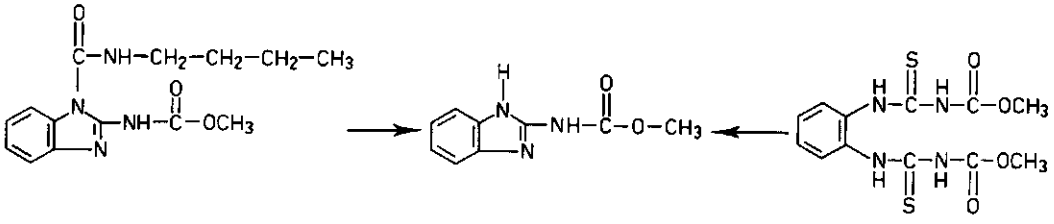

Figure 3. Transformation of benomyl and thiophanate-methyl to carbendazim.

plodia natalensis and *Phomopsis citri*. They are somewhat more effective against *P. digitatum* than *P. italicum* (Gutter, 1975), and they are inactive against sour rot (*Geotrichum candidum*), *Alternaria* rot (*Alternaria citri*), and brown rot (*Phytophthora citrophthora*). These diseases may become more prominent in fruit lots that have been treated with benzimidazole fungicides and then stored for several weeks (Brown, 1977).

Resistance to diphenyl and sodium o-phenylphenate

Farkas & Aman (1940), in Israel, reported that mutants with stable resistance to diphenyl arose spontaneously in pure cultures of *P. digitatum* that were exposed to diphenyl vapors. Littauer & Gutter (1953) observed that resistant mutants of *P. italicum* and *Diplodia natalensis*, as well as *P. digitatum*, appeared under similar conditions. Investigators in the United States have described strains of *P. digitatum* and *P. italicum* with different degrees of resistance to diphenyl. These variants were observed in cultures exposed to diphenyl vapor and in isolates collected in citrus groves and packing houses where neither diphenyl nor related fungicides had ever been used (Duran & Norman, 1961; Harding, 1964; Harding, 1965; Smoot & Winston, 1967).

The existence of diphenyl-resistant mutants of *Penicillium* spp. was not regarded as a practical problem until the late 1960s, when certain shipments of Californian lemons packed with diphenyl-impregnated papers arrived at distant markets with high levels of *Penicillium* decay and sporulation. Harding (1962, 1965) observed that many of these shipments originated in packing houses where the lemons had been treated with sodium *o*-phenylphenate (SOPP) before storage for several months prior to shipment. Harding (1962) found that strains of *P. digitatum* that were resistant to diphenyl were also resistant to SOPP, as would be anticipated from the structural similarity of the two fungicides (Figure 2). Harding (1962) further demonstrated that residues of *o*-phenylphenol on SOPP-treated lemons were sufficient to suppress the growth of diphenyl-sensitive strains, thereby allowing the diphenyl-resistant mutants in the population to proliferate on the fruit. In a survey of Californian citrus-growing areas, Harding (1964) observed diphenyl-resistant variants of *P. digitatum* at a frequency of about 0.5 % of the isolates collected in citrus groves and packing houses that had never been exposed to SOPP. Packing houses where SOPP had been used had a resistant strain frequency of 14-18 % of the *P. digitatum* population. Sixty percent of the *Penicillium* isolates were resistant to diphenyl in certain packing houses that had used SOPP continually over a period of years. These observations explained the failure of the diphenyl treatment to control green mold on fruit shipped from these packing houses. Diphenyl-/SOPP-resistant isolates of *P. italicum* were rarely recovered from the atmosphere of Califor-

nian packing houses, which explains why this species was not involved in the diphenyl-resistance problem. Diphenyl-resistant strains of *P. digitatum* have been reported in Florida, but these isolates have not caused a significant commercial problem because SOPP-treated fruits are not stored routinely in the packing houses there (Smoot & Winston, 1967; Houck, 1977). Thus, there is little opportunity for selection and dissemination of diphenyl-/SOPP-resistant mutants in Florida.

Beraha and Garber (1966, 1980) investigated the genetics of SOPP resistance in *P. italicum* and *P. expansum* (an apple-fruit pathogen) by means of the parasexual cycle in these species. In *P. italicum*, SOPP resistance is due to a single Mendelian gene that is dominant in heterozygous diploids. Although the pathogenicity of the SOPP-resistant mutants of *P. italicum* was not reported, earlier work (Beraha et al., 1964) had shown that approximately 44 % of color and auxotropic mutants of *P. italicum*, and 67 % of the mutants of *P. digitatum*, were virulent when inoculated into orange fruit. SOPP resistance in *P. expansum* has been attributed to three unlinked genes that are recessive in heterozygous diploids. Three mutants resistant to concentrations of SOPP of 50 µg/ml were avirulent to apple fruit, whereas two mutants resistant to 30 µg/ml were virulent (Beraha & Garber, 1966).

Resistance to sec-butylamine

An aqueous solution of *sec*-butylamine phosphate (pH 9) is applied to oranges and lemons after harvest to control *Penicillium* molds during storage. The treatment may be applied to oranges before degreening with ethylene gas and to lemons before storage (Dawson & Eckert, 1977; Eckert, 1977, 1978). Both applications create a situation that is highly favorable for the selection and proliferation of *sec*-butylamine-resistant strains of *P. digitatum* and *P. italicum* in the natural population. Therefore the severe resistance problem that developed within several years of the introduction of this fungicide should have been anticipated.

Resistance to benzimidazole fungicides

Benzimidazole fungicides have been used extensively over the past 10 years to control post-harvest decays of citrus and other fruits (Eckert, 1977; Eckert, 1978). The best known compounds in this group of fungicides are benomyl, thiabendazole, carbendazim and thiophanate-methyl (Figures 2 and 3). Benzimidazole-resistance in *Penicillium* spp. was observed in Californian citrus packing houses about 15 months after thiabendazole was incorporated in the wax applied to lemons before storage (Houck, 1977). Harding (1972) reported that thiabendazole-resistant isolates of both *P. digitatum* and *P. italicum* occurred with high frequency in the atmosphere

of lemon packing houses that used thiabendazole intensively as a pre-storage treatment for lemons. These isolates were not controlled by treating inoculated fruit with high concentrations (3 g/l) of thiabendazole. Benzimidazole-resistant isolates of *P. digitatum* and *P. italicum* have been isolated also in Florida (Smoot & Brown, 1974), Israel, Australia (Muirhead, 1974; Wild & Rippon, 1975) and Japan (Kuramoto, 1976). A survey of the frequency of occurrence of benzimidazole-resistant isolates of *Penicillium* on decayed citrus fruit in all major production areas has revealed the magnitude of the problem (Table 1). Benzimidazole-resistant strains of *P. expansum* have been reported on harvested apples and pears in Oregon (Bertrand & Saulie-Carter, 1978), New York (Rosenberger & Meyer, 1979; Rosenberger et al, 1979), and Australia (Wicks, 1977; Koffman et al., 1978). Most observations of benzimidazole-resistant strains of *Penicillium* spp. have followed intensive use of thiabendazole or benomyl over a period of some months, but Kuramoto (1976) reported a severe resistance problem on satsuma mandarins in Japan following application of a single annual spray of thiophanate-methyl immediately before harvest. Several investigators have recorded the isolation of benzimidazole-resistant strains from citrus groves and packing houses where benzimidazole fungicides had never been used (Harding, 1972; Kuramoto, 1976; Wild, 1980).

Benzimidazole-resistant isolates of *Penicillium* spp. found in packing

Table 1. Benzimidazole-resistant strains of Penicillium digitatum isolated from citrus fruits shipped to Rotterdam.

Origin of fruit	Number of isolates	Proportion of isolates resistant to thiabendazole (%)	Proportion of isolates resistant to benomyl (%)
Algeria	27	37	30
Argentina	142	49	39
Australia	2	100	100
Brazil	86	33	19
California	388	55	50
Chile	13	15	8
Cuba	19	37	26
Cyprus	50	14	12
Egypt	22	27	14
Florida	65	28	15
Greece	57	16	9
Honduras	24	54	29
Israel	99	90	80
Italy	73	49	44
Morocco	56	57	43
South Africa	115	77	32
Spain	146	31	16
Texas	57	35	26
Turkey	7	29	14
Uruguay	43	58	51

Source: McDonald et al. (1979).

houses are usually more sensitive to benomyl than to thiabendazole (Harding, 1972; Houck, 1977; Wild, 1980) although some isolates of *P. digitatum* and *P. italicum* are less sensitive to benomyl than to thiabendazole (Muirhead, 1974; Wild & Rippon, 1975). Furthermore, some thiabendazole-resistant mutants of *P. expansum* produced by mutagenic agents in vitro were more sensitive to benomyl than the wild type strain (van Tuyl, 1975; van Tuyl, 1977). This phenomenon is known as negatively correlated cross-resistance. Most of the benzimidazole-resistant mutants that have been isolated in fruit packing houses are sensitive to the other post-harvest fungicides, sodium *o*-phenylphenate, *sec*-butylamine, and imazalil (Smoot & Brown, 1974; Wild & Rippon, 1975; Harding, 1976; Wicks, 1977). Less frequently, isolates of *Penicillium digitatum* that are multiply-resistant to benzimidazole fungicides, SOPP and *sec*-butylamine have been reported (Davé et al., 1980, Harding, 1976; Wild, 1980).

In general, benzimidazole-resistant strains of *Penicillium* spp. that have been isolated from packing houses have been as virulent as benzimidazole-sensitive isolates of the same species (Muirhead, 1974; Wild & Rippon, 1975; Wicks, 1977; Koffman et al., 1978). However some benzimidazole-resistant strains of *P. expansum* collected from rotting apples and pears were less virulent than sensitive strains, especially when tested on certain varieties of apples (Bertrand & Saulie-Carter, 1978; Rosenberger & Meyer, 1979). Beraha & Garber (1980) produced mutants of *P. italicum* by treatment of conidia with ultra-violet light and nitrosoguanidine. They isolated mutants with low (L), intermediate (I) and high (H) resistance to thiabendazole based upon the ability of the mutants to grow on a medium amended with concentrations of thiabendazole of 0.5, 7.0 or 500 µg/ml, respectively. Thiabendazole resistance in this fungus involved two or three linked genes. Beraha & Garber (1980) found that a sensitive (wild) strain could not be mutated directly to an intermediate or high level of thiabendazole resistance. Rather, it was obligatory that gene L first undergo mutation to give low-level mutants that could be mutated again to give strains with intermediate and high resistance.

Levels of benzimidazole resistance in natural populations of P. digitatum

Wild (1980) investigated the characteristics of the natural population of benzimidazole-resistant strains of *P. digitatum* in California. Approximately 130 carbendazim-resistant isolates of *P. digitatum* were collected in packing houses and citrus groves in California and classified according to dosage required to alter colony appearance, ED_{50} for inhibition of growth rate, minimum growth-inhibiting concentration (MIC) and dosage required to inhibit sporulation. The ED_{50} values (µg carbendazim/ml) for the recognized categories of resistance were: Category 0 (wild type) - 0.03; Category I - 1.3; Category II - 5.5; Category III - 6.5; Category

IV - 40. Isolates resistant to carbendazim were cross-resistant to thiabendazole at appropriate concentrations, but the relative resistance of each strain to the two fungicides was not constant. Isolates that belonged to carbendazim resistance Category II were the most resistant to thiabendazole (ED_{50}=60 µg/ml). None of the isolates were negatively cross-resistant. Isolates in Category II and III could not be separated solely on the basis of ED_{50} or dosage required to alter colony appearance. However, these categories were readily differentiated when the additional criteria of MIC and sporulation inhibition were applied to the classification. Approximately 5 % of the resistant isolates obtained from packing houses fell into Category I, 33 % into Category II, 58 % Category III and 4 % in Category IV. In a similar survey of packing houses in Japan, Kuramoto (1976) found that the MIC of benomyl for the great majority of resistant isolates was 400-800 µg/ml (which transforms into 263-527 µg/ml carbendazim), approximately 9-18 times greater than for the most abundant category (Category III, MIC = 30 µg/ml) of resistant strains in California. Wild (1980) also collected spores from citrus groves where benomyl and thiabendazole had never been applied, to assess the range of carbendazim-resistance in the wild population of *P. digitatum*. Only a few carbendazim-resistant isolates (Category III) were recovered when 10^8-10^9 spores from three citrus groves were plated on medium ammended with carbendazim at a concentration of 1 µg/ml.

Isolates belonging to resistance Category IV were less infectious than isolates belonging to Categories 0, I, II or III when spores (10^5/ml) from a pure culture of each strain were inoculated into untreated oranges. Although the benzimidazole-sensitive strains of *P. digitatum* were well controlled by the treatment of inoculated fruit with carbendazim (as benomyl) concentrations of 0.03 g/l or thiabendazole concentrations of 0.08 g/l, none of the benzimidazole-resistant strains, irrespective of resistance category, were controlled by carbendazim at 1-2 g/l (as benomyl) or thiabendazole at 2-4 g/l - concentrations used in commercial packing houses.

Competition between sensitive and resistant isolates in diseased fruit

The low frequency of carbendazim-resistant isolates in the natural population of *P. digitatum* in citrus groves prompted an investigation of the competition between carbendazim-sensitive and -resistant isolates in infected oranges (Wild, 1980). Healthy oranges were inoculated with a standarized mixture of two or more strains of *P. digitatum*, incubated at 26 °C for 5 days, and a representative sample of the spore population was collected from the surface of the diseased fruits. A second lot of healthy oranges was then inoculated with a suspension of these spores (first generation). The relative abundance of fungicide-resistant and -sensitive

spores in the population of each spore generation was determined by plating a sample of the spores into potato-dextrose agar amended with carbendazim or another fungicide. In some experiments the fruit were treated with a fungicide after they had been inoculated with each spore generation. Treatment of oranges with a benomyl concentration of 0.5 g/l (which transforms into 0.33 g/l carbendazim), after inoculation with a mixture of carbendazim-resistant and -sensitive spores, resulted in almost total selection for the resistant isolate in the first spore generation (Figure 4). The addition of a second unrelated fungicide to the benomyl treatment, as recommended by some authorities to control resistance development, had no measurable influence upon the selection and proliferation of the resistant strain. Treatment of the inoculated fruit with a non-benzimidazole fungicide alone, or no fungicide at all, usually resulted in a rapid decline in the frequency of the benzimidazole-resistant strain in subsequent spore generations (Figure 4). The rapid disappearance of the resistant strains from the spore population was unexpected because the resistant isolates were as virulent as the carbendazim-sensitive isolates.

These investigations show that resistant strains can be rapidly selected from a natural population of *P. digitatum* spores under the selection pressure of a benzimidazole fruit treatment. Furthermore, the weak competitive behaviour of resistant strains in mixture with sensitive strains in infected fruit (not treated with a benzimidazole fungicide) could explain the low frequency of benzimidazole-resistant strains in the natural spore populations of citrus groves. Benzimidazole fungicides have never been used for control of field diseases of citrus trees in California, but enormous numbers of benzimidazole-resistant spores have been discharged into the atmosphere from packing houses.

Despite the competitive advantage of benzimidazole-sensitive strains over resistant strains in untreated fruit, it has been observed that benzimidazole-resistant strains do not disappear from the spore population of packing houses after the benzimidazole fungicides have been replaced by sodium *o*-phenylphenate (SOPP) or *sec*-butylamine. Wild (1980) found that many carbendazim-resistant isolates collected in these packing houses were also resistant to *sec*-butylamine or SOPP. Oranges were inoculated with a mixture of a strain sensitive to carbendazim, *sec*-butylamine and SOPP and a strain that was doubly-resistant to both carbendazim and *sec*-butylamine. Treatment of fruit inoculated with this spore mixture with either benomyl or *sec*-butylamine resulted in an immediate increase in the frequency of the doubly-resistant strain in the population of the first spore generation (Figure 4, RRS), whereas treatment with unrelated fungicides did not create selection pressure for either of the resistance genes for carbendazim or *sec*-butylamine. Treatment of oranges inoculated with a mixture of sensitive and carbendazim/SOPP doubly-resistant spores

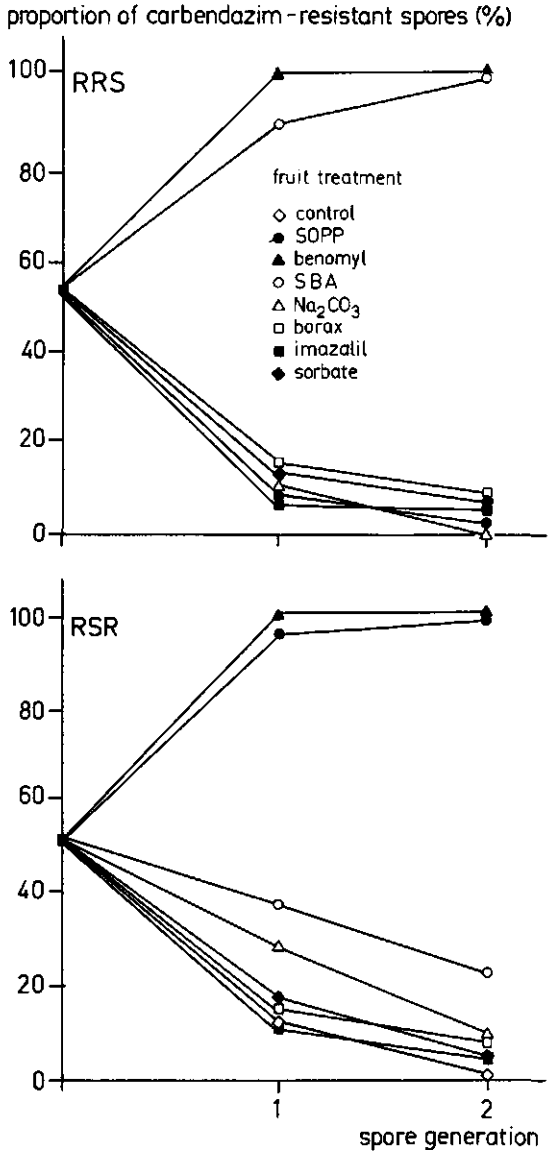

Figure 4. Effect of treatment of several lots of Valencia oranges with the same fungicide upon the frequency of carbendazim-resistant spores of P. digitatum in the population after two disease cycles (spore generations). The fruit were treated with the specified fungicides after infection was established by inoculation with a spore mixture containing: Upper plot, a fungicide-sensitive strain and a strain doubly-resistant to carbendazim and sec-butylamine but sensitive to SOPP (RRS); Lower plot, a fungicide-sensitive strain and a strain doubly-resistant to carbendazim and SOPP but sensitive to sec-butylamine (RSR) (Wild, 1980).

with either carbendazim or SOPP alone had an analogous influence on the build-up of SOPP/carbendazim doubly-resistant strains in the population (Figure 4, RSR).

Factors promoting the build-up of a fungicide-resistant population

The development of a serious resistance problem depends upon the rapid multiplication and dissemination of the resistant strain to many other hosts treated with the same fungicide. The number of resistant propagules (spores) of *P. digitatum* can increase by about a factor of 10 to the power 8 in 10 days, and they are readily disseminated to other selective environments, since all fruits are treated uniformly with the same fungicide. The points in the handling sequence for citrus fruit that are most favorable to the development and proliferation of fungicide-resistant strains of *Penicillium* are shown in Figure 1.

Early-season oranges are drenched with a solution of thiabendazole, benomyl, or *sec*-butylamine and then placed in an ethylene degreening room at 22-25 °C. The fungicide residues suppress the sensitive strains that are dominant in the field population of *Penicillium* spp., thereby permitting the fungicide-resistant strains to colonize the fruit and sporulate after several days. When, after degreening, the fruit are dumped from the field boxes the spores of the resistant strains are borne by the air to the newly-harvested fruit as they are placed in the degreening room. After de-greening, the fruit contaminated with benzimidazole-resistant spores receive a weak disinfectant treatment and then pass into the packing house where they receive a second treatment with a benzimidazole fungicide in 'wax', before shipment to market. Inevitably, the first benzimidazole treatment selects for resistant strains and, therefore, the second treatment fails to control fruit decay.

Figure 1 shows that lemon fruit are washed in a bath of SOPP or sodium carbonate, coated with a storage 'wax' containing 2,4-D and thiabendazole, benomyl or *sec*-butylamine, and stored at 15 °C for several months. When the lemons are removed from storage, benzimidazole-resistant strains that are selected during the storage period contaminate the sound fruit, leading to the failure of their second benzimidazole treatment (in wax). Similarly, the application of SOPP to lemons before storage may select for SOPP-/diphenyl-resistant strains that may greatly reduce the effectiveness of the diphenyl treatment during shipment. Another serious problem, and perhaps more difficult to solve, is the contamination of lemons entering storage with spores of resistant strains that have been selected by fungicide residues on the preceeding lot of fruit during storage.

A final scenario for the development of a serious fungicide-resistance problem is when lemons or oranges are packed for market, but cannot be sold because of weak demand. These fruit remain in cold storage for several weeks before they are returned to the packing house for removal of decayed fruit from the boxes prior to shipment. Fungicide-resistant *Penicillium* spp., selected during storage by the fungicide deposits on the

fruit, contaminate all fruit passing through the packing house, resulting in a great reduction in effectiveness of the final fungicide treatments.

Detection and evaluation of the fungicide-resistance problem

It is common practice to monitor the level of fungicide resistance in the population of *Penicillium* spores in packing houses. Petri dishes containing potato-dextrose agar, amended with concentrations of benomyl of 10 µg/ml or *sec*-butylamine (HCl salt) of 500 µg/ml, are exposed to the atmosphere of the packing house for a length of time that depends upon the size of the *Penicillium* spore population. Dicloran at a concentration of 3 µg/ml may be added to the medium to restrict the radial growth of the *Penicillium* colonies. The exposed plates are incubated for several days at 25 °C and the colonies counted. The number of colonies that develop on the fungicide-treated medium compared with the colonies that appear on paired plates containing basal medium only indicates the level of fungicide-resistance in the population in the packing house. Diphenyl and sodium *o*-phenylphenate resistance can be monitored in a similar fashion, except that it may be more convenient to place crystals of diphenyl in the lid of the inverted petri dish than in the growth medium.

Strategies to avoid build-up of resistant strains

Since the effective use of the benzimidazole fungicides is essential for the success of current procedures in marketing citrus fruits, several strategies for the control of resistant strains of *Penicillium* are being investigated.

Sanitation

During periods of the year when fruit are not harvested, packing houses can be disinfested with broad-spectrum antimicrobial agents (e.g., formaldehyde, quarternary ammonium compounds) that cannot be used when fruit are present. This assures a very low population of spores in the packing house at the beginning of the harvest period, and results in high efficiency of all post-harvest fungicide treatments. However, within a month of operation of the packing house the level of fungicide-resistance in the *Penicillium* population begins to build-up, accompanied by a gradual deterioration in effectiveness of one or more fungicide treatments. Eventually, a point is reached where the decay control programme is no longer cost-effective. This economic threshold can be delayed by excluding contaminated fruit from the packing house and by a rigorous sanitation programme inside the packing house, consisting of a daily disinfestation of the fruit-handling equipment with a broad-spectrum sanitizing agent.

These practices do not reduce the frequency of resistant strains existing in the population, nor do they influence intrinsically the selection process. But sanitation does reduce the absolute number of resistant spores in the packing house to the same extent that it reduces the total spore population. A reduction in the number of resistant spores should delay the development of a fungicide-resistance epidemic because the probability of successful infection (by a resistant strain) depends upon the spore concentration (in relation to the numerical inoculum threshold of infection) at a limited number of injured sites on the fruit surface. A reduction in total spore population also should delay the build-up of resistance, since fungicide-sensitive spores can increase the probability of infection of benzimidazole-treated fruit by a benzimidazole-resistant strain (Wild, 1980).

Non-selective treatments

The elimination of selective fungicides and structurally-related compounds from the post-harvest fungicide programme should produce a decrease in the frequency of the resistance strains, because of the competition of the sensitive strains (Figure 4). This strategy would be counter-productive, however, since heavy decay losses would arise through fruit infection by the uncontrolled fungicide-sensitive strains of *Penicillium*. Furthermore, reduction of the fungicide-resistant population to a level that could be controlled by a benzimidazole treatment would require several disease cycles, and restoration of the benzimidazole treatment could cause an immediate increase in the frequency of the resistant strains (Figure 4).

Heated solutions of borax and sodium carbonate reduce fruit infection by *Penicillium*. Moreover, they have not been implicated in a fungicide-resistance problem. The application of borax is rather limited today because of the possibility that it will pollute the ground-water, but sodium carbonate (30 g/l, at 43 °C) is an effective treatment for disinfecting citrus fruit before they are brought into the packing house. None of the other well-known multi-site fungicides (e.g., captan, maneb, chlorothalonil) are effective against *Penicillium* decay.

Mixtures of selective and non-selective fungicides

This strategy has been suggested by several authorities to delay the build-up of resistant strains of pathogens in the field. Unfortunately, there is little published data to support this contention. Mixtures of systemic fungicides (e.g. benomyl) and protective fungicides (e.g. captan) may prevent penetration of benzimidazole-resistant strains into treated fruits, thereby reducing the total pathogen population, including the

benzimidazole-resistant component (Koffman et al., 1978; Rosenberger et al., 1979). However, the combination of benzimidazole and protective fungicides does not appear to be a suitable strategy for control of resistant strains of *Penicillium* on citrus and other fruits that may be infected with a mixture of resistant and sensitive strains before the fungicide treatment is applied. Non-systemic fungicides cannot eradicate deep-seated infections nor inhibit sporulation efficiently (Brown, 1977; Eckert et al, 1979). Thus, the rapid build-up of benzimidazole-resistant strains following treatment of infected fruit with a mixture of a benzimidazole and a protective fungicide is predictable. The combination of two unrelated systemic fungicides, such as benomyl and imazalil (Figure 2), each with a unique mechanism of action, may be a more promising, but expensive, strategy for the control of resistant strains (Laville et al., 1977; Koffman et al., 1978; Rosenberger et al., 1979). The possibility of combining two synergistic compounds or two fungicides that exhibit negatively correlated cross-resistance is an intriguing, but unproven, strategy for controlling fungicide-resistant plant pathogens (Dekker, 1977; Georgopoulos, 1977).

Alternation of two or more unrelated fungicides

This strategy involves the application of two or more unrelated fungicides in a sequence that, hopefully, will suppress strains of the pathogen that are resistant to the final fungicide in the series. This procedure is feasible for citrus fruits because they are already treated with several fungicides after harvest. For example, lemons may be treated with SOPP and/or *sec*-butylamine before storage and, after storage, with SOPP, a benzimidazole fungicide, and diphenyl (Dawson & Eckert, 1977; Eckert, 1978). This sequence is required because the benzimidazoles are the only approved fungicides that can control sporulation of *Penicillium* on diseased fruits. The success of this schedule for lemons depends upon an efficient sanitation programme, since the build-up of a large population of SOPP- or *sec*-butylamine-resistant strains during storage could result in the selection of mutants that are doubly-resistant to one of these compounds and the benzimidazole fungicides (Figure 4). A pre-storage sequence of SOPP followed by *sec*-butylamine might be effective in suppressing in *Penicillium* population, since double-resistance to these two fungicides is relatively rare, which indicates that such doubly-resistant (SOPP/*sec*-butylamine) mutants may be poorly adapted as fruit pathogens (Wild, 1980). The multiple sites of action of SOPP and *sec*-butylamine should further discourage the development of strains resistant to both of these fungicides. At low concentrations, *sec*-butylamine inhibits pyruvic dehydrogenase in *P. digitatum* (Yoshikawa & Eckert, 1976; Yoshikawa et al., 1976); at higher concentrations the transport of amino acids into

the hyphae is inhibited (Bartz & Eckert, 1972). SOPP increases mitotic abnormalities in diploid strains of *Aspergillus nidulans*, which suggests that this inhibitor may adversely effect hereditary processes in other fungi as well (Georgopoulos et al., 1976; 1979). SOPP also inhibits several tricarboxylic acid (TCA)cycle enzymes in *P. italicum* (Rehm, 1969).

Fungicides that the pathogen cannot escape easily by mutation

Tests conducted over the past several years have demonstrated that imazalil (Figure 2) is highly effective in preventing infection of citrus fruits by strains of *Penicillium* spp. that are resistant to other fungicides, and also highly effective in suppressing sporulation of these fungi on diseased fruits (Harding, 1976; Laville et al., 1977). Imazalil-resistant strains of *P. digitatum* were not observed following treatment of spores with mutagenic agents that produce mutants resistant to other fungicides (van Hoorn & Eckert, unpublished data). van Tuyl (1977b) found that the mutational frequency for resistance to imazalil in *P. expansum* was high (70×10^{-6}) compared to that for benomyl (1×10^{-6}), but that the increase in resistance level accompanying a single gene mutation was rather small. The increase in resistance level (ED_{50} mutant ÷ ED_{50} wild type) for a single mutation was 3 300 for benomyl and 4 for imazalil. Mutations for imazalil resistance at different loci yielded strains that were pleiotropically resistant or hypersensitive to other fungicides. Imazalil inhibits ergosterol synthesis in *P. italicum* (Siegel & Ragsdale, 1978), *P. expansum* (Leroux & Gredt, 1978), and *Ustilago avenae* (Buchenauer, 1977). The striking pleiotropic effects accompanying imazalil resistance in *A. nidulans* may be related to a site of action involving the cell membrane (van Tuyl, 1977A). Other fungicides with a similar mechanism of action have not yet encountered a practical problem with pathogen resistance (Dekker, 1977).

A recent report of imazalil-resistant strains of *P. expansum* causing decay of pears in Israel (Prusky et al., 1980) makes it clear that even this fungicide must be used cautiously to avoid the development of resistance. Nonetheless, genetic and biochemical investigations of imazalil and related inhibitors in fungi provide hope that the development of resistance in *Penicillium* spp. will be slow and involve mutants with diminished virulence. Relatively high dosages of imazalil should be applied to fruit to suppress the development of low-level resistant mutants, which, if allowed to persist in the population, might acquire additional resistance genes, resulting in strains with a higher level of resistance.

All strategies for use of imazalil should include a strict sanitation programme to minimize the resistance-gene pool available for selection by imazalil. Trials are now under way in lemon packing houses to evaluate several fungicide sequences including imazalil and benzimidazoles. Ta-

Table 2. Estimated decay losses in 1979 of Californian citrus fruits treated with currently used fungicides or imazalil.

Citrus fruit	Decay losses (number of 35-pound cartons)		Decrease in decay (%)
	currently used fungicides [a]	imazalil [b]	
Lemons	5 323 400	129 108	98
Oranges	4 424 000	1 659 000	63
Grapefruit	627 000	235 000	63
Tangerines	110 400	55 200	50
Total	10 484 800	3 240 280	

a. SOPP, sec-butylamine, thiabendazole and benomyl.
b. Imazalil was not used extensively in California in 1979. Estimates of decay losses, if all fruit had been treated with imazalil, were made by California Citrus Quality Council (Anonymous, 1980).

ble 2 contains estimates of the potential reduction in decay anticipated if all California citrus fruits had been treated with imazalil in 1979. A major portion of the beneficial effects of imazalil would result from the control of *Penicillium* strains that are resistant to other fungicides currently in use.

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Index