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BEHAVIOUR OF A BARLEY POWDERY MILDEW STRAIN TOLERANT TO ETHIRIMOL

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Summary A strain of barley powdery mildew has been characterized which can tolerate 10-100 times more ethirimol than a standard sensitive strain. Attempts during 1975 to isolate similar stable ethirimol tolerant strains from the field have not yet been successful. Under laboratory conditions this tolerant strain did not compete with the sensitive one. Tolerance was not transferred to the sensitive strain during vegetative growth, and it does not appear to behave as a mendelian character. Asexual selection experiments failed to alter the response of either strain to ethirimol. Cross-tolerance to dimethirimol was observed, but not to M.B.C. or tridemorph.

INTRODUCTION

Ethirimol (Milstem*; 5-n-butyl-2-ethylamino-4-hydroxy-6-methyl pyrimidine) has been used commercially since 1970 in the U.K. to control barley powdery mildew (Erysiphe graminis DC f.sp. hordei Em. Marchal). In 1973 tolerance to ethirimol was observed (Wolfe, 1973) and extensive monitoring since then has confirmed its existence, although ethirimol continues to give significant yield increases in fungicide trials (Shephard and Bent, 1975). Despite extensive epidemiological studies the nature of this tolerance is poorly understood. This paper describes laboratory investigations, using two mildew strains, into some fundamental aspects of the problem.

MATERIALS AND METHODS

The two single pustule strains (genetically homogeneous) used here were isolated from field samples collected in 1973 by Dr. M.S. Wolfe (Plant Breeding Institute) and Mr. M. Woolner (Plant Protection Ltd.). Details of the methods used to maintain and bioassay these strains are given elsewhere (Hollomon, 1975). When whole plants were inoculated these were maintained in an isolated propagator (Jenkyn et al, 1973). Field samples collected in 1975 were bioassayed at one discriminating dose. After collection samples were placed on agar in small polystyrene boxes and any conidia present removed. Conidia that then formed after 24 hours were tapped onto cellophane membranes overlaying nutrient agar (2 µg ethirimol/ml agar). Germination, expressed as a percentage of that occurring in the absence of fungicide, was used as a measure of the tolerance level of each sample.

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RESULTS

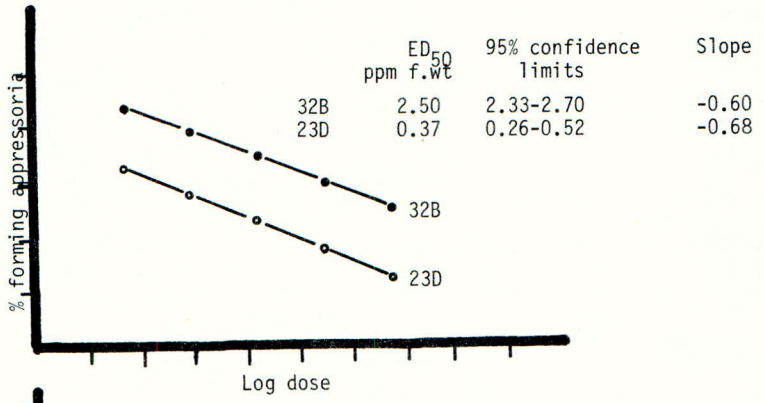
Characterization of standard sensitive and tolerant strains

One effect of ethirimol on mildew is to inhibit the formation of appressoria. Fig. 1a shows the dosage response lines for each strain in an in vivo bioassay of appressorial formation at different fungicide doses. Data from an in vitro bioassay of germ-tube length are given in Fig. 1b. The dosage response lines for each strain have similar, but shallow, slopes and the difference in ethirimol tolerance can be adequately expressed by the ED_{50} values. Differences between these two strains were also observed on seedlings grown from ethirimol dressed seed. The response of these two strains to ethirimol has remained stable for two years.

Figure 1

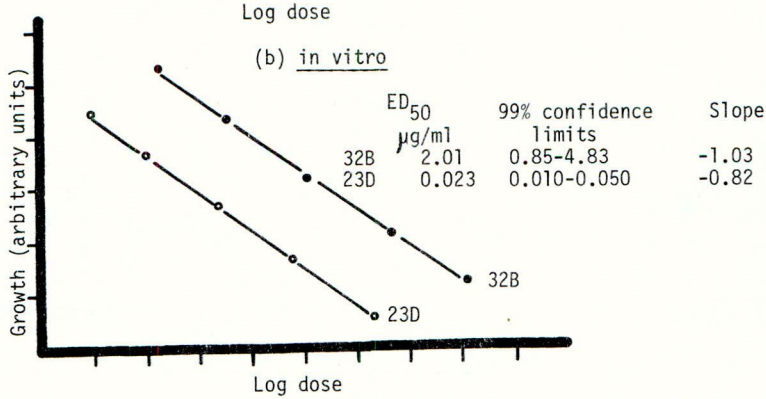
Dose response lines for ethirimol activity from two bioassays. 32B tolerant strain; 23D sensitive strain

(a) in vivo



	ED_{50} ppm f.wt	95% confidence limits	Slope
32B	2.50	2.33-2.70	-0.60
23D	0.37	0.26-0.52	-0.68

(b) in vitro



	ED_{50} µg/ml	99% confidence limits	Slope
32B	2.01	0.85-4.83	-1.03
23D	0.023	0.010-0.050	-0.82

Field samples collected in 1975

Using the discriminating dose assay, several samples were found with tolerance levels comparable with that of the standard tolerant strain (Table 1). Ten-day-old Proctor seedlings grown from ethirimol dressed seed (Table 2), and exposed overnight at site 1, became heavily infected indicating that the *in vitro* discriminating dose bioassay provided a reliable indication that tolerance is present. These results, however, give no indication of the effectiveness of Milstem; for example, at site 6 pustules were found only after an extensive search although there was up to 25% mildew in an adjacent field of Proctor. Some samples were subsequently maintained for several generations on fungicide free material. In two cases tolerance declined rapidly (Fig. 2) to a level not much greater than that of the sensitive strain; for other samples tolerance declined less rapidly. In two instances tolerance remained high but growth was poor, and the samples were soon lost. Mildew isolated from fungicide containing plants (12,800 ppm ethirimol) exposed at site 1, was maintained for five generations on detached leaves in the absence of fungicide. It was then used to inoculate seedlings grown from ethirimol treated seed. Less than 10% of individuals were still able to infect plants receiving the highest ethirimol seed dressing (Table 2). Despite the ease with which tolerance can be detected in field samples, I have so far been unable to isolate from this material a strain which has maintained this level of tolerance in the absence of the fungicide.

Table 1

In vitro test for ethirimol tolerance in field samples

	Site	Date	Variety	Milstem treatment	Germination at 2 ppm ethirimol (% Control - no fungicide)
1	Rothamsted	12/6	JULIA	+	79.0
2	Rothamsted	18/6	MARIS CONCORDE	-	3.6
3	Boxworth(Cambs.)	25/6	JULIA	-	23.4
4	Boxworth(Cambs.)	25/6	JULIA	+	40.0
5	Barley(Herts.)	27/6	PROCTOR (Dec.sown)		40.0
6	Barley(Herts.)	27/6	PROCTOR (March sown)	+	82.5
7	Little Wymondley (Herts.)	27/6	HASSAN	+	73.3*
8	Rothamsted	10/7	MARIS CONCORDE	-	44.2*
9	Rothamsted	10/7	JULIA	+	63.5
10	Rothamsted	10/7	SULTAN	-	87.2
11	Stoke Priors (Worcs.)	9/7	ZEPHYR	+	64.4
	Standard tolerant strain		JULIA	+	80.0±9.0
	Standard sensitive strain		ZEPHYR	-	9.0±7.0

* Calixin treated before sample was collected

Figure 2

Stability of ethirimol tolerance in field samples

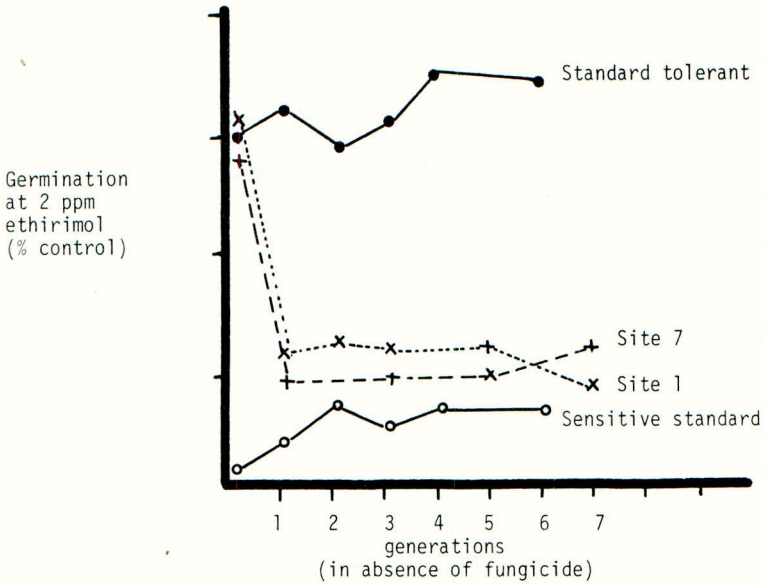


Table 2

Behaviour of field tolerance after a period in the absence of ethirimol

Proctor plants grown from seed dressed with	Number of pustules on prophyll eight days after inoculation	
	Plants exposed at site 1 overnight	Plants inoculated with ethirimol tolerant mildew maintained in the absence of fungicide for five generations
No ethirimol	61 (100)	85 (100)
6,400 ppm	61 (100)	24 (28)
12,800 ppm	25 (41)	7 (8)

Competition

The standard sensitive strain is virulent on the barley variety Armelle whereas the tolerant strain is not. On proctor, germination, appressorial formation, and growth of both strains appear identical. Changes in the relative proportions of these two strains in a mixture can, therefore, be monitored by observing the number of pustules formed on both Armelle and Proctor following their inoculation with conidia from the mixture. When a mixture, initially containing equal numbers of each strain, was monitored in this way, the tolerant strain could not be detected after only two generations in the absence of fungicide (Fig. 3a). Measurement of tolerance in this mixture using both an *in vitro* and *in vivo* discriminating dose bioassay also indicated that the tolerant strain did not compete with the sensitive one under these conditions (Figs. 3b, 3c).

Genetical studies

After each generation conidia from the mixture were used to inoculate whole plants of Armelle grown from ethirimol dressed seed (12,800 ppm a.i.). These plants remained free from mildew suggesting that the ability to grow at this ethirimol level was not transferred from the tolerant strain to the Armelle sensitive one during vegetative growth. Identical results were obtained using detached leaves rather than whole plants.

Attempts to cross these two strains failed to yield cleistothecia, and both strains were assumed to be of the same mating type. A few cleistothecia formed from a cross between the tolerant strain and a different sensitive one. Seven ascospore progeny yielded sufficient material for bioassay but all were sensitive.

Dose response relationships shown in Fig. 1 indicate that individual conidia of the same strain vary widely in their response to ethirimol. When single pustule progeny of the sensitive strain were bioassayed using both the *in vitro* and *in vivo* techniques, significant differences between some of the progeny were observed, although none differed significantly from the parent (Hollomon, 1975). Further asexual selection did not reduce this variability, but did result in the loss of aggressiveness of some progeny. Bioassay of progeny derived from the least sensitive of the first generation lines showed that insensitivity to ethirimol was not readily selected for in this way (Table 3).

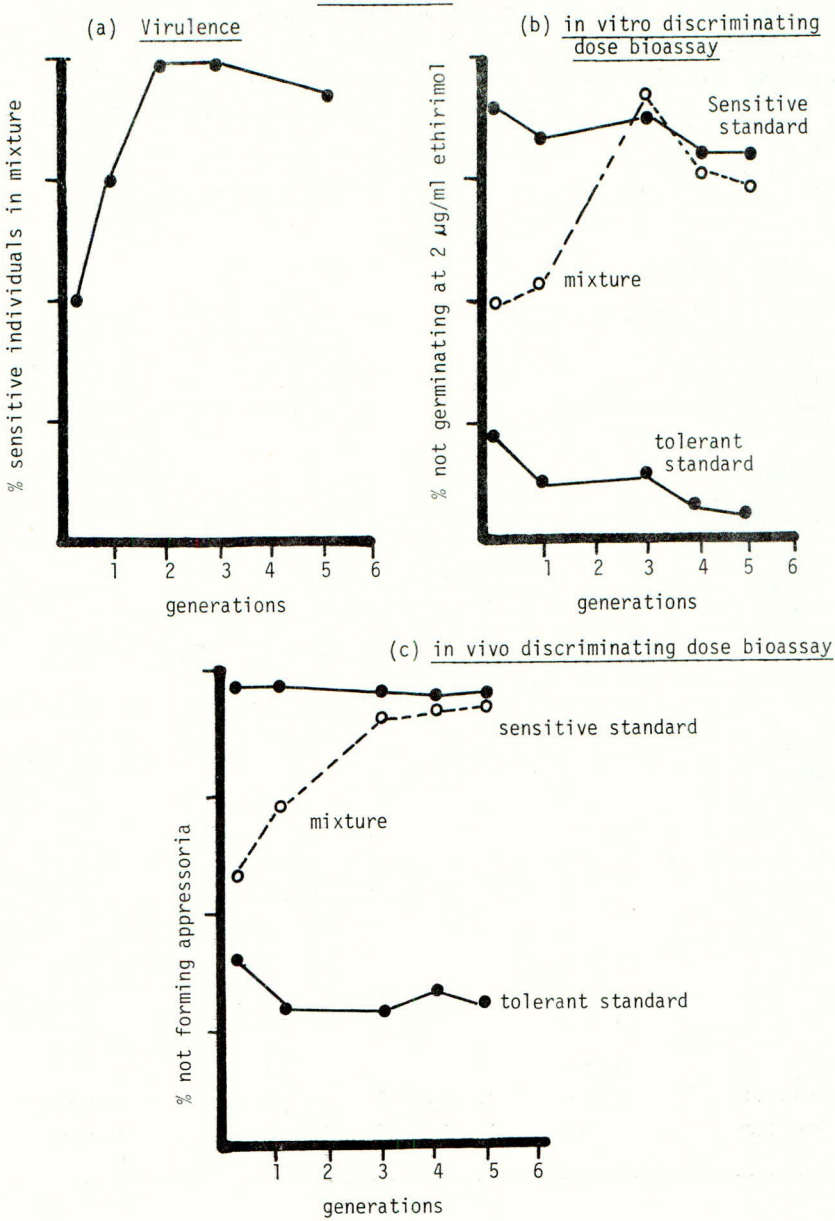
Table 3

Asexual selection for altered response to ethirimol. Selection was for the least sensitive progeny

	(a) *in vitro*		(b) *in vivo*	
	ED_{50} (μg/ml)		E_{50} (ppm fresh wt)	
	Mean for all progeny	Range	Mean for all progeny	Range
Parent	0.023		0.37	
1st generation	0.019	0.006-0.052	0.16	0.08-0.38
2nd generation	0.026	0.007-0.056	0.54	0.28-0.76

Figure 3

Behaviour of a mixture of sensitive and tolerant in the absence of ethirimol



Cross-tolerance to other fungicides

Methyl benzimidazole-2-yl-carbamate (M.B.C.) and tridemorph act on mildew after penetration of the leaf has occurred, and consequently appressorial formation cannot be used to bioassay their activity. Both fungicides affect germ-tube growth in vitro, but using this bioassay cross-tolerance with the ethirimol tolerant strain was not observed. Growth of established colonies is inhibited by both M.B.C. and tridemorph; when bioassayed in this way both the ethirimol tolerant and sensitive strains responded similarly to each fungicide. However, this bioassay would also not detect tolerance to ethirimol. Not surprisingly, cross-tolerance to dimethirimol was observed in appropriate bioassays.

DISCUSSION

Ethirimol tolerance can be readily demonstrated in field samples and may be associated with both effective and ineffective mildew control. Nevertheless, stable tolerant strains were not readily isolated from such samples. There may be many reasons for this, but it remains uncertain how widespread strains resembling the stable tolerant one used here are amongst the mildew population. A sensitive strain exhibits a wide range of phenotypic response to ethirimol; some individuals are able to withstand quite high fungicide levels and would be selected for under conditions of Milstem use. After several generations in the absence of fungicide the whole range of phenotypic response might again be espressed, and sensitivity to ethirimol would increase.

Despite its apparent ability to germinate, infect, and grow as well as the sensitive strain, the tolerant strain shows poor competitive ability in a mixture. The significance of this is difficult to evaluate as laboratory conditions may differ greatly from those found in the field. However, any members of the tolerant strain remaining after Milstem treatment will have to compete with those sensitive phenotypes which also survive, and increasing numbers of these as fungicide levels decline.

Whatever the genetic basis for this tolerance is it seems not to be transferred to the sensitive strain during vegetative growth. Asexual progeny may differ in their response to the fungicide but these differences are not heritable, and may be lost in subsequent generations. The limited data available from progeny of a sexual cross provide no evidence as yet that tolerance is controlled in a Mendelian way, or that it is linked with any virulence gene in the pathogen. The genetical basis of tolerance therefore, remains in doubt.

In the mildew strain used here tolerance is expressed at appressorial formation, and since neither M.B.C. nor tridemorph act on this developmental step, it is perahps not surprising that cross-tolerance to these fungicides was not observed. At present, the stable type of tolerance investigated here may not be widespread in the field, where selection of sensitive phenotypes able to withstand higher fungicide levels may be more important. The biochemical basis of this type of tolerance may be different and these less sensitive phenotypes might well show cross-tolerance to tridemorph and M.B.C.

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SENSITIVITY TO ETHIRIMOL OF POWDERY MILDEW FROM UK BARLEY CROPS

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Summary In view of the extensive use of 'Milstem' seed treatment in the UK, powdery mildew samples from many barley fields were examined for sensitivity to ethirimol. Sensitivity varied within and between untreated fields. It was also variable but generally lower where ethirimol had been used. Lower sensitivities probably arose by preferential removal of the more sensitive individuals during disease control. Regional differences were noted in 1973 but these were less marked in 1974.

Fluctuations in sensitivity were detected in individual fields, with rapid responses in either direction as selection pressures changed. Use of ethirimol on winter barley has been discouraged so as to avoid additional selection pressure. Yield responses and mildew control on spring barley have been essentially the same throughout the period 1968-74.

INTRODUCTION

For the past six years ethirimol has been used widely in the UK as a seed treatment ('Milstem'®) for the control of mildew (Erysiphe graminis DC f. sp. hordei) on spring barley. Substantial increases in yield resulting from 'Milstem' treatment have been recorded each year (Table 1). Sprays of ethirimol and some other mildew fungicides have given similar increases under optimum conditions, but the need for critical timing can cause problems for the user. The advantages of seed treatment have been reflected in an increasing usage of 'Milstem', so that by 1974 over 0·6 million ha of spring barley were treated in the UK.

Table 1

Yield increases following use of 'Milstem' seed treatment

(Mean values for ICI trials)

	1968	1969	1970	1971	1972	1973	1974
% mildew (untreated)	21·1	19·1	23·1	33·1	19·9	18·8	16·0
% yield increase with 'Milstem'	12·2	10·0	11·0	16·7	11·0	11·0	11·3

With such widespread usage, and with the knowledge that adaptation of some fungi to other fungicides has caused practical difficulties of disease control, it seemed prudent to test samples of mildew from barley crops for their sensitivity to ethirimol. By 1973 reasonably reliable methods were established, and in May-June 1973 initial observations at Jealott's Hill and at the Plant Breeding Institute (PBI), Cambridge, indicated that mildews from different field sources varied appreciably in sensitivity to ethirimol. Some of the PBI results were reported by Wolfe (1973). General surveys in the United Kingdom were organised subsequently by both research centres, aimed at determining the frequency and the degree of the

variation among mildews in different areas, seasonal fluctuations, and the relationships between sensitivity and 'Milstem' performance. The main results of surveys conducted by Imperial Chemical Industries Ltd. (ICI) in 1973 and 1974 are reported here.

•'Milstem' is a Registered Trade Mark of Imperial Chemical Industries Ltd.

METHODS

The first survey in late June-August 1973 covered approximately 300 fields or plots in the main barley growing areas of England and Scotland, and included a number of cultivars. During the following winter a smaller survey was conducted on 100 fields of Maris Otter in East Anglia and more detailed observations were made on a few individual fields. The second general survey, in the summer of 1974, based on the area of barley grown in each county covered over 400 fields mainly of Proctor, Julia and Golden Promise. In 1975 a limited survey has been conducted on 90 fields or plots of Julia and Golden Promise in East Anglia and Scotland.

In the winter survey and 1974 summer survey pairs of fields were selected, 'Milstem' treated and untreated. Some pairs were adjacent parts of the same field, but others were up to four miles apart. In 1975 adjacent areas were specially sown with treated or untreated seed from the same source.

Where possible fields were sampled twice. Mildew levels were recorded and pustules collected from at least 25 points in each field. Mildew was assessed either on the whole plant or the 3rd or 4th leaf according to the stage of growth. For field mildew and laboratory tests the following grades were used:

4 = No disease present
3 = 0-5% leaf area infected (mean 2·5%)
2 = 6-25% " " " (" 16%)
1 = 26-60% " " " (" 43%)
0 = 61-100%" " " (" 80%)

Isolates were cultured on untreated Proctor barley leaves, in Petri dishes, generally one bulk isolate per field, occasionally as single pustule or spore chain isolates. Isolates were kept at 19°C under 'daylight' fluorescent light (approx 3000 lux, 16 h per day), subcultured every 7 days, and when sufficient spores were available (usually after two generations) tested as follows. Proctor barley seed treated with 'Milstem' at concentrations between 8000 and 100 ppm (µg a.i. per g seed) and untreated seed was grown in John Innes Potting Compost No 1, 50 per 12·5 cm pot, in a controlled environment (day 20°C, RH 70%; night 15°C, RH 95%; daylength 16 h 7500 lux). After 10-11 days, two leaf pieces 2·5 cm long were cut from the middle of the prophyll of seedlings of uniform appearance. Five replicate leaf pieces for each concentration were mounted with one end embedded in tap water agar in a square dish. Spores (24 h old) from the test isolate were then blown (using a jet of air at c 50 g per cm^2) into a small settling tower, about 40 cm high and 10 cm square with air entry via a 4 cm tube inserted near the top, and allowed to settle over the dish for 1 minute before replacing the lid. After 6 days at 19°C infection on each segment was recorded as indicated above. High inoculum pressures were used so that disease levels on untreated leaf pieces were normally greater than 60%. Five concentrations of ethirimol were normally used and the sum of the mean disease scores was taken as the sensitivity of the isolate, with a maximum score of 5 x 4 = 20 if no growth of mildew occurred and a minimum of 5 x 0 = 0 if over 60% mildew developed at all rates. Each isolate was tested on at least two occasions. Results generally agreed within 2 units on the sensitivity scale. Marker isolates were included in every test.

The survey data have been analysed statistically where possible, but owing to differences in the distribution of samples significance tests could not be applied to the differences between seasons.

The concentration of ethirimol in test leaf pieces was assessed by treating seed with 'Milstem' containing ^{14}C-ethirimol, ring-labelled at carbon 2. After growth under standard conditions the ^{14}C content of leaf pieces was assayed by conventional combustion procedures.

RESULTS

When tested in the laboratory, mildews which survived in ethirimol treated fields were usually less sensitive than those from untreated fields (Tables 2 and 3). Samples from Scotland tended to be more sensitive than those from the east of England but regional differences were less marked in 1974. There may have been a general decrease in sensitivity from 1973 to 1974, but this is uncertain owing to differences in the sampling areas. Over the same period there may have been a slight overall increase in sensitivity of the untreated mildews in Norfolk and Suffolk, with the highest sensitivity in the winter when little 'Milstem' was in use. There was no shift in the range of sensitivities encountered in the two years; 1973, treated fields 4·6–19·0, untreated 5·6–20·0; 1974 treated 5·2–18·7, untreated 7·8–19·0. In 1974, of the 660 isolates examined, 3·3% had sensitivities below 7. Mildews from the different cultivars appeared to follow the general trends but regional differences in popularity precluded meaningful analysis.

Table 2

Patterns of sensitivity in untreated and treated fields 1973–74

Sampling period**	Mean sensitivity of isolates					
	National average		Scotland		Norfolk/Suffolk	
	Untr.	'Milstem'	Untr.	'Milstem'	Untr.	'Milstem'
late June – Aug 1974	15·3 (113)*	12·7 (186)	17·1	15·4	11·9	10·5
Dec 1973 – Jan 1974	–	–	–	–	14·0	12·5
Feb 1974	–	–	–	–	12·7	11·3
May – June 1974	13·6 (171)	10·0 (166)	14·8	10·5	12·3	9·3
July – Aug 1974	–	–	13·8	10·7	14·2	10·0

( )* Number of fields sampled

** Earliest samples were taken in the south and east

Table 3

Sensitivity of mildews from different areas of the UK (May 1974)

Region	No. of site pairs	Mean sensitivity of isolates	
		Untreated fields	'Milstem' treated fields
East	40	12·9	9·3
West	15	13·3	9·7
North	43	13·5	9·9
South	21	13·7	10·0
Midlands	21	14·0	10·3
Scotland	35	14·5	10·8

Difference for significance between treated and untreated fields within a region 1·7 (P=0·05).

Difference for significance between regions: 1·03-1·59.

Good control of mildew was recorded in all areas at both early and late samplings (Table 1). Lower sensitivities did not appear to be linked with poor mildew control. Yields from ICI trials in 1974 followed the normal pattern (Table 1). Yields in 14 trials by independent cooperators from Hants to Aberdeen averaged 5·11 t/ha, of which 0·54 t/ha was attributable to ethirimol treatment.

Detailed consideration of individual sites indicated that sensitivity could change rapidly during the season. This is illustrated by data from a trial in Essex (Table 5). Early in the year, when disease control was very good the surviving mildew had very low sensitivity. In mid-season, under very heavy inoculum pressure and severe drought conditions, mildew levels increased and the sensitivity increased also. Towards the end of the season, mildew levels on the untreated plots changed little as the leaves began to die, but mildew continued to develop on the still green areas of the treated crop. Final levels of sensitivity on the treated crops showed a slight decrease, possibly associated with the onset of rain and a consequent enhancement of ethirimol uptake. Ethirimol treatment resulted in a large increase in yield. Similar fluctuations in sensitivity were observed in other trials, and are reflected to some degree in the overall survey figures.

Table 4

Field mildew levels (1974)

Region	Mean % leaf area with mildew					
	Early May			Late June**		
	Untreated		'Milstem'	Untreated		'Milstem'
East	3·5	(40)	1·0	29	(26)	13
West	1·4	(15)	0·2	11	(16)	2·5
North	4·3	(42)	1·6	7·1	(27)	1·4
South	2·5	(21)	0·6	17	(16)	6·0
Midlands	3·0	(21)	0·9	21	(17)	7·1
Scotland	1·8	(35)	0·3	9·8	(32)	2·5

Differences between treated and untreated within each region and on each date were significant (P=0·05).

**Sample size generally decreases at second sampling owing to necessity for spraying some of the 'untreated' fields.

( ) Number of site pairs assessed.

Table 5

Pattern of sensitivity, mildew control and yield on one trial in Essex

Date	Leaf assessed	% Mildew		Laboratory sensitivity		Yield+ (t/ha)	
		Untreated	'Milstem'	Untreated	'Milstem'	Untreated	'Milstem'
May 22	W*	16	2·5	11·5	6·5		
June 6	4	67	22	13·2	12·4		
July 7	3	72	45	17·5	9·1	4·14	4·79

W* = whole plant Standard error for sensitivity 1·66, and for yield 0·07.

+ Data from Davies and Adams (1974)

At the time of submission the results of the 1975 survey were not complete. Early control of mildew as generally good and the sensitivity of those isolates tested lies within the range found previously. Differences between treated and untreated fields in thetwo regions were similar to 1974. It is hoped to present some data at the Conference.

Populations in individual fields were mixed. For example, the sensitivity of 47 isolates from single pustules in an untreated field of winter barley in Worcestershire ranged from 6·4 to 19·0 with a mean of 14·3, and co-efficient of variation of 24%. Other examples of within-field variations are quoted by Wolfe (1973). The spread of response in typical sensitivity tests with bulk isolates is illustrated in Table 6. An isolate derived from a single chain similarly exhibited wide variation in dosage response (a 75-fold range of ethirimol concentration gave an incomplete range of response - Table 7).

Table 6

Relationship between application rate, disease control and sensitivity in laboratory tests

ppm a.i. on seed (nominal)	Mean grade disease control		
	Isolate 212W (3 tests)	St 1 (8 tests)	425 H (3 tests)
8000	2·6	3·3	4·0
2000	2·1	3·0	3·7
1000	1·0	2·2	3·2
250	0·23	1·4	2·9
100	0·07	1·0	1·6
0	0	0	0
Mean sensitivity	6·0	10·9	15·4
Standard error (sensitivity)	1·36	0·83	1·36

Table 7

Dosage response [+] of isolate derived from single spore chain

ppm a.i. in spray	Mean grade disease control* (2 replicate pots)
30·0	3·5
10·0	3·5
3·3	3·0
1·1	2·0
0·4	1·0

* Standard grades (see methods section)

[+] Ethirimol applied to barley seedlings as a foliage spray 2 days before inoculation with E.graminis.

Analysis of the ethirimol content of leaf pieces used in sensitivity tests (as ^{14}C) showed that concentrations in the ranges 1·9–8·3 and 0·06–0·10 ppm could be expected with seed treated at 8000 and 100 ppm respectively. Proximal pieces contained 30–50% less than distal pieces from the same leaves. It is noted that the wide range of concentrations did not quite cover the full range of disease control 0–100%.

DISCUSSION

Mildews from many fields were examined so as to determine overall trends. As it was impracticable to test multiple samples from each site, pustules from 25 sampling points were bulked so as to give one reasonably representative isolate per field. An alternative approach, in which test leaf material is placed in the field to receive inoculum directly (Wolfe 1975), is attractive because it avoids culturing the fungus before testing and the inoculum pressures are natural. However, it was rejected for our surveys because of its high labour requirement, difficulty in obtaining satisfactory test material over a wide area, dependence on the weather, and the relatively high risk of failure. Other methods, such as observations of spore germination and the early stages of infection as used by Holloman (1975), although providing excellent tools for detailed studies would be too tedious for a large survey and might apply to only part of the full process of disease control. The % leaf area diseased compared with an untreated control was chosen as a simple and direct measure of fungicide performance. Control of inoculum levels and ethirimol content of the leaf pieces was important.

The small settling tower provided very uniform inoculation within tests. Provision of large numbers of ethirimol treated leaf pieces proved difficult. By use of a pressurised constant environment and careful regulation of all manual operations, the concentrations of ethirimol in the leaf pieces were regulated between broad limits. Reliability was further increased by the use of 5 replicates and repeating tests on two occasions.

Little is known about the concentrations of ethirimol in the leaves of 'Milstem' treated barley in the field at the time when mildew control is critical. It is known that concentration varies within single leaves, between plants and between crops, and declines as the season advances. In one field test concentrations ranging between 0·2 and 4·5 ppm were encountered within 50 days of sowing (Shephard unpublished data), so that the concentrations used in the test leaf pieces are probably fairly realistic.

In the laboratory tests, although different degrees of control were obtained with the different isolates, all were controlled to a large extent by the higher concentrations. However, great caution must be exercised in any attempt to link application rate with field performance simply by looking at sensitivity grades. The relationship is complex and is likely to be influenced by such factors as soil condition, temperature, inoculum pressure, generation time and metabolic rate. At the moment there is no satisfactory basis on which to make such a correlation. Apart from these factors, sensitivity is unsatisfactory as a direct indicator of field performance because its measurement takes no account of the changes in field population which gave rise to the isolate under consideration. These surveys have demonstrated conclusively that direct measurements of disease control and yield are necessary to determine field performance. Trends in sensitivity, may however, provide useful advance warnings of changes in mildew populations. For instance, in 1973, the area which had been subjected to continuous and extensive use of ethirimol for the longest period, Norfolk and Suffolk, had the lowest overall sensitivity. In view of the importance of the generations of mildew on winter barley in determining the life of a resistant variety, and after consulting Dr Wolfe, it was decided to avoid such influence by withdrawing recommendations for use of ethirimol on winter barley. Subsequent observations in the same region show no further decrease in sensitivity, and probably a slight increase on the untreated crops, in spite of continued extensive use of the product. Over the same period in Scotland the frequency of occurrence of the most sensitive isolates appears to have decreased. The Norfolk - Suffolk results suggested that trends in sensitivity might be readily reversible.

With the broad spread of dosage response shown in Tables 6 and 7, it is understandable that treatment with ethirimol should result in preferential control of the most sensitive members of the population. The surviving pustules in a treated field would then have a lower sensitivity than the original population. This hypothesis is supported by the data. Sensitivity generally decreased in fields where ethirimol had been used and the mildew had been controlled. Examination of events in particular fields, as illustrated by Table 5, showed that although an isolate of abnormally low sensitivity was detected early in the season, there was no abrupt cessation of mildew control, only the normal build-up which accompanies the lower availability of the fungicide and extended life of the crop. There was no suggestion of dominance by the isolate of low sensitivity. Later samplings indicated that rapid and reversible response of the mildew population to prevailing pressures were possible.

Prediction of future trends in sensitivity of field performance is difficult. There are no satisfactory examples from which to draw analogies. With resistant cultivars the fungus is continuously exposed to the 'resistance factor' throughout the life-span of the crop. With some fungicide disease situations, e.g. Sphaerotheca fuliginea (powdery mildew of cucumber) and dimethirimol (Bent et al, 1971) and Cercospora beticola (leaf spot of sugar beet) and benomyl (Georgopoulos, 1972) individuals of low sensitivity rapidly dominated the population and caused major failures in disease control. However, the present situation seems different, with rapid shifts in sensitivity in both directions. From experience so far it seems likely that the sensitivity of E.graminis to ethirimol will continue to oscillate between the limits already observed, and the benefits of treatment will be maintained. However, it seems important to restrict application to once per year.

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STUDIES OF THE CARBOXYLESTERASES OF MYZUS PERSICAE RESISTANT AND SUSCEPTIBLE TO ORGANOPHOSPHORUS INSECTICIDES

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Summary An electrophoretic technique is described for separating and detecting the carboxylesterases from individual Myzus persicae. The improvement in sensitivity over previously described methods was primarily achieved by incorporating the non-ionic detergent, Triton X-100, into the aphid homogenates and polyacrylamide gels to solubilize membrane-bound esterases. This treatment affected only the intensity of the esterase bands on the gels without influencing their electrophoretic mobility. All resistant aphids had increased activity in one of the 7 esterase bands detected, and in some cases, other bands were also more active, or had a different electrophoretic mobility. The total esterase activities of the strains also differed, and were measured by a simple colorimetric method, and the advantages of both techniques for characterizing resistant aphids are discussed.

Résumé A partir d'un seul puceron Myzus persicae sept carboxylestérases peuvent être séparées et identifiées par analyse électrophorétique grâce à l'emploi du détergent Triton X-100 qui solubilise les estérase des fractions membranaires. Ce traitement intensifie la réaction colorimétrique sans modifier les R_M diagnostiques. La reaction colorimétrique d'une des sept estérases est particulièrement intense uniquement dans les souches résistantes aux insecticides derivés de l'acide phosphorique. Les souches susceptibles et résistantes different dans l'activité totale de leurs estérases et les avantages de ces deux techniques pour caracteriser la sensibilité des pucerons à certains insecticides sont discutés.

INTRODUCTION

The incidence of resistance of Myzus persicae (Sulz) to insecticides is increasing and there is an urgent need for improved laboratory techniques to detect resistance. Present methods depend on carefully controlled bioassay with many aphids (Needham & Devonshire, 1976), especially when resistance is small. Needham and Sawicki (1971) suggested a possible technique for detecting resistance in individual aphids based on the increased activity in resistant aphids of carboxylesterases hydrolysing 1-naphthyl acetate. The correlation they reported between resistance and increased activity of these enzymes has been confirmed

in subsequent work (Sudderuddin, 1973; Beranek, 1974; Needham & Devonshire, 1975), but it has not been definitely established that the increased enzyme activity is a cause of resistance. This year, in a survey of M.persicae from sugar beet, good agreement between resistance and esterase activity was again found, but there were some cases where the bioassay and enzyme assay were in conflict (Devonshire & Needham, 1975).

The esterase activity in M.persicae has been resolved by electrophoresis into several component enzymes (Sudderuddin, 1973; Beranek, 1974) using homogenates of 5 aphids or more per separation. This is very useful for examining aphid clones, but would not be suitable for characterizing individuals in mixed populations of resistant and susceptible aphids. The present paper describes an electrophoresis technique for resolving and detecting the enzymes from individual aphids, and compares this with an in vitro assay for total carboxylesterase activity for screening field populations of M.persicae.

METHOD AND MATERIALS

Aphids The origin of most of the aphid strains (Devonshire & Needham, 1974; Needham & Devonshire, 1975) and the rearing and bioassay techniques (Needham & Devonshire, 1973) have been described previously. Of the other aphids used, strain PirR had survived treatment with several organophosphorus compounds and pirimicarb in a glasshouse, a strain T1 clone V was established from a resistant population from sugar beet in 1975 (Devonshire & Needham, 1975), and strain SP was collected from peaches in Spain.

Carboxylesterase assay This was based on the method described by Gomori (1953). Individual aphids were weighed on a Beckman LM-500 microbalance and homogenized in 20mM phosphate buffer, pH 7.0 using a glass homogenizer with a PTFE pestle. Portions of the uncentrifuged homogenates equivalent to 0.25 aphid (for susceptible or slightly resistant strains) or 0.1 aphid (for very resistant strains) were incubated at 25°C with 1-naphthyl acetate (0.25mM) in a total volume of 3.0 ml buffer. After 30 min, Fast Blue B salt (0.3%) in 3.5% aqueous sodium lauryl sulphate (0.5 ml) was added, and the resulting complex measured colorimetrically at 605 nm 15 min. later. Under these conditions E_{605} was proportional to enzyme activity.

Electrophoresis was performed on 7.5% polyacrylamide gel rods (70 mm x 5 mm diameter) using the discontinuous buffer system described by Williams and Reisfeld (1964), but omitting the sample gel and spacer gel. After preliminary experiments, Triton X-100 (0.2%, w/v) was incorporated into the gels. The buffer system of Davis (1964) was also used, but its higher pH resulted in greatly increased background staining.

Individual aphids were weighed and homogenized in 15µl of water containing (by weight) 10% sucrose, 0.5% Triton X-100 and 0.001% bromocresol purple. The homogenizers were made by drawing and sealing 5 mm I.D. glass tubing to form conical tubes with tips closely fitting sealed 25µl Microcaps which were used as pestles. The homogenates were layered on the surface of the gels under the top reservoir buffer using either a microsyringe or narrow bore nylon tubing in a Microcap holder. After 2h of electrophoresis at 2mA/tube (approximately 10V/cm), the gels were stained for esterase activity at 25°C for 90 min. The stain was prepared by adding 30mM 1-naphthyl acetate in acetone (1 ml) to 0.2% (w/v) Fast Blue BB salt (Sigma) in 0.2M phosphate buffer, pH 6.0 (50 ml). The stained gels were stored in 7% acetic acid and photographed as described by Oliver and Chalkley (1971).

Electrophoretic mobilities were measured relative to the movement of the buffer interface in the gel. This was indicated in gels without detergent by the marker dye, bromocresol purple, but in gels containing 0.2% Triton X-100, the dye trailed behind the interface which was clearly visible as a refractive boundary. The position of this interface was marked on the tubes containing gels immediately after electrophoresis, and when calculating electrophoretic mobilities, allowance was made for any swelling of the gels during staining.

RESULTS

Total carboxylesterase activity Table 1 shows the ranges of total esterase activity in several strains of aphid from glasshouses and outdoors.

Table 1

Resistance factors and total esterase activities of several strains of Myzus persicae

Strain	Resistance factor to dimethoate [a]	Esterase activity μmol $hr^{-1}mg^{-1}$	Esterase $R_{/S}$
Susceptible			
SH[b]) US1 clone L[c])	-	0.20–0.35	-
Resistant			
US1 clone D[c]) MS1 clone G[c]) S5 clone H[c]) US6 clone C[c])	7–10	0.36–0.65) 0.36–0.76) 0.45–0.74)	2.0
T1 clone V[c]	c.40	1.50–2.70	7.6
$DDTR_b$[b]	c.150	1.25–1.62	5.2
$PirR_b$[b]	c.250	2.70–4.68	13.2
SP[d]	c.40	1.54–2.34	7.1

a. by topical application; b. from glasshouses, Great Britain;
c. from sugar beet, Great Britain; d. from peaches, Spain.

Activities were expressed per unit weight of the aphids because the resulting correlation between replicates was better than when expressed in terms of "activity per aphid". As in previous studies, the very resistant strains had much greater esterase activity than the susceptible strains, while the less resistant clones from sugar beet had approximately twice the esterase activity, sufficient to distinguish them from susceptible aphids. Esterase activity is given as the total range within the populations, and even in the slightly resistant clones this does not overlap with the range in susceptible aphids.

With the exception of strain DDTR, total esterase activities correlated well with resistance factors, and it may generally be possible to estimate the level of resistance on this basis.

Electrophoretic separation of esterases Previous work has shown that much of the esterase activity of M. persicae is associated with the "microsomal fraction" (Needham & Sawicki, 1971). Such membrane-bound enzymes would not be expected to migrate into polyacrylamide gels during electrophoresis. In the present study, 60-70% of the activity in crude aphid homogenates in buffer was membrane-bound, and was sedimented by centrifuging at 175,000g for 1 hr. However, other membrane-bound enzymes have been solubilized using the non-ionic detergent, Triton X-100 (Fishman, 1974; Devonshire, 1975), and this was also found to be effective with aphid esterases. Homogenates in 1% Triton X-100 in buffer contained 70-100% of the enzyme activity in a form which was not sedimented by centrifuging at 175,000g for 1 hr.

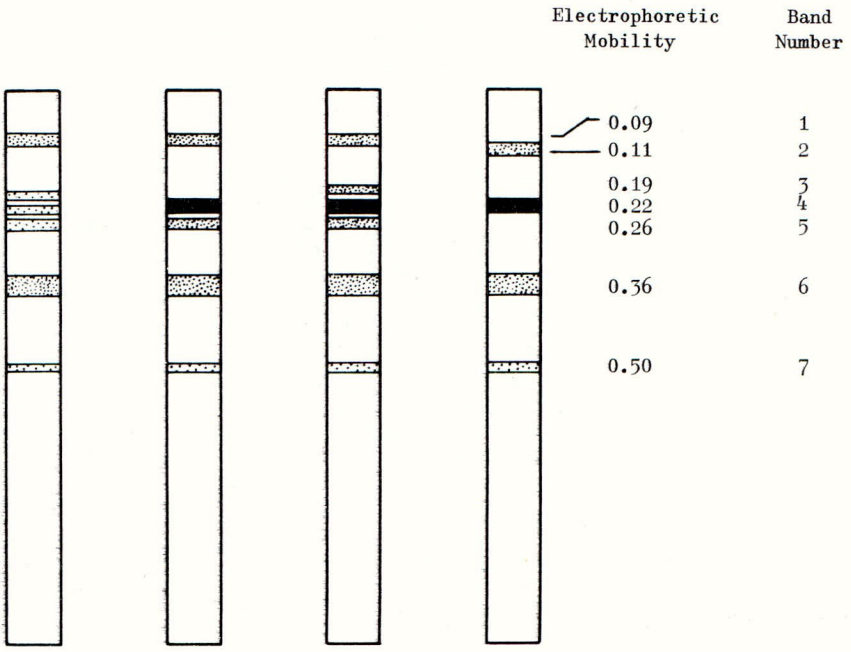
These preliminary results were supported by electrophoretic separations in the presence and absence of Triton X-100. With no detergent in the homogenate or gel, several esterase bands were detected, but most of the activity remained as a diffuse area on the top of the gels. However, little insoluble enzyme remained on the origin when Triton X-100 was incorporated into the homogenates and gels, and there was a corresponding increase in the intensity of some of the bands (mainly bands 4, 5 and 6 in figure 1). In the absence of detergent, some of the bands were visible only when homogenates containing 4-6 aphids were examined on a single gel, but all could be detected when individual aphids were homogenized in buffer containing 0.5% Triton, and 0.2% Triton was incorporated into the gels. The electrophoretic mobilities of the enzymes in the presence and absence of detergent were identical, indicating that its effect was only to solubilize the membrane-bound enzymes, without affecting their charge or molecular weight.

All aphids were therefore examined by electrophoresis in the presence of detergent and gave one of the esterase zymograms shown in Figure 1. There was no difference between strains in the activities of bands 6 and 7, and susceptible aphids from both glasshouse and field gave identical patterns. All resistant strains examined differed from susceptible aphids in having increased activity of esterase 4 (equivalent to esterase 2 described by Beranek, 1974).

Most of the resistant strains from the field were of type I, i.e. band 3 was absent, and bands 4 and 5 were both more intense than in susceptible strains. One field strain (S5 clone H) was of type II, having bands 3, 4 and 5 present, all with increased activity. All the resistant aphids from glasshouses, and one strain from sugar beet (T1 clone V) lacked bands 3 and 5, and only band 4 had increased activity. This third group also differed from susceptible aphids in having esterase band 2 instead of band 1.

Although strain DDTR had more total esterase activity than the resistant clones from sugar beet (US1D, MS1G, etc.), band 4 from all of these strains was of similar intensity. This enzyme had greater activity in strains SP and T1 clone V, and was most active in strain PirR.

Figure 1. Zymograms of esterase activity of susceptible and resistant M. persicae



DISCUSSION

We have used both total esterase activity, and the activities of electrophoretically resolved enzymes to distinguish resistant and susceptible aphids, and each technique has certain advantages. The total esterase assay is simple and gives quantitative results; for much of the work, we have used a very simple spectrophotometer, which gave identical results to more elaborate equipment. Resistant aphids with more than four times the esterase activity of susceptible aphids are readily recognised by visual examination of the esterase assay. In many cases an indication of resistance may be obtained simply by crushing individual aphids with a glass rod in 0.2 ml substrate in the wells of staining tiles, adding 0.05 ml dye solution after 10 minutes, and assessing the colour visually. The electrophoretic technique requires more elaborate equipment and greater expertise.

Furthermore, total esterase activity is measured in solution rather than in a gel matrix where limited penetration of the reactants into the gel could affect the activities of the separated enzymes to different extents. This might explain the discrepancy between the two methods when applied to the very resistant DDTR strain and the slightly resistant MS1G strain. The former had approximately twice the total esterase activity of the latter, but in polyacrylamide gels, esterase band 4 was equally intense in both strains, and MS1G also had greater activity in band 5.

The electrophoretic technique is more sensitive to small differences in activity than total esterase assay and distinguished more clearly between susceptible aphids and the slightly resistant strains from sugar beet. The intensities of the bands on gels were only slightly affected by weight variation between individual aphids. However, because of the small difference in total esterase between susceptible and slightly resistant aphids, it was necessary in these cases to correct for their weight, and this complicated the assay.

Both techniques are therefore suitable for distinguishing resistant and susceptible aphids, and both are sensitive enough to detect resistance levels as low as 7-10 fold. They are complementary with each other and with bioassays for assessing resistance, but the esterase methods have the advantage that they determine the proportion of resistant (i.e. high esterase) individuals in a mixed population.

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