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Sensitivity of Drechslera teres and Septoria nodorum to sterol-biosynthesis inhibitors

H. OLVÅNG

Department of Plant and Forest Protection, Swedish University of Agricultural Sciences, P.O. Box 7044, S-750 07 Uppsala, Sweden

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Abstract

The sensitivity of 282 isolates of *Septoria nodorum* was tested on prochloraz, propiconazole and guazatine, and of 129 isolates of *Drechslera teres* on prochloraz, propiconazole, imazalil and triadimefon. There was a great variation in sensitivity between isolates particularly at relatively low concentrations of the fungicides. However, none of the isolates was considered resistant towards the sterol biosynthesis inhibitors tested. Some isolates of *S. nodorum* grew on concentrations of guazatine that may present difficulties in controlling them under practical conditions.

Additional keywords: fungicide resistance, guazatine, imazalil, prochloraz, propiconazole, triadimefon.

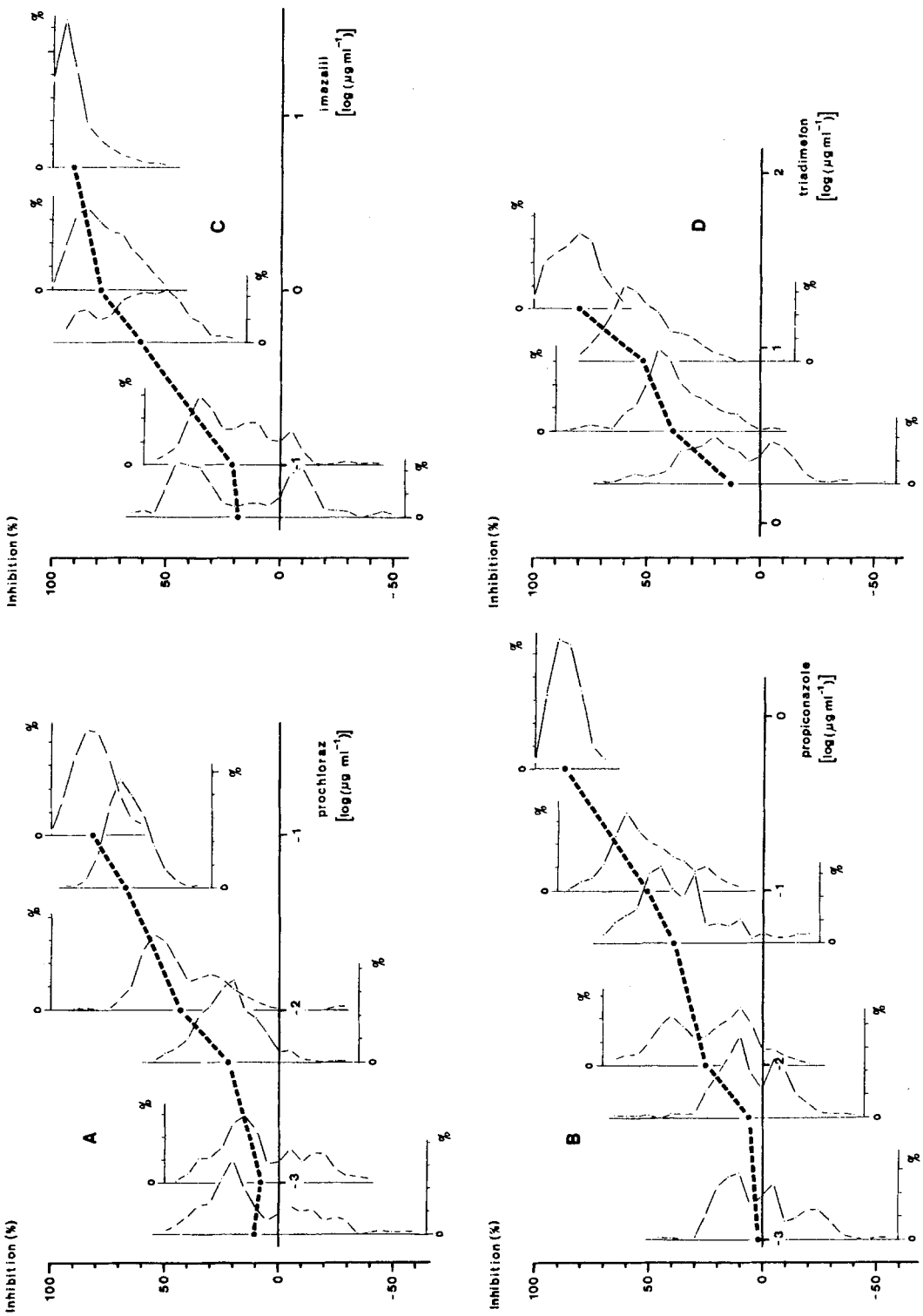
Introduction

Bayleton (triadimefon) was introduced as the first member of the sterol-biosynthesis inhibitors (SBI) on the Swedish market in the mid-seventies. It was essentially used as a mildew fungicide in wheat and barley. In 1979 imazalil was put on the market in the seed-dressing compound Panoctine Plus (guazatine, 300 g l^{-1}; imazalil, 20 g l^{-1}) against *Drechslera* spp. in spring sown cereals. Until 1982 the use of SBIs in winter wheat was rather limited, as was the foliar application in spring barley. Approximately 50% of the spring barley seed was treated with imazalil during the period 1980-1982.

In 1983, propiconazole (Tilt) and prochloraz (Sportak) were expected to be registered for use in Sweden, with a possibly sharp increase in the use of SBI fungicides. Before a strong selection pressure was applied on the fungal populations, a survey was undertaken to obtain baseline data on the sensitivity of *Drechslera teres* (Sacc.) Shoem. and *Septoria nodorum* (Berk.) Berk. to various SBIs. Isolates of the two fungi from seed on the 1982 crop of spring barley and winter wheat were used as material for the investigations. The sensitivity of *S. nodorum* to guazatine, which is not a SBI, was included in the investigations because of its frequent use as a seed disinfectant.

Materials and methods

Seed lots. Seed lots of winter wheat with high infestation of *S. nodorum* and of spring barley with high infestation of *D. teres* were obtained from the Swedish Seed Testing Institutes. The seed lots were collected from the entire wheat growing district from the 1982 harvest. Barley seed was collected in 1982 from the southern part of Sweden.


Inhibition (%)
A
prochloraz
[log(µg ml⁻¹)]
B
propiconazole
[log(µg ml⁻¹)]
C
imazalil
[log(µg ml⁻¹)]
D
triadimefon
[log(µg ml⁻¹)]

Fungicides. In agar tests, commercial formulations of the following fungicides were used: prochloraz (Sportak 45 EC), propiconaloze (Tilt 250 EC), triadimefon (Bayleton 25 WP), imazalil (experimental formulation, 25 g l^{-1}) and guazatine (Panoctine 40, experimental formulation, 400 g l^{-1}). Freshly prepared solutions were added to the agar just before use. Technical prochloraz (96.2% a.i.), kindly provided by Kemi-Intressen AB, Sweden, was used in the tests of one experiment.

Fungal isolates of D. teres. Spring barley with high natural infestation of *D. teres* was sown in a sand-peat mixture in shallow 20 cm diameter plastic pots. The pots were kept in the dark at 6 °C for 10 days. Then they were placed in a greenhouse until the 2-3 leaf stage. Leaves with primary symptoms of *D. teres* were collected and air dried. Spores were produced by placing leaves with long stripe lesions on wet filter paper in plastic dishes for 30 h at room temperature. Single spores (one per leaf) were picked with a sterile needle and grown for 5 days at 20 °C on PDA (Oxoid) with 100 μg streptomycin sulfate per ml agar. Mycelium plugs, 4 mm diameter, were then taken from the margins of the cultures and tested on freshly prepared malt extract agar (MEA, malt extract, Difco, 10 g l^{-1} and technical agar, Oxoid no. 3, 12 g l^{-1}) with various concentrations of the fungicides.

The colony diameters were measured after 3 days at 20 °C. Using only one plate per concentration, all fungicides were tested simultaneously. The tests comparing the effect of Sportak and technical prochloraz were done with three replicates.

Fungal isolates of Septoria nodorum. Winter wheat kernels with natural infestation by *S. nodorum* were surface sterilized for one minute in 70% ethanol and rinsed in running tap water. They were placed on heavy, wet blotting papers at 20 °C till the coleoptiles had grown out approximately 10 mm. Discoloured coleoptiles were cut off and placed on PDA (Oxoid) in 5-cm plastic dishes at 20 °C under near UV light 12 h day^{-1}. After approximately 2 weeks, ripe pycnidia of *S. nodorum* were formed in the mycelium. A spore suspension was made from one pycnidium per isolate and spread on PDA. Single spores were isolated (one per kernel) and the isolates were tested in an agar plug method as above. Mycelium was taken from 7-day-old cultures on MEA. Final colony diameters were measured after 7 days at 20 °C.

Statistical analysis. The frequency curves at each concentration in Figs 1 and 2 were obtained by grouping the degree of inhibition (%) for individual isolates into classes with 10%-units class width. The midpoints were selected so that values were obtained with 5% increments. The frequency of isolates was plotted versus the inhibition.

The isolates were divided into two groups according to their degree of inhibition at the lowest concentration used. In *D. teres* the distinction between isolates with inhibitions greater and smaller than +5% was made for prochloraz, imazalil and triadimefon.

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Fig. 1. Dosage - response curves of prochloraz (A), propiconazole (B), imazalil (C) and triadimefon (D) for 129 isolates of *D. teres* from 13 seed lots grown on MEA (thick dotted lines). The variation in response between isolates at each concentration is illustrated by frequency curves of the inhibition (thin lines). Axes: vertical = degree of inhibition (%); horizontal = % isolates (scale unit = 10%).

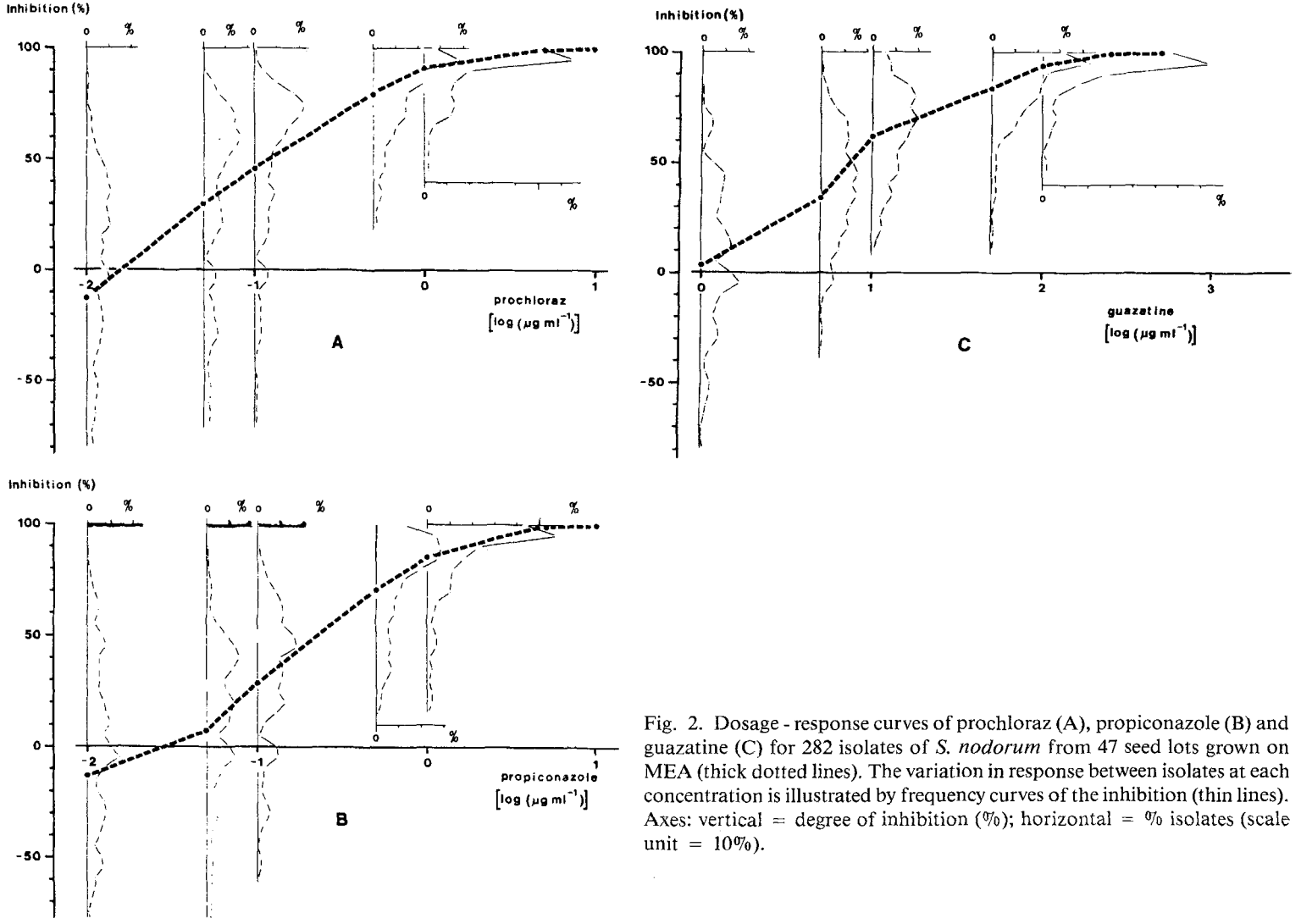

Fig. 2. Dosage - response curves of prochloraz (A), propiconazole (B) and guazatine (C) for 282 isolates of *S. nodorum* from 47 seed lots grown on MEA (thick dotted lines). The variation in response between isolates at each concentration is illustrated by frequency curves of the inhibition (thin lines). Axes: vertical = degree of inhibition (%); horizontal = % isolates (scale unit = 10%).

For propiconazole the division was made between isolates with inhibition greater than +5% and less than −10%. In *S. nodorum* the distinction was made between isolates that were inhibited and stimulated at the lowest concentration. The differences between groups at each concentration were tested for significant differences with Student's test. This test was also performed in comparision of groups of isolates with different growth rates in the control plates of *S. nodorum* and in comparision of the effect of prochloraz and Sportak.

Results

The average inhibition of 129 isolates of *D. teres* from 13 seed lots caused by prochloraz, propiconazol, imazalil and triadimefon is presented in Fig. 1. The variation in sensitivity between isolates is demonstrated by frequency curves of the response (% inhibition) in the individual isolates. The effect of prochloraz, propiconazole and guazatine on 282 isolates of *S. nodorum* from 47 seed lots is shown in dosage-response curves in Fig. 2. The variation in sensitivity is demonstrated in the same way as for *D. teres*.

The variation in response was rather large in both species. In *D. teres* there seemed to be a tendency for a bimodal distribution of the sensitivity, which was noticeable even at fairly high concentrations. For these reasons the populations were divided into two groups according to the sensitivity at the lowest concentrations used. In Figs 3 and 4 the average dosage-response curves for the different fungicides in the two groups are presented. In Table 1 the EC_{50} values for the different groups are listed.

Total inhibition of growth occurred in all isolates of *S. nodorum*. The minimal inhibitory concentrations (MIC) are presented in Fig. 5. Particularly for guazatine the values were high and the variation great. In *D. teres* only a few isolates were completely inhibited by those concentrations used.

Table 1. EC_{50} values for groups of isolates with different sensitivity to sterol biosynthesis inhibitors at low concentrations.

Fungus/Fungicide	EC_{50} value ($\mu g\ ml^{-1}$)[1]		Q-value[2]
	sensitive isolates	less sensitive isolates	
S. nodorum			
prochloraz	0.04	0.26	6.7
propiconazole	0.12	0.35	3.0
guazatine	5.32	10.38	2.0
D. teres			
prochloraz	0.009	0.026	2.9
propiconazole	0.065	0.24	3.7
imazalil	0.44	0.28	0.6
triadimefon	4.79	10.28	2.1

[1] Calculated from Figs 3 and 4.

[2] EC_{50} value of less sensitive isolates/EC_{50} value of sensitive isolates.

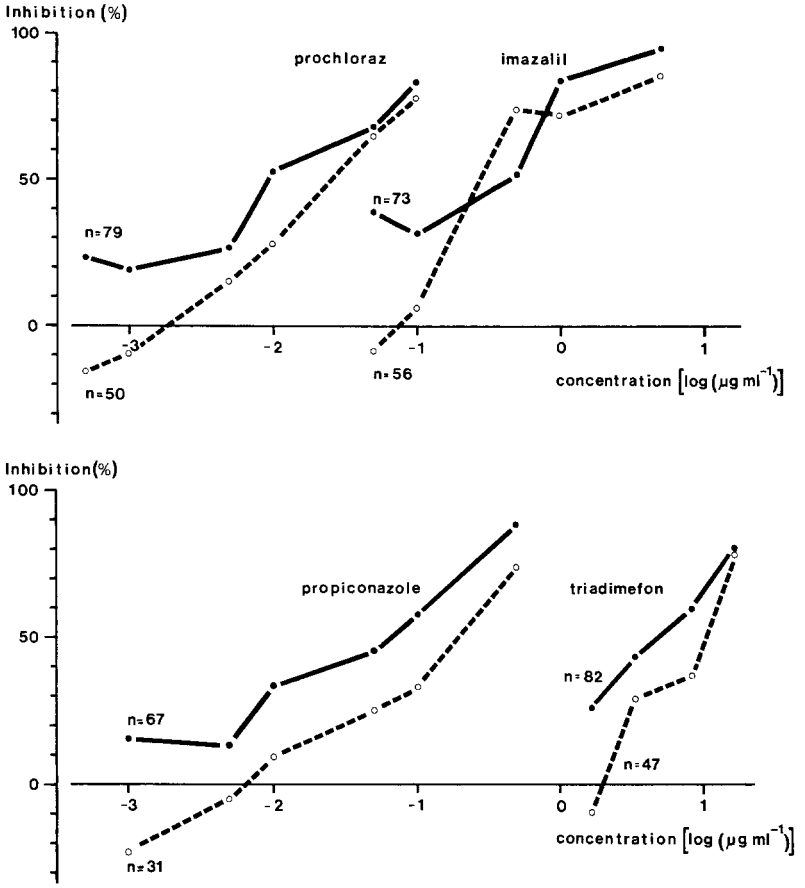

Fig. 3. Dosage - response curves for isolates of *D. teres* with different sensitivity to low concentrations of sterol biosynthesis inhibitors. Solid lines: isolates with strong inhibition at low concentrations. Broken lines: isolates with weak inhibition at low concentrations. For explanation see text. Significant differences between groups (t-test, $P < 0.05$) exist for all concentrations but prochloraz at 0.05 μg ml^{-1} and triadimefon at 16.7 μg ml^{-1}.

The growth rate in the control plates varied considerably between isolates in both species. For *S. nodorum* the colony diameter was between 7 and 32 mm in 7-day-old cultures and for *D. teres* the corresponding values were 12 and 26 mm after 3 days. A division of the *S. nodorum* isolates into two groups according to the size of the diameters in the control plates showed a significant difference in response between groups (Fig. 6). The slow-growing isolates had dosage-response curves almost identical to those of the isolates that were stimulated at low concentrations of the fungicides. The curves for the fast-growing isolates corresponded well with those for isolates that were inhibited at low concentrations (compare Figs 4 and 6). A similar division of the *D. teres* isolates did not reveal any difference in response between growth rate and inhibition at low concentrations (not presented).

In the tests mentioned above, commercial formulations of the fungicides were used.

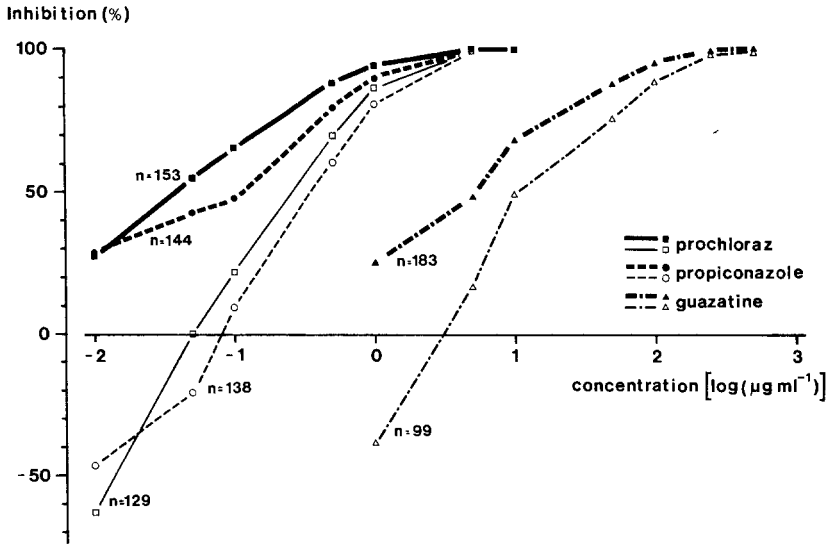

Fig. 4. Dosage - response curves of prochloraz, propiconazole and guazatine for isolates of *S. nodorum* that were inhibited (thick lines) or stimulated (thin lines) by low concentrations of the fungicides. Significant differences (t-test, $P < 0.05$) between groups existed for all concentrations but prochloraz at 5-10 μg ml^{-1}, propiconazole at 10 μg ml^{-1} and guazatine at 500 μg ml^{-1}.

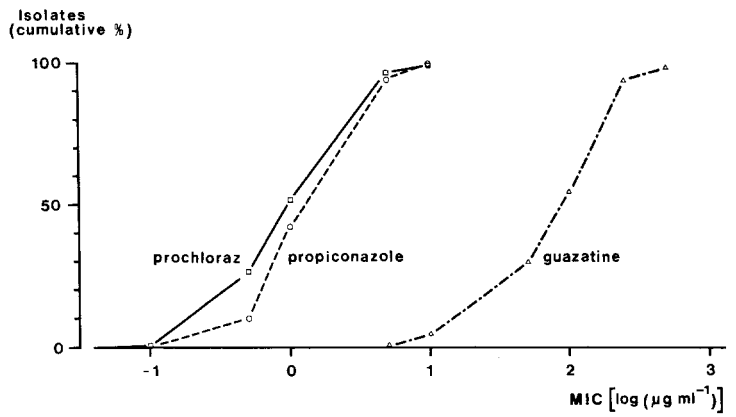

Fig. 5. Distribution curves of MIC values of prochloraz, propiconazole and guazatine for 282 isolates of *S. nodorum*.

These contain solvents and surface-active substances which may affect the growth of the fungus. Technical prochloraz (96.2% a.i.) was used in comparative tests between Sportak 45 EC and prochloraz. Sportak 45 EC stimulated the growth slightly in 5 of 9 tested isolates of *D. teres* at a concentration of 0.0005 μg prochloraz ml^{-1}, while no isolate was stimulated by technical prochloraz at this concentration. On the other hand, the inhibition of the growth was consistently stronger for Sportak 45 EC than for technical prochloraz at 0.5 μg ml^{-1}. The results are presented in Fig. 7.

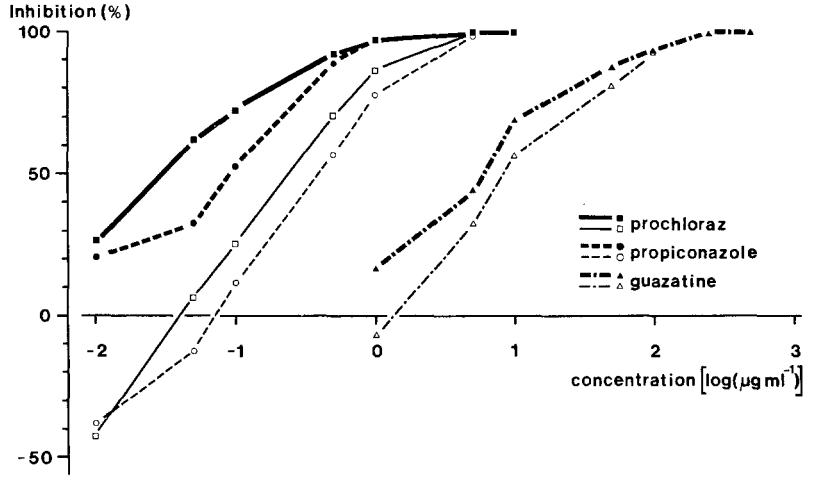

Fig. 6. Dosage - response curves of prochloraz, propiconazole and guazatine for isolates of *S. nodorum* with slow (thin lines, n = 164) and rapid growth (thick lines, n = 118) in the control plates. Significant differences (t-test, $P < 0.05$) between groups exist for prochloraz at 0.01-1.0 $\mu g\ ml^{-1}$, propiconazole at 0.01-5.0 $\mu g\ ml^{-1}$ and guazatine at 1.0-50 $\mu g\ ml^{-1}$.

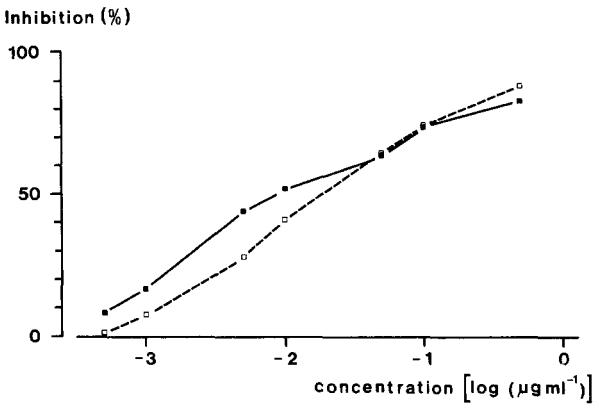

Fig. 7. Comparison of the inhibitory effect of equal concentrations of prochloraz as technical prochloraz (■ ——) or Sportak 45 EC (□ ------) on the growth of nine isolates of *D. teres*. Average of three replicates on PDA. Significant differences between formulations (t-test, $P < 0.05$) exist for all concentrations but 0.05 and 0.1 $\mu g\ ml^{-1}$.

Discussion

The SBIs affect sterol synthesis and in this manner cause a disturbance of cell-membrane functions. At least three different sites of action in sterol-biosynthesis are known: inhibition of C-14 demethylation, Δ7-Δ8 isomerisation and C-14 double-bond reduction (Fuchs et al., 1984). The SBIs used in this investigation are all supposed to interfere with the demethylation at the C-14 position, so-called demethylation inhibitors (DMIs). Resistance to one of these, imazalil, was shown to be controlled by at least 10 different

genes in *Aspergillus nidulans* (Van Tuyl, 1977). Our knowledge of the mechanisms at the enzyme level is still limited. For fenarimol, De Waard and Van Nistelrooy (1979, 1980) suggested an energy-dependent efflux mechanism for the resistance, but in other cases no mechanisms have been found.

In the present study a great variation in sensitivity in both species was recorded. In *S. nodorum* the sensitivity was evenly distributed (Fig. 2), but in *D. teres* subpopulations with different sensitivity to the DMIs, which could be noticed even at high concentrations of the fungicides, seemed to exist (Fig. 1). The ratio between the EC_{50} values in the two sensitivity groups (Q, Table 1), as calculated from Figs 3 and 4, varied between 0.6 and 6.7 and would in most cases indicate 'resistance' in the least sensitive group in comparison to the more sensitive ones according to the definition of Fuchs et al. (1984).

However, in *S. nodorum* the two curves merged at the point where total inhibition was obtained. All isolates were completely inhibited by 10 μg ml^{-1} prochloraz and propiconazole and only a few strongly inhibited (> 90%) isolates grew at 5 μg ml^{-1} of these two fungicides. There were no isolates that drastically differed in sensitivity from the rest of the population at high concentrations and, thus, would be considered resistant towards the DMIs. Total inhibition was obtained for only a few isolates of *D. teres*, but also here there was a tendency for the curves to merge with increasing concentrations of the fungicides. Nor did there exist any isolates that clearly differed in sensitivity from the population at the highest used concentrations in *D. teres*.

At the start of this investigation technical products of the fungicides were unfortunately not available at our department. It was later noticed that low concentrations of the formulated fungicides stimulated growth in a large fraction (½-⅓) of the isolates in both species. This stimulation could be caused either by the fungicides themselves or by additives (surface-active substances and solvents) in the commercial products. As the SBIs affect the membrane functions, it is possible that low concentrations of the fungicides in some isolates stimulated nutrient uptake and growth without harming the cell functions. Surface-active substances could also give such an effect. To test a possible difference in response between the pure fungicide and the formulated compound, nine isolates of *D. teres* were tested at the same concentration of prochloraz as Sportak 45 EC and technical prochloraz. At low concentrations, Sportak 45 EC inhibited the growth of *D. teres* significantly less than prochloraz did. For Sportak the slope of the curve was steeper so that at the highest concentration the inhibition was stronger for Sportak 45 EC than for prochloraz (Fig. 7). This indicates that the additives in the formulated fungicides seem in part to be responsible for the stimulation effect that was recorded in this investigation. However, the effect was relatively small for Sportak 45 EC, and it seems justified to assume that the dosage-response curves recorded here will only differ slightly from those of the pure fungicides.

In *S. nodorum* the stimulation of the fungicides at low concentrations was found in isolates with a slow growth rate in the control plates (Fig. 6). In *D. teres* such a correlation was not observed. There is no explanation for the observed phenomenon. The stimulation occurred at such low concentrations that it probably is of little importance under practical conditions.

A stimulation effect was also recorded for guazatine. Guazatine by itself is a surface-active substance and seems also to act on the membrane functions (Meller and Buchenauer, 1986); therefore the stimulation may have the same cause as the SBIs.

Resistance to guazatine was detected in *Penicillium digitatum* in Australia (Wild, 1983). Resistant isolates had EC_{50} values of approximately 0.5 $\mu g\ ml^{-1}$ compared with approximately 0.05 for the wild-type population. Meller and Buchenauer (1986) could induce resistance in *Fusarium culmorum* with UV radiation and mutagenic substances. The resistance factor was between 3.4 and 5.7. However, they considered the resistance risk low due to guazatine's mode of action and the low mutation rate. In the present investigation the average EC_{50} value for *S. nodorum* was approximately 7.2 $\mu g\ ml^{-1}$. There were isolates that still grew at 500 μg guazatine ml^{-1} agar and approximately 50% of the isolates had MIC values of 100 $\mu g\ ml^{-1}$ or more. It seems likely that the least sensitive types will be difficult to control with seed treatment. Since the end of the seventies, guazatine (0.7 $g\ kg^{-1}$) has dominated as seed-dressing fungicide in winter cereals in Sweden.

As no material of untreated origin was available in Sweden, it is unknown whether this low sensitivity for guazatine occurs naturally in *S. nodorum* or whether the use of guazatine has selected for less sensitive types. There have been no complaints of decreased efficiency in practice nor have reduced effects in field and laboratory experiments been observed in the course of time. It is suggested that the first possibility is most likely. However, since small changes are difficult to detect in this type of experiment, selection for less sensitivity types cannot be ruled out.

Resistance to SBIs is easily induced in vitro (Van Tuyl, 1977; De Waard and Fuchs, 1982). In early studies it was observed that resistant types were less fit than wild-types and that resistance under practical conditions was unlikely to develop (Fuchs and Drandarevski, 1976; Fuchs et al., 1977). However, in the course of time resistance in practice has been reported in *Sphaerotecha fuliginea* (Huggenberger et al., 1984; Schepers, 1985a) and *Pyrenophora teres* (Sheridan and Grbavac, 1985). In the Netherlands triforine was successfully used against *S. fuliginea* during the whole growing season when first introduced. After the more persistent DMIs fenarimol and imazalil had been used, the weaker triforine lost much of its effect (Schepers, 1983). A similar phenomenon was observed in New Zealand where the weak triadimenol lost its effect on *D. teres* after the introduction of new barley varieties from Europe. However, the stronger imazalil still gave good control as seed treatment (Sheridan and Grbavax, 1985). Later they discovered isolates of the spot form of the fungus (*D. teres* f. *maculata*) with reduced sensitivity to imazalil (Sheridan and Nendick, 1987).

No failure in disease control was observed in *Erysiphe graminis* f.sp. *hordei*, but it was suspected that the duration of control measures may be decreased due to reduced sensitivity in the fungal population (Fletcher and Wolfe, 1981; Fehrmann, 1984). However, the proportion of isolates with reduced sensitivity has increased between 1981 and 1984 (Wolfe et al., 1984), which shows that less sensitive types of the fungus seem to increase under high selection pressure with the risk of loss in fungicide efficiency. Apparently the effective dose of fungicide was high enough to control also resistant types (cf. Wolfe et al., 1984; Fig. 1). Increased use of DMIs in the Netherlands was correlated to an increase in isolates of *E. graminis* f.sp. *tritici* with reduced sensitivity to DMI fungicides. An observed decline in field performance of the DMIs was suggested to be partly caused by the reduced sensitivity in the fungal population (De Waard et al., 1986).

Isolates resistant to SBIs have been considered less fit and conducive to slowing down the build-up of resistant populations under practical conditions (Fuchs and Drandarev-

ski, 1976; Fuchs et al., 1977; De Waard and Gieskes, 1977). Schepers (1985a, 1985b) studied the fitness parameters of *S. fuliginea* from commercial greenhouses and found that for many isolates the fitness was hardly reduced. He concluded that resistance to SBIs can gradually develop under prolonged and exclusive selection pressure of SBIs. A similar warning was made by De Waard et al. (1982) who studied resistance to SBIs in *P. italicum*.

The variation in sensitivity between isolates in the present study was high, although the MIC values and the dosage-response curves did not reveal any isolates of *S. nodorum* and *D. teres* that could be considered resistant to the DMIs. Nevertheless, a warning for problems with reduced efficiency of the DMIs in the future is still called for. The selection pressure applied to the crop will select for the least sensitive types in the populations. With their increased fitness, there is a great risk for development of populations which are more difficult to control. For *D. teres* almost all barley seed is treated with imazalil and a growing proportion of the barley crop acreage is treated against mildew and/or net blotch. A large part of the wheat acreage is treated with SBIs against mildew and glume blotch. The SBIs are used almost exclusively for these treatments and will cause a strong selection pressure for resistance in both pathogens.

Aknowledgements

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Samenvatting

De gevoeligheid van Drechslera teres en Septoria nodorum voor remmers van de sterolbiosynthese

Isolaten van *Septoria nodorum* (n = 282) werden getoetst op gevoeligheid voor prochloraz, propiconazool en guazatine en van *Drechslera teres* (n = 129) voor prochloraz, propiconazool, imazalil en triadimefon. Er waren grote verschillen in de gevoeligheid van de isolaten, in het bijzonder bij lage concentraties van de fungiciden. Geen van de isolaten kan echter als resistent tegen sterolbiosynthese-remmers worden opgevat. Het feit, dat sommige van de isolaten van *S. nodorum* zich ontwikkelden op media die hoge concentraties guazatine bevatten, kan echter duiden op moeilijkheden bij de bestrijding onder praktische omstandigheden.

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