SENSITIVITY OF ERYSIPHE GRAMINIS F.SP. TRITICI TO DEMETHYLATION INHIBITING FUNGICIDES IN EUROPE

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ABSTRACT

To study the sensitivity of the wheat mildew pathogen to demethylation inhibiting fungicides (DMIs) on a European scale, a monitoring programme has been followed since 1986. Single-colony isolates from different regions were analysed. In relation to standard isolates with wild-type sensitivity, the resistance factor (RF) and for samples the mean/median RF (MRF) were calculated. The current results show evident regional differences in the DMI sensitivity of the pathogen, as well as differences in its resistance level to several DMI compounds. The sensitivity distribution within Europe is devided into three large areas. There is the north-west with the highest MRF level, the east with lower MRFs, and the south with a sensitivity level close to that of the standard isolates. Results are postulated to be due to selection pressures and wind dissemination of the pathogen. In recent years the DMI sensitivity of wheat mildew has partly stabilized at a reduced level in North-western Europe. Genetic recombination of the pathogen is considered to be the main reason.

INTRODUCTION

Powdery mildew on wheat, caused by Erysiphe graminis f.sp. tritici, appears periodically from year to year in all European wheat-growing areas. Because of its frequently epidemic occurrence, wheat powdery mildew is often one of the main target pathogens for chemical disease control. In the early 1980s, new fungicides with modern active ingredients, namely the demethylation inhibitors (DMIs) triadimenol and propiconazole, promised highly successful mildew control, and this led to their wide spread and common use. However, it was soon realised that the pathogen showed unexpectedly good adaptability towards DMIs. First reports of a decrease in DMI-sensitivity (Bennet & van Kints, 1982; Buchenauer, 1983), as well as different regional observations and experiences, led to intense discussions at the beginning of this specific gradual evolution of fungicide resistance. Up to now, reduced DMI sensitivity of the wheat mildew pathogen has not, in general, led to disease control failures, but rather a change in efficacy of DMIs under some field conditions.

To determine the sensitivity situation of wheat powdery mildew towards different active compounds and to study the changes in sensitivity of pathogen populations with time, due to selection and wind dispersal of the pathogen, a European-wide monitoring programme, based at Weihenstephan, was started in 1986. A survey of current data on sensitivity to triadimenol, tebuconazole, cyproconazole and propiconazole is presented in this paper, as well as comparison with data from previous years.

MATERIALS AND METHODS

To produce representative data from different regional mildew populations, random samples were taken from the air above areas of interest. Conidio-spores were collected with a jet spore trap (Schwarzbach, 1979) mounted on the roof of a car. While passing through the regions, trapped spores fall onto segments of primary leaves of a highly susceptible

wheat variety placed in Petri dishes on water agar (0.6 % agar, 45 mg/l benzimidazole). The trapping distance within an area was approximately 100 km on average.

In the laboratory, the sampled spores grew up to single colony isolates (climate chamber: 18 °C, 10 µEinstein/m²s continuous light). They were transferred onto fresh leaf segments (water agar: 0,6 % agar, 35 mg/l benzimidazole) for storage and multiplication before testing.

For propiconazole, current sensitivity results were obtained from field samples. Mildew-infected leaves were sent from different sites in Ireland, UK, France and Germany by co-workers of Ciba. In the laboratory, conidio-spores of freshly-sporulating colonies were transferred onto leaf segments, and their sensitivities assayed as described below.

The sensitivity of each single colony progeny to triadimenol was determined on a test set of 3 cm long leaf segments. These were cut from the first leaf of ten day old seedlings grown from Baytan treated seed (200 µE/m²s continuous light, 20 °C). Only the middle sections of the primary leaves were used, because of uneven distribution of the fungicide in the leaf. Fungicide treatment was graded logarithmically by a factor of 2. Each test set was inoculated with one isolate using a mini inoculation tower. After 10 days' incubation (18 °C, 10 µE/m²s continuous light), disease coverage was scored relative to the untreated control, and the highest dose allowing $\geq$ 50 % sporulation was determined for each isolate. If available at least 30 isolates per sample (region) were analysed in this way.

In order to analyse sensitivity to the other active ingredients, seedlings were sprayed with different fungicide solutions, containing concentrations graded as above, one day before cutting and inoculation of the test sets. To avoid gas phase interactions among differently treated leaf segments, separate disposable Petri dishes of 6 cm diameter were used for each concentration for every single test set; each Petri dish contained leaf segments of 5 replicates. Thus a test set for analysing one isolate, involving e.g. 10 fungicide concentrations (including untreated control) consisted of 10 Petri dishes. Only during inoculation, the dishes of a test set were placed next to each other under a settling tower, and the leaves exposed to conidia for about 60 seconds. In this way, 10 isolates per sample were tested. After 10 days' incubation, each test set was scored for sporulating diseased area, and the LD50 of each test isolate was calculated by probit analysis.

Standard (wild-type) isolates were included in the sensitivity tests. They were obtained from the field in the 1970s, before the fungicides in question were commercialized, and therefore represent the sensitivity of the fungus in original, unselected populations. If the sensitivity of each test isolate is related to that of the standard isolates, a resistance factor, RF, can be calculated. In order to characterize each random sample, the median resistance factor was determined for triadimenol (seed treatment) and the mean (geometrical mean) resistance factor for the other DMIs (leaf treatment). The abbreviation MRF will be used below for both.

RESULTS

Since 1986, an extensive monitoring programme has been carried out to measure triadimenol sensitivity. When investigations were started, selection pressure had been present for some years. For North-western Europe, results from 1986 showed MRF values of about 15 for the most part, with a range of about 5 to 30 (Felsenstein, 1991; Felsenstein *et al.*, 1991). Only in the southern European regions investigated (South of France, North of Italy) MRFs still remained close to 1. In the following years, the evolution of resistance continued, mainly in North-western Europe, and led to the 1989 situation shown in Figure 1, with MRF values up to 90 and enormous regional differences in triadimenol sensitivity within Europe. Populations with sensitivities close to the level of the wild-type isolates could only be found south of the Pyrenees and the Alps. Apparent differences were also obtained between North-western and Eastern Europe, where MRFs remained at a level of about 10. The current sensitivity situation is presented in Figure 2. On the whole, only few striking

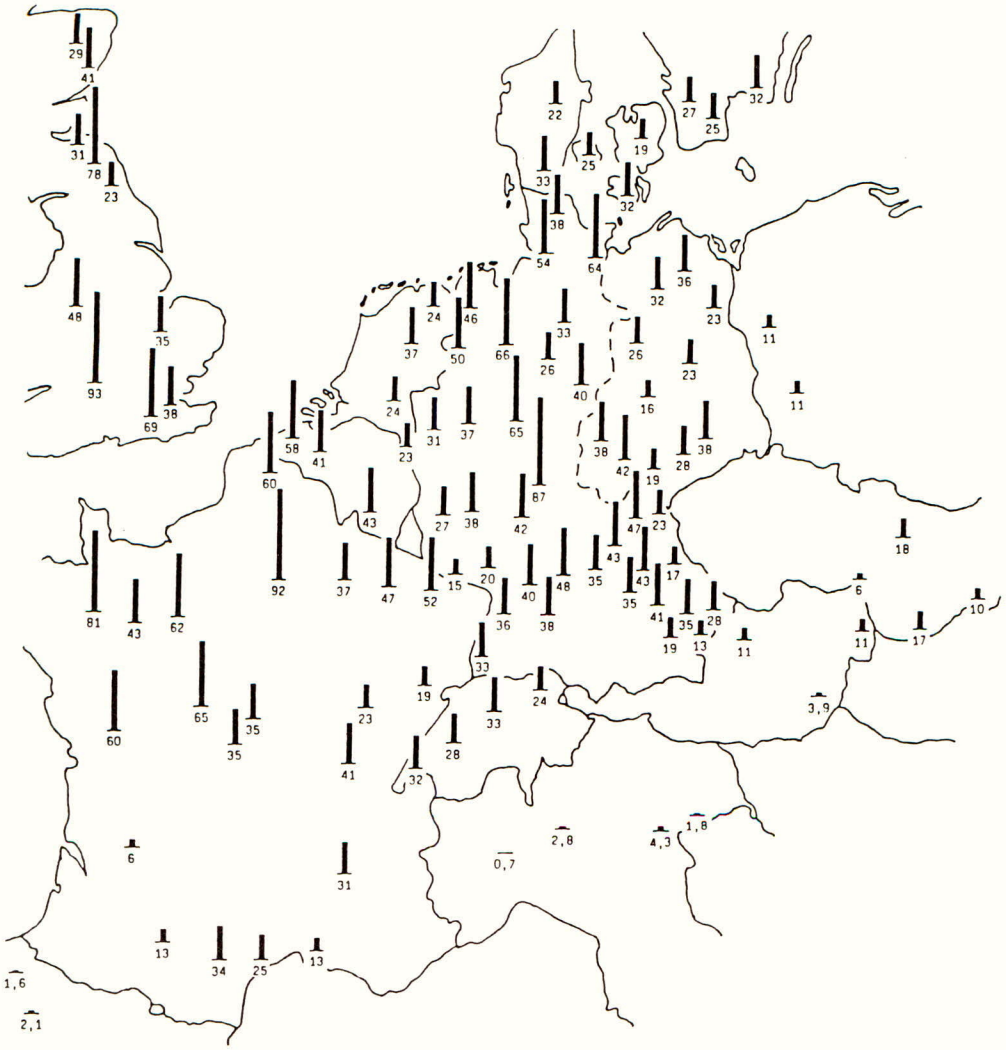

FIGURE 1. Regional differentiation of MRFs of wheat powdery mildew towards triadimenol, 1989

sensitivity changes have occurred on a European scale since 1989. Only in some areas of North-western Europe, namely Denmark and Eastern Germany, the resistance levels detected increased up to a MRF range from 30 to 70. Up to 1993 there were remarkable differences in resistance level between the north-west, east and south of Europe.

For tebuconazole MRF values of random samples from different regional wheat mildew populations are shown in Table 1. Investigations were started in 1990 as tebuconazole was launched commercially in Europe. At this time, MRF values were between approximately 10 and 15 in North-western Europe. MRF values in the east and south were distinctly lower. In the following years up to 1993, a slow shift in tebuconazole sensitivity has occurred for a number of populations. At present the MRFs of most investigated populations in North-west Europe vary around 20 and are still in contrast to those of the east and

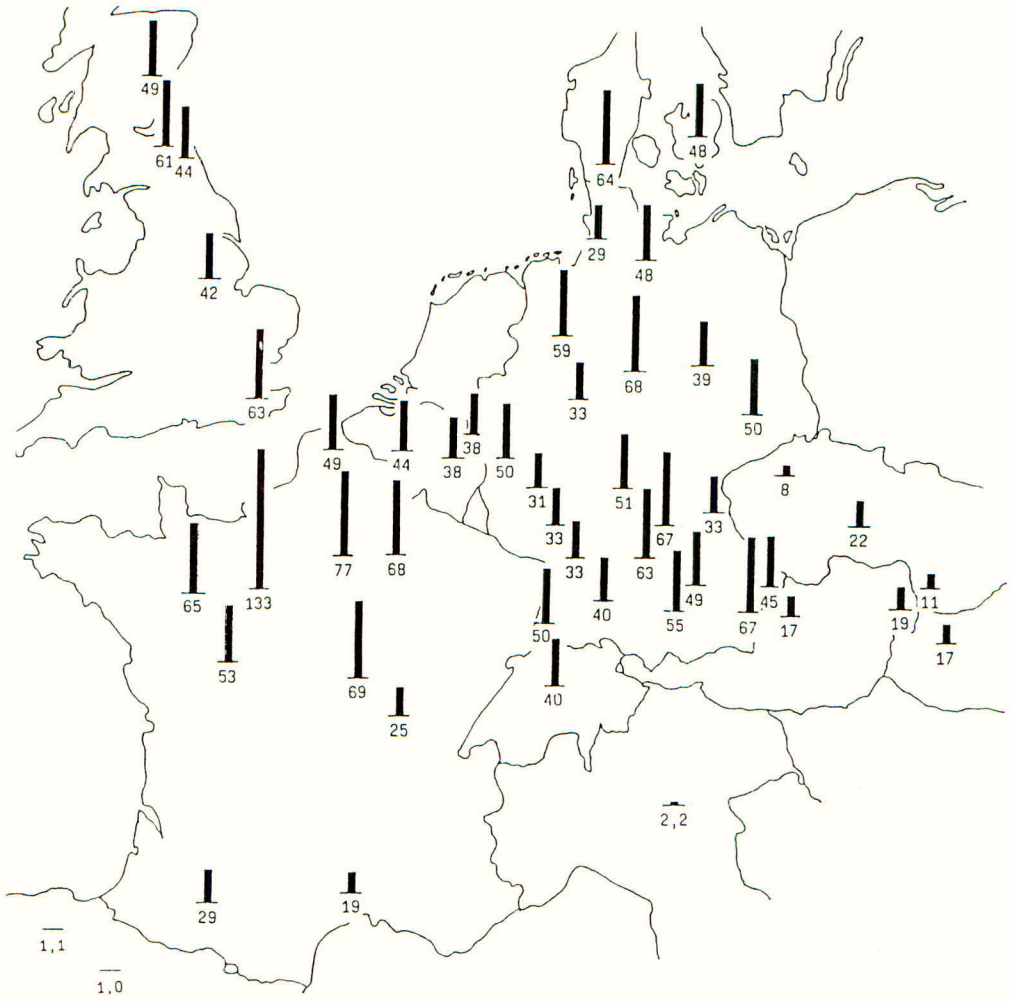

FIGURE 2. Regional differentiation of MRFs of wheat powdery mildew towards triadimenol, 1993 (values in northern Spain from 1992)

south. In comparison with the triadimenol-sensitivity data (see above), tebuconazole resistance factors are, in general, lower.

Cyproconazole is also an active compound which was introduced on the European market more recently than triadimenol. The MRF values of random samples from 5 different European wheat mildew populations between 1990 and 1993 are listed in Table 2. As with the results obtained with tebuconazole, MRF values had a level of about 10 in North-western Europe before the fungicide was launched. There is a clear difference between the north-west and the south of Europe as well. From 1990 to 1993, only slight changes in the populations investigated were observed.

Like triadimenol, propiconazole was first sold in Europe in the early 80s, and it has also been widely used. In Table 3, current data obtained from field samples are presented for North-west Europe, including the average MRF values of each country. Comparison between the four countries shows a relatively homogeneous sensitivity situation, with MRFs

TABLE 1. Mean resistance factors (MRFs) of random samples out of regional wheat mildew populations towards tebuconazole within Europe, 1990-1993

Region	1990	1991	1992	1993
GB:				
Edinburgh-Grantsh.	-	10.4	24.0	31.3
Cambridge-Dover	14.5	9.0	15.3	22.7
F:				
Calais-Mons/Lille	13.8	-	-	21.3
Paris-Reims	16.0	10.4	20.0	22.5
Bourges-Nevers	16.5	11.6	20.0	23.7
Narb./Auch-Toulouse	-	8.2	10.2	15.2
DK:				
Nyborg-Kopenhagen	12.8	8.2	12.3	14.0
D:				
Hamburg-Neustadt	16.0	15.2	20.0	23.2
Hannover-Kassel	-	22.0	17.0	21.0
Magdeburg-Halle	8.0	13.4	15.5	18.3
Nürnberg-Freising	11.8	9.2	11.3	17.2
A:				
Marchfeld/b. Wien	3.8	3.4	5.3	5.8
I:				
Verona-Venedig	1.5	1.4	2.7	3.8

TABLE 2. Mean resistance factors (MRFs) of random samples out of regional wheat mildew populations towards cyproconazole within Europe, 1990-1993

Region	1990	1991	1992	1993
GB:				
Edinburgh-Grantsh.	-	11.5	11.9	10.5
F				
Paris-Reims	10.9	9.8	10.9	12.5
D				
Hamburg-Neustadt	12.0	13.6	12.1	10.2
CH				
Baden-Bern	6.0	8.8	9.4	8.0
I				
Verona-Venedig	1.9	2.4	2.7	1.6

TABLE 3. Mean resistance factors (MRFs) of field samples of wheat mildew from different sites of Ireland, Great Britain, France and Germany towards propiconazole, 1993

Country	No of samples	MRFs (min. - max.)	average-MRF
IRE	10	10.1 - 23.1	18
GB	10	9.9 - 23.8	17
F	10	10.4 - 25.8	18
D	10	10.1 - 23.1	16

varying in each country between nearly 10 and 25. Results from air-borne spore samples, collected mainly in Southern Germany, confirm those obtained with field samples. Their MRF values seldom reached a level of 30 (Felsenstein, unpublished). Thus, despite selection pressure for more than one decade, European wheat mildew populations reached only a level of resistance to propiconazole similar or a little higher than to other fungicides which were introduced on the market recently. Comparison with triadimenol-sensitivity data shows that there is still a difference in resistance behaviour of the pathogen towards these two triazoles. However, for both compounds, only a few sensitivity changes have occurred in the populations of North-west Europe in the last few years, and resistance evolution has stopped in some areas.

DISCUSSION

The results show a clear picture of the current resistance situation of the wheat mildew pathogen towards DMI fungicides on a European scale. It has to be emphasized that within Europe there are populations with a relatively high level of resistance, as well as populations with wild-type DMI sensitivity. Data show a distinct division of Europe into 3 large areas. One of them is the north-west. In this area a more or less evident shift in sensitivity took place during the 1980s. The reason was partly a high selection pressure due to regionally extensive use of DMI fungicides. Until 1993, the highest median/mean resistance factors were obtained mainly in important wheat growing areas, even though it seems that wind dispersal of the pathogen causes increased mixing of neighbouring populations. Furthermore, comparison of data between the active ingredients investigated shows that the wheat mildew pathogen has reached different resistance levels towards the different compounds. It is also true for azoles which have been used on a similar scale and over a similar time period, like triadimenol and propiconazole. In contrast to reduction of sensitivity mainly caused by selection pressure, MRF values towards tebuconazole and cyproconazole, found at the beginning of the 1990s, can only be explained by positive cross resistance of the pathogen towards azoles. These relationships have been described for cereal mildews in several reports (Butters *et al.*, 1984; Buchenauer & Hellwald, 1985; Gisi *et al.*, 1986; de Waard *et al.*, 1986), and their findings are confirmed in this report. Moreover, data presented indicate that since the end of the 1980s, there is an evident reduction in the rate of sensitivity change of most wheat mildew populations in North-western Europe. For some regions it seems that a balance is now reached at a reduced sensitivity level, between forces which promote and impede resistance development.

The second one of the 3 large areas in Europe mentioned above is the east, where clearly lower MRF values (still) predominate. A relatively low fungicide input in the past is responsible. Whilst in Austria, varieties carrying effective mildew resistance were commonly used, the other Eastern countries did not use fungicide treatments because of a lack of foreign currency. A normally broad spectrum of sensitivity in each population (Felsenstein, 1991) infers not only a lower level of fungicide use, but also an influence by wind spread of the pathogen out of western neighbouring regions. In particular, if economic situations change in the future, there might also be a change in the sensitivity level, equivalent to that seen in North-western Europe. The wheat mildew populations in the area of the former GDR provide a clear example.

In contrast to the two large areas described above, there is a third one in Southern Europe, south of the Alps and Pyrenees. There, sensitivity of the isolates tested is predominently the same as the wild-type (and the standards), and the MRFs still remain around one. There are two reasons for the wide-spread unchanged levels of sensitivity. Firstly, low selection pressure because of low fungicide input due to low priority in controlling the pathogen has to be taken into account. Secondly, there are the high mountains, namely the Alps and Pyrenees, which seem to protect the populations in the south of Europe from an influx of spores from the north and north-west, respectively. It is evident that the high mountains act as an epidemiological barrier and that they allow little pathogen exchange between neighbouring populations on either side. This observation is

confirmed by investigations into the virulence situation of wheat and barley powdery mildew in Europe (Felsenstein, 1991; Limpert *et al.*, 1991). Therefore, if local selection pressure does not increase, the sensitivity of wheat powdery mildew to DMIs is expected to change more moderately and over a longer time scale in Southern Europe beyond the Alps and Pyrenees.

Finally, if attention is concentrated again on the north-west of Europe, the question arises as to why in the recent past (since 1989) sensitivity levels have regionally stabilised and resistance evolution has been relatively moderate. There are three main points which have to be discussed: Firstly, there is increasing diversification in the use of azole derivates. With regard to the single DMI compounds, there is now less specific selection pressure. Indeed, there is positive cross resistance of the pathogen towards DMIs on the one hand, but on the other hand, there are also variations concerning its expression (see above). Treatment with different azoles is supplemented by an increased use of fungicide mixtures and morpholines, partly within the implementation of appropriate anti-resistance strategies. Also there is no cross-sensitivity between azoles and morpholines /piperidine compounds, which show a different mode of action from the DMIs.

Secondly, there is a possible reduction in fitness of the pathogen, due to increased DMI resistance. In the 1980s, some reports suggested to this relationship (Buchenauer, 1983; Buchenauer & Hellwald, 1985), leading to the opinion that evolution of resistance in the pathogen might have no practical effect in the field. As other investigations could not confirm these results and conclusions (Butters *et al.*, 1984; Wolfe, 1985; Porras *et al.*, 1990), this question was much discussed. Today, on consideration of all available information, it seems that the phenomenon of reduced fitness based on an increase of DMI resistance plays only a subordinate role in the stabilized sensitivity levels observed in North-western Europe.

Thus, a third factor seems to be mainly responsible: Poly-(oligo-)genic control of the gradual (quantitative) resistance evolution towards SBIs (Butters *et al.*, 1984; Hollomon *et al.*, 1984; Skylakakis, 1985), combined with evident genetic recombination of the pathogen. It has to be considered that the polygenic background of DMI resistance encompasses an effect which acts against unimpeded, continued decrease in sensitivity. The more resistant the pathogen becomes, the more genetic changes are necessary. But the polygenic model does not automatically guarantee limited resistance development, as is illustrated e.g. by the barley powdery mildew pathogen, E. graminis f.sp. hordei, where, in general, much higher resistance factors have been obtained (Limpert, 1991; Felsenstein, unpublished). Thus, in the specific case of wheat mildew, the polygenic control of resistance evolution, combined with genetic recombination due to the sexual stage (ascospores) of the pathogen has to be taken into account. The latter yields a large number of isolates with different sensitivities (Hollomon *et al.*, 1984; Butters *et al.*, 1986). In particular, data on virulence and pathotypes indicate that within the yearly reproduction cycle, ascospores are a much more important factor in the life cycle of wheat powdery mildew (Felsenstein, 1991) than they are for barley powdery mildew (Welz & Kranz, 1987; Brown & Wolfe, 1990). Because of more intensive yearly redistribution of genes responsible for DMI resistance, maintenance and in particular multiplication of pathotypes with an exceptionally low DMI sensitivity (high RFs) are probably not possible to the same extent as reported for barley powdery mildew (Welz & Kranz, 1987; Brown & Wolfe, 1990). Thus, the phenomenon of genetic recombination in the wheat mildew pathogen is considered to be the main reason for the observed reduction in DMI sensitivity changes in North-west Europe.

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EVALUATION OF ANTI-RESISTANCE STRATEGIES

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ABSTRACT

The feasibility and the success of any anti-resistance strategy depend not only on the anti-resistance strategy itself, but on several additional factors.
One of these is the availability of rapid and reliable monitoring methods which allow control of the efficacy of a certain strategy. Another crucial point is the availability of companion partners. As a result of increased development costs and political and legislative hurdles, the number of active ingredients in plant protection has decreased in a dramatic manner. Under these circumstances, existing anti-resistance strategies are mostly based on the use of preventive fungicides as companion partners of DMIs.
In addition to this narrow range of options concerning the available fungicide partners anti-resistance strategies have to fit economical, ecological and legislative requirements.

INTRODUCTION

The first DMI-fungicides were introduced nearly twenty years ago. They rapidly became the most important group of fungicides, representing a new standard of modern specific fungicides. Although the risk of resistance for DMIs was initially considered to be low to moderate, first reports on decreased sensitivity of powdery mildew fungi against DMIs were published in the early eighties.
Today, growers, agronomists and scientists have learned to deal with resistance towards DMIs and a lot of experience with resistance development against DMI fungicides has been gained.

In spite of these favourable assumption, there are still many unsolved questions concerning the most appropriate anti-resistance strategies for DMIs. Most of the difficulties have their origin in the fact that DMIs have an extremely broad spectrum of fungicidal activity in a wide variety of crops. This results in a wide variation in

a) pathogens or pathogen complexes to be controlled in a given crop.
b) the number of treatments with DMIs and with fungicides from other (non cross resistant) chemical classes in one season in a crop.

Many biochemical studies have elucidated the biochemical mode of action of DMIs in several different fungi. However, studies on the mechanisms of DMI resistance in fungi are much rarer. It is still unclear whether changes in target sensitivity, in the uptake of fungicide into the fungus or in other compensating biochemical alterations are the main cause of resistance development in those pathogens which have meanwhile shown potential to develop more or less pronounced resistance to DMIs.

Some indications suggest that resistance of fungi to DMIs has a multigenic basis. This means that only an accumulation of several independent mutational changes would allow the

development of a high degree of resistance. Moreover, if this is true, it is probable that different combinations of different resistance mechanisms exist in different fungal species. For that reason, it is unlikely that only one theoretical model describing the molecular basis of resistance to DMIs in all fungi will be determined in the near future.

The following analysis aims, therefore, to show
- the difficulties in evaluating the success of anti-resistance strategies
- the practical limitations which hinder the use of an optimal anti-resistance strategy
- the availability of suitable companion partners from non-cross resistant fungicides
- the status of practically existing anti-resistance strategies in several crops and countries.

DIFFICULTIES IN EVALUATING THE SUCCESS OF STRATEGIES

Under ideal circumstances, the implementation of an anti-resistance strategy has to be accompanied by monitoring methods which allow rapid and reliable feed-back of the efficacy of a given strategy.

With DMIs, there are several hurdles which usually make it very expensive to optimise the success of a given anti-resistance strategy with the aid of simultaneous sensitivity monitoring.

Many of the target fungi are either obligate parasites (e.g. powdery mildews or rusts) or they are very slow growing fungi in ordinary *in vitro* cultures (e.g. *Mycosphaerella fijiensis*, *Venturia inaequalis*). This necessitates the use of more time consuming and/or more expensive monitoring methods compared to those which can be used for monitoring sensitivity in other fungicide classes.

The fact that resistance of fungi to DMIs is typically characterised by a relatively slow and continuous selection process (shifting type), requires the determination of sensitivity profiles of fungal populations. In order to guarantee statistically sound results, large sample numbers are the consequence. Additionally, it is usually not sufficient to differentiate the sensitivities of a fungal population using only one discriminatory concentration. The use of several concentrations covering a wide range is necessary to determine LC_{50} values or equivalent data.

ECONOMICAL LIMITATIONS

Every anti-resistance strategy must overcome a basic problem. As long as no actual resistance problems are obvious, an anti-resistance management is of secondary priority for the farmer who is primarily concerned with economical problems. Why should the farmer not use the fungicide or the fungicide class with the best price / efficacy ratio all the time? Educational efforts from officials and industry can only in part open the mind to the fact that anti-resistance strategies are mostly a profitable investment in the long term. The implementation of an anti-resistance strategy has, therefore, to be a compromise between the technical need and the narrow economical and legislative framework.

Usually, every anti-resistance management strategy is more expensive than conventional farming methods. For example, the use of two-way mixtures at full rates, which is often

recommended by researchers, is clearly more expensive than alternation or the use of mixtures at reduced rates.

Economical reasons are also one of the main causes of numerous efforts to reduce the cost of chemical input by using reduced and split dosages. In some regions, practical farming has, generally, adopted this habit (Jørgensen and Nielsen, 1992; Bosse *et al.*, 1991). Although a potentially negative influence on the effectiveness of anti-resistance strategies was presumed from the beginning, evaluation of the effect of split and reduced rates on the selection of less sensitive fungal strains has been carried out only recently. Experiments by FRAC members gave clear indications that the use of reduced and split doses of fungicides may increase the selection of less sensitive fungal strains (Anonymous, 1994).

LEGISLATIVE LIMITATIONS

Any good anti-resistance strategy should include all factors which are usually described by terms such as "Integrated Pest Management" (IPM) or "Good Agricultural Practice". This includes, for example, the use of resistant cultivars, adequate fertilisation, crop rotation etc.

In most cases, an anti-resistance strategy based on chemicals is additionally necessary. The basis of any anti-fungicide-resistance strategy is the availability of active ingredients from non cross resistant fungicide classes which are effective against the pathogens of interest.

In recent years, the number of registered active ingredients has decreased dramatically in all countries. In Germany, for example, the number of registered active ingredients has decreased by more than 30 % since 1986. On a world-wide basis, the number of newly introduced active ingredients has decreased in a similar way.

The reasons for this decline are multifarious:

- dramatically increasing costs for development of new pesticides and for the re-registration of older compounds have seriously narrowed the variety of options especially in minor and midsize crops.
- public and political pressure to reduce pesticide usage.

AVAILABILITY OF COMPANION PARTNERS

The benefits of the use of companion products for DMIs do not only include anti-resistance strategies. In apple scab, for example, the simultaneous use of preventive and curative products improves the performance in a significant manner. In cereals, the broadening of the activity spectrum of DMIs against several secondary diseases is a reason for the use of companion products (Urech, 1988).

In many crops, DMIs are the only available fungicide class which is highly active and which can be used curatively. Examples include economically important pathogens such as

-powdery mildew in grapevine
-apple scab
-*Septoria* diseases in cereals

If only non-systemics are available as potential companion partners, it is evident that the evaluation of possible advantages or disadvantages of the use of systemic partners (which have a resistance risk on their own) versus the use of non systemic multisite inhibitors (with a very low risk of resistance) is only of theoretical value. Curative products need curative companion partners (Urech, 1988). For that reason the use of DMIs in mixture with preventive fungicides

has to be preventive. In alternation programs, the application intervals have to be adapted to take into account the lasting effect of the preventive partner.

Only in a minority of cases are systemic fungicides available which on the one hand are suitable companion partners for DMIs, and which on the other hand do not have severe resistance problems on their own. One of the most successful examples is the use of morpholines and of DMIs for the control of cereal powdery mildew in Europe. The regular use of both fungicide groups has without doubt considerably retarded the development of DMI resistance in this pathogen.

STATUS OF EXISTING ANTI-RESISTANCE STRATEGIES FOR DMIS

Generally speaking, producers, officials, and farmers have learned to treat the phenomenon of DMI resistance development in fungi in an objective and technical manner. At the basis of this was the realisation that resistance of fungi to DMIs is a continuous not a sudden event which is normally correlated with gradually decreasing efficacy in the field.

In face of the limited choice of companion partners, several general rules have found a broad acceptance. These are well reflected in the recommendations of the FRAC SBI-Working Group (Anonymous, 1994).

Generally, with "high risk" pathogens, repeated applications of DMIs alone should be avoided. The use of DMIs should be reserved for the critical parts of the season. The use of mixtures, or alternation with non cross-resistant fungicides are equally recommended.

In crops where several pathogens have to be controlled simultaneously, such as in cereals, the use of mixtures with morpholines or with preventive fungicides is well established. Mixtures are also mostly used in the control of scab on apple, an example of a crop where relatively small failures of disease control can cause severe economic losses.

Alternation programs with tridemorph and other (mostly preventive) fungicides are normally used in Sigatoka control in bananas.

In grapevine, DMIs are preferably used in the most critical period of the season (around flowering), whereas preventive fungicides dominate the early and the later season treatments.

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THE EFFECT OF REDUCED DOSE ON THE EVOLUTION OF FUNGICIDE RESISTANCE IN *SEPTORIA TRITICI*

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ABSTRACT

Genetic variation in flutriafol resistance of *Septoria tritici* exists, and reduced fungicide rates produced reduced control of the pathogen population in field experiments. Despite this, no reproducible shifts in resistance appear to have occurred in response to selection by flutriafol applications at either full-rate or 1/4 rate, or to a mixture of flutriafol and chlorothalanil, or in plots sprayed with water only. Possible explanations are discussed.

INTRODUCTION

There are three hypotheses as to how reduction of the rate of a single fungicide application might affect the rate at which resistance evolves. The reduced dose might increase the rate of evolution, because partly resistant forms could survive and subsequently give rise to more resistant individuals by crossing or mutating; the reduced dose might, over the range within which fungal growth was affected at all, do nothing; or the reduced dose might reduce the rate of evolution, because more sensitive forms survive to breed in the future. In the latter case, a quantitative understanding is needed to decide the overall effect on resistance evolution when alternative treatments are, say, several small doses or one large dose.

The theoretical justification for the use of maximal doses to retard the evolution of resistance is clearest in the case of diploid sexually reproducing organisms with resistance inherited as an allele at a single locus. Here a large dose should minimise the expression of resistance in heterozygotes. Since almost all resistant genes are in heterozygotes initially, because of Hardy-Weinberg assortment, it is on the fitness of these that the rate of evolution of resistance depends. This argument has been strongly made by, for example, Mani (Mani, 1989). However, the effectiveness of this has been disputed by others, not least because a pesticide must pass through all concentrations less than the application rate as it decays, and therefore there will be stages at which there is an advantage to the heterozygote (R T Roush, pers. comm., 1991). Even if applicable to organisms like insects, there are few fungi which are diploid and sexually reproducing during the epidemic phase when selection occurs.

Shaw (Shaw, 1989) studied a model of the evolution of polygenic resistance in haploid or clonal fungi, which suggested that dose was irrelevant to the evolution of resistance. This prediction depended on the assumption in the model that intrinsic growth rate of both forms was similarly affected by fungicide application, but that of the resistant form was always greater around reasonable field doses. Shaw (Shaw, 1989) acknowledged that this could be at best an approximation, roughly true around field doses, but showed that the approximation

was likely to be quite good between about 1/4 and 4 times a reference dose.

The argument that an individual reduced dose should have less effect on the genetic composition of the pathogen population than a standard dose is a common-sense one. It can be based on comparisons with plant or animal breeding, where weaker selection, not unreasonably, means slower selective progress. What is much less clear is that the reduction will be proportional to the dose: if the selective effect of a half dose is more than half that of a full dose, but two half doses are applied, the evolution of resistance will clearly be speeded up overall.

We are trying to test these hypotheses about the relative effect per application of full and reduced doses, using the wheat-*Septoria tritici* (*Mycosphaerella graminicola*) pathosystem. This seems to be well-suited to the work. The population in a field is believed to be initiated by widely dispersed and fairly abundant sexually generated ascospores (Shaw & Royle, 1989). Genetic evidence based on molecular polymorphism supports this view: the populations surveyed so far are extremely variable on a very fine scale and contain many clones, and almost all the variability in a population is contained within a field (McDonald & Martinez, 1990; McDonald & Martinez, 1991). This means that the past history of a site has a very minor effect on the population within it, and that plots should be representative of the surrounding farming district and normal populations. Infection in the autumn is certain, and artificial inoculation unnecessary to augment the population. Multiplication thereafter is rapid through splash-dispersed, clonally produced spores, which disperse only over a few metres, so immigration into a field should be numerically negligible after the autumn (Shaw & Royle, 1989), and plots of 100 m^2 or so should behave as independent populations over a period of one year (Shaw & Royle, 1993). Thus, sites separated by several km, or sampled in different years, should serve as true replicates (Hurlbert, 1984), encompassing the variability in the founding populations, and allowing us to make inferences about agricultural populations of the pathogen.

We report here partial results from the first two years' work in this system.

MATERIALS AND METHODS

Experimental Design of Field trials

Field experiments have now been planted for three consecutive years at two locations, Sonning Farm (Reading University) and Jealott's Hill Experimental Station (Zeneca), about 20 km apart. However, we shall be concerned with only the first two years' crops. At Sonning the crop was on a new site, not sown to wheat the year before, every year while at Jealott's Hill plots (and treatments) were on the same site in the same position for the first two seasons (1991/92, 1992/93).

The lay-out at each site was a split-plot design, using two winter wheat cultivars (Mercia and Riband) and four different fungicide treatments. Riband is very susceptible to *S. tritici* while Mercia is moderately resistant. Each site had two main plots composed of four split plots of the same cultivar. Each split-plot was separated from all the others by 3 m wide strips of winter barley to minimise gene flow between treatments. Each split-plot was at least 12x20m. Plots were sprayed at around GS 37 (flag leaf just visible) with one of four

treatments at a spray rate of 250 l/ha: water; ¼ l/ha Impact (125 g/l flutriafol); 1 l/ha Impact or 2.66 l/ha Impact Excel (47 g/l flutriafol + 300 g/l chlorothalonil). The formulations used were made up every year and the same batch was used at both locations.

Field performance of the sprays was assessed on a regular basis by taking random samples of the leaf below the flag at about 0.5m intervals on two diagonal walks across the field. Disease incidence was expressed as percentage leaves with one or more lesions bearing pycnidia.

Sampling of pathogen population

Wheat leaves bearing pycnidia were collected from each plot in May, just before spraying, The youngest leaves with pycnidia were taken, since these were most likely to infect the top leaves. Within 6 weeks after spraying, another sample was taken, this time from the leaf below the flag. This six weeks limit was set to avoid sampling the second generation of pycnidia after spraying. Depending on the amount of disease present in a plot, 75-100 leaves were taken at a time. Dirty leaves were washed before they were dried for several hours at room temperature. Subsequently, leaves were stored frozen at -20°C.

Assay of Fungicide Resistance

Details of the assay are being published (Pijls et al., 1994), so only a brief outline will be given here. Leaves were surface sterilised in a 1% NaOCl-solution for 30 s, and incubated in a sandwich box containing a thin layer of tap-water agar. The box was then covered by paper tissue and put away for 24-48 hours at 17°C after which a single dry, curly cirri could be picked off each sampled lesion, and suspended in sterile water. These spores were used in an assay of flutriafol resistance based on light absorbance in liquid medium.

Flat bottomed microtitre plates with 8 rows of 12 wells were used. Each row was filled with pycnidiospores of a single S. tritici isolate and medium containing different fungicide concentrations. The range of final fungicide concentrations was: 0, 0, 0.010, 0.0316, 0.100, 0.158, 0.251, 0.398, 0.631, 1.0, 1.78, 3.16 μg A.I. ml^{-1}. Preliminary experiments had shown that most field isolates had an EC_{50}-value between 0.056 and 0.56 μg A.I. ml^{-1}, and therefore the range was made more precise in this interval. Two rows in every plate were used for control isolates, R12 and S27 (D.W. Hollomon, Long Ashton Research Station) with a known sensitivity to flutriafol. After 10 days incubation in the dark at 17°C, growth was measured using absorbance of light at 405 nm. A dose response curve was then fitted to the absorbance data to estimate the fungicide concentration reducing absorbance by one-half (EC_{50}). EC_{50}-values based on this method seem to be adequately correlated with preliminary results from a bio-assay on wheat seedlings (GS12-13).

RESULTS

Field performance of sprays

The results on Riband for the first season are shown in Tables 1 and 2. These show that some multiplication of disease was possible under all fungicide treatments, and that substantially more was possible under the reduced dose than the full dose. Notwithstanding

this, the fungicide very greatly reduced the incidence of disease. There was also a difference in disease severity (proportion of leaf area infected). Infected leaves from plots untreated (0) or treated with reduced (¼) dosage usually had more and bigger lesions than those from plots treated at the full rate (1) or with the mixture with chlorothalonil (1+C). This suggests that the pathogen was not only growing in leaves which had remained by chance completely free from fungicide. Findings in the second season were similar.

TABLE 1. Sonning, 1992: Incidence of disease caused by *S. tritici* on leaf 2 of wheat cv. Riband at various dates after spraying on 14 May.

		Incidence (%)		
Cultivar	Spray	23 May	2 June	9 June
Riband	0	6.1	30.3	72.1
	1/4	7.1	15.2	43.5
	1	3.3	6.9	22.0
	1+C	0	3.4	18.6

TABLE 2. Jealott's Hill, 1992: Incidence of disease caused by *S. tritici* on leaf 2 of cv Riband at various dates after spraying on 6 May.

	Incidence (%)			
Spray	17 May	23 May	2 June	9 June
0	14.3	74.6	94.2	100
1/4	5.7	42.6	89.0	98.9
1	1.4	27.3	72.2	92.5
1+C	* 5.7	27.1	48.3	52.9

*: high incidence due to uneven plant development

The results of fungicide assays conducted so far on isolates from Riband are presented in Figures 1 and 2, as 'box-and-whisker' plots of the distributions. Each plot shows the extremes, the quartiles and the median of the distribution for the plot. The Kolmogorov-Smirnoff test for differences between probability distributions was used to test for differences between the distributions before and after treatment (Siegel, 1956).

Several points are striking in these data. First, there is no systematic change in sensitivity after spraying, at any dose. Second, such significant changes as are present are as likely to be to lower levels of resistance as to higher, and occur equally with the water and fungicide sprays. Third, plots appear to differ in fungicide sensitivity distribution before spraying, certainly across years. For example, in Sonning in 1993 the distributions before spraying were all much narrower than in 1992.

FIGURE 1. 1992, cv Riband: distribution of fungicide sensitivity (EC50) in each plot before and after spraying. The sample size (N) is shown in parentheses to the right of each plot; the plot is identified to the left: 0 - water spray; 1/4 - 1/4 rate spray with flutriafol; 1 - spray with full recommended dose of flutriafol; 1+C - sprayed with a mixture of flutriafol and chlorothalanil. A scale appears in the middle of the figure. Each plot shows the extremes, quartiles and median of the distribution of the observed EC50. ★ : significant ($P \leq 0.05$) difference between before and after, according to a Kolmogorov-Smirnoff test.

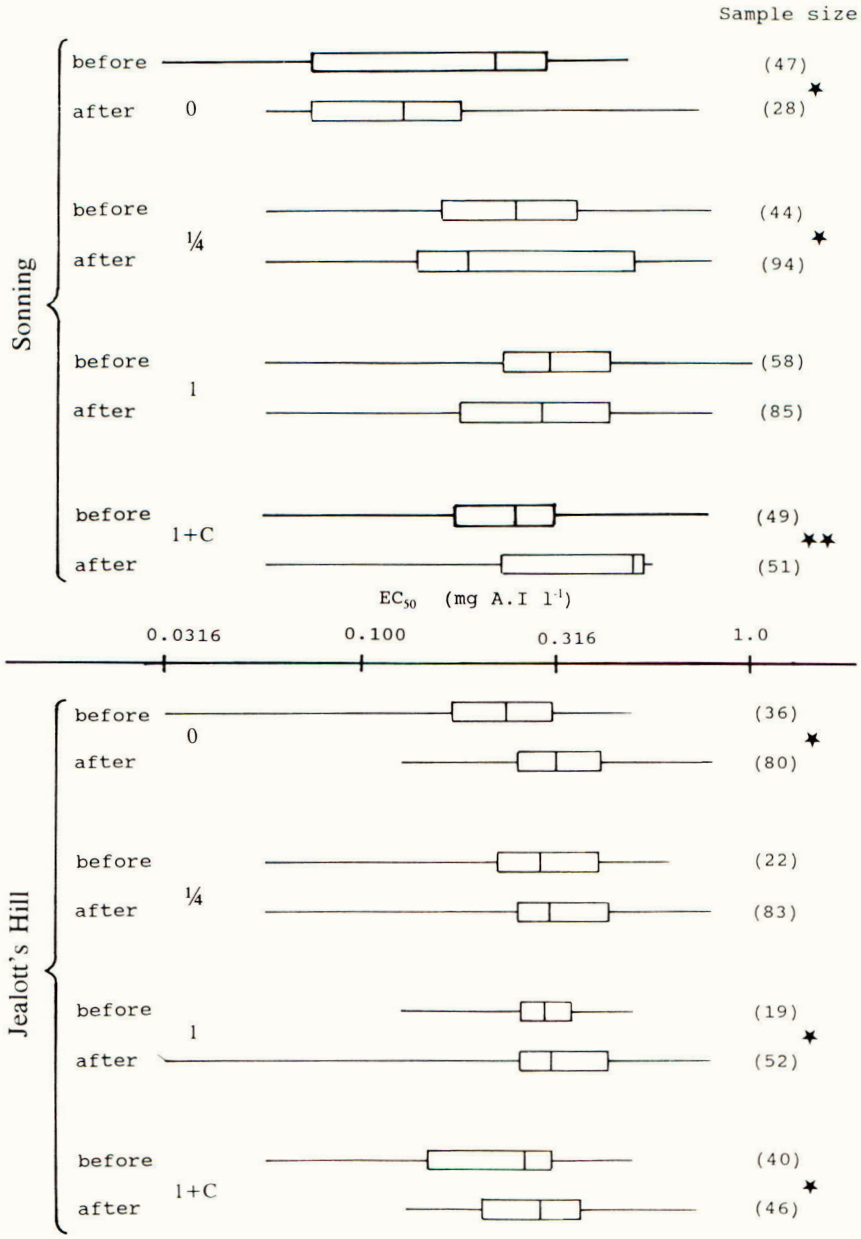
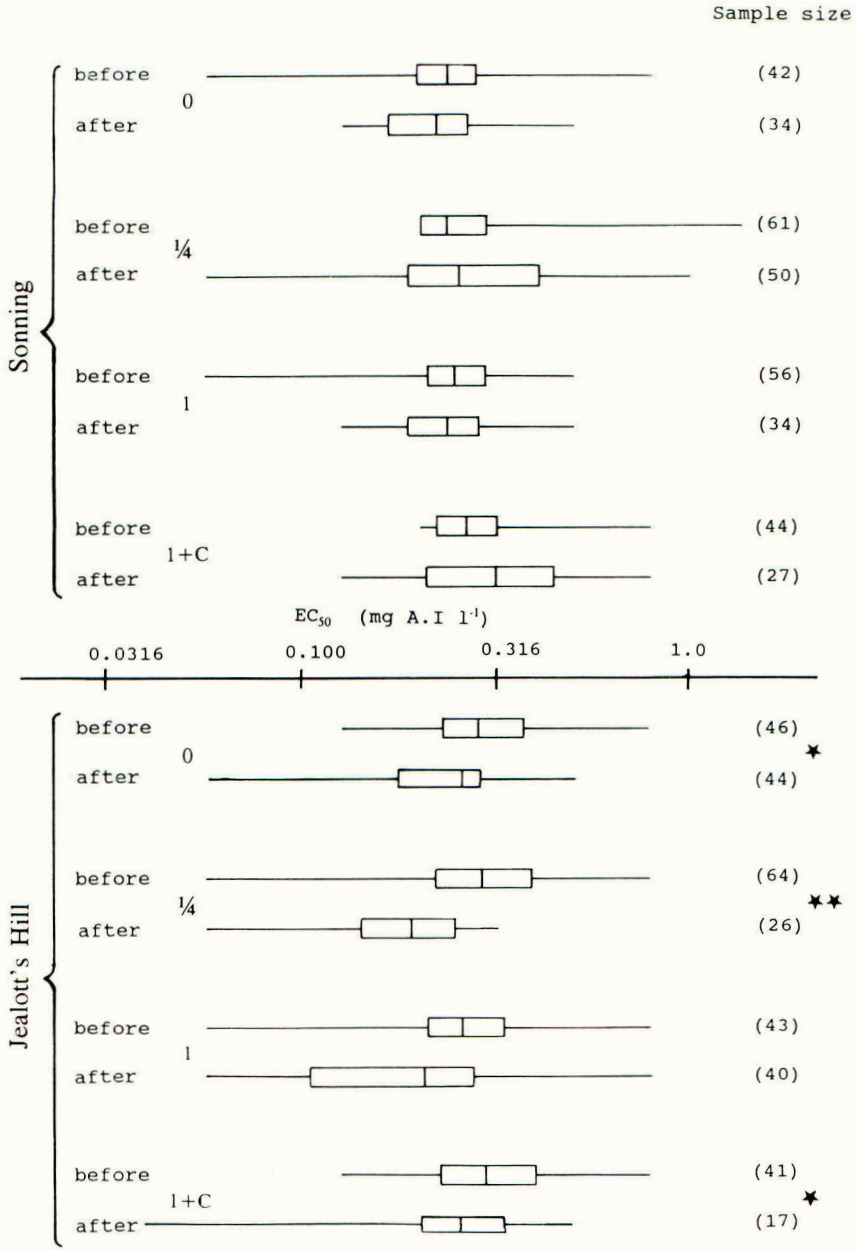


FIGURE 2. 1993, cv Riband: distribution of fungicide sensistivity (EC50) in each plot before and after spraying. The sample size (N) is shown in parentheses to the right of each plot; the plot is identified to the left: 0 - water spray; 1/4 - 1/4 rate spray with flutriafol; 1 - spray with full recommended dose of flutriafol; 1+C - sprayed with a mixture of flutriafol and chlorothalanil. A scale appears in the middle of the figure. Each plot shows the extremes, quartiles and median of the distribution of the observed EC50. ★,★★ : significant ($P \leq 0.05$ or $P \leq 0.01$) difference between before and after, according to a Kolmogorov-Smirnoff test.



DISCUSSION

The results are more surprising than may appear at first sight. There is genetic variation in fungicide sensitivity present in the population, spanning a range of about 30-fold in EC_{50}, although most of the variation is contained within a roughly 3-fold range, form 0.2 to 0.6 mg/l. The EC_{50} of an isolate is stable following reinoculation onto plants and reisolation. Thus, the character measured is heritable and reflects the ability of the fungus to cause disease in the presence of fungicide. Selection was applied by the fungicide treatments, because the population size of the pathogen was substantially reduced. Therefore, the fungicide sensitivity distribution should have changed after spraying; the median sensitivity should have decreased. This did not happen, to the accuracy of our experiments.

There are a number of possible explanations of our observations, some of which can be excluded fairly quickly using data we already have. We shall consider some of the simplest explanations in turn.

First, selection could have been so strong that the variation we have seen was irrelevant: disease found after treatment was in leaves or parts of leaves without fungicide, and the area available to a more resistant isolate for successful disease expression would be no larger than for a more susceptible isolate. However, as we noted, lesions in the 1/4 rate and water treated plots tended to be larger than in the full rate and mixture plots, and control was better with full rate than with 1/4 rate. This explanation therefore requires that a graph of area of foliage with a given fungicide concentration against fungicide concentration have a slope of 0 near the maximum concentration at which growth is possible, but also that in the 1/4 rate plots much more disease is expressed. This implies that the change in disease incidence caused by reducing the rate to 1/4 was entirely due to an increased area of the foliage containing no fungicide at all . However, with a fully systemic and quite mobile chemical like flutriafol, this seems improbable.

Second, perhaps more resistant isolates are systematically less able to cause infection than more susceptible ones. However, in this case, resistance should have decreased in the water treated plots where no selection by fungicide occurred.

Third, perhaps the origin of the inoculum for the disease on the upper leaves was not within the plots. This is not consistent with what we know of the epidemiology of the disease, and, in any case, such immigrating inoculum should have been similar for all plots, so there should be differences in fungicide sensitivity distribution between the water and fungicide treated plots.

Fourth, perhaps selection for ability to infect the particular variety used under the particular conditions prevailing completely outweighs selection for resistance, so that resistance shifts appear random. This argument works only if the proportion of genotypes capable of infecting at any given time is actually very small, so that selection by fungicide is negligible compared to selection by the environment and the host. But this flies in the face of the excellent control achieved by fungicide and the difference between full and 1/4 rates, which demonstrate at least the potential strength of selection by fungicide.

Fifth, if only one or a few clones could best infect cv. Riband under the prevailing environmental conditions, then random association between the virulence characteristics of the isolate and its fungicide sensitivity could generate essentially random shifts in fungicide sensitivity. This requires effective population sizes in each plot to be small. In the US this seems not to be the case (McDonald & Martinez, 1990; McDonald & Martinez, 1991), and our preliminary, unpublished, evidence concurs in the UK. However, this explanation is the only one of those put forward which can explain the increase in resistance in the water treated plot at Jealott's Hill in 1992, or the decrease in the 1/4 treated plot at Jealott's Hill in 1993.

To sum up, insofar as our results provide an answer to the question we have posed, it suggests that resistance is no more likely to be selected by reduced rates than by full rates; but since the results suggest that no effective selection for resistance is happening in the system, this result needs substantial further investigation before it can be regarded as a trustworthy example.

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