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Fungicide sensitivity of South African net- and spot-type isolates of *Pyrenophora teres* to ergosterol biosynthesis inhibitors

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Abstract. *Pyrenophora teres*, the causal agent of net blotch of barley (*Hordeum vulgare*), is a foliar pathogen that occurs as two distinct types as indicated by symptom expression on differentially susceptible cultivars. *P. teres* f. *teres* produces a net-type symptom while *P. teres* f. *maculata* produces a spot-type symptom. Fungicide sensitivities (IC_{50} values) of 89 monoconidial isolates of *P. teres* to sterol-demethylation-inhibiting fungicides were determined, based on the inhibitory effect on radial mycelial growth. These isolates were evaluated *in vitro* to determine their sensitivity to triadimenol, bromuconazole, flusilazole, propiconazole and tebuconazole. Infected leaves displaying either net- or spot-type symptoms were sampled from four fields, with two fields representing each respective symptom type. Both net- and spot-type isolates revealed strong resistance to triadimenol, the mean IC_{50} value being 25.7 μg/mL. Flusilazole was shown to be the strongest inhibitor of fungal growth with a mean IC_{50} value of 0.71 μg/mL. Spot-type isolates showed a higher resistance than net-type isolates to all five fungicides screened. Significant differences in fungicide sensitivities were found. The overall conclusion of this study is that spot-type isolates show a higher degree of resistance to commercially used fungicides than net-type isolates.

Introduction

Sterol-demethylation inhibitors (DMIs) constitute a modern class of fungicides with a broad spectrum of fungal activity (Scheinpflug and Kuck 1987). Irrespective of their diverse chemical structures, all DMIs have been identified as effective inhibitors of the C-14 demethylation of 24-methylenedihydrolanosterol, a precursor of fungal sterol biosynthesis (Buchenauer 1987). It is generally accepted that a small proportion of a population contains naturally occurring resistant genotypes in a pathogen population before the first fungicide applications (Brent 1992). However, under continuous selection pressure due to fungicide application, a fungal population can shift towards a state of reduced sensitivity, and the proportion of resistant phenotypes may reach a level where satisfactory disease control is no longer achieved.

Pyrenophora teres (anamorph *Drechslera teres*) the causal agent of net blotch disease of barley (*Hordeum vulgare*), is an economically important disease of this crop in South Africa and throughout most other barley growing regions in the world (Shipton *et al*. 1973; Steffenson *et al*. 1991; Louw *et al*. 1996). Yield losses attributed to net blotch, ranging from 26–77%, have been reported from different countries (Deadman and Cooke 1987; Steffenson *et al*. 1991). Two types of leaf symptoms are associated with net blotch disease, namely a net-type symptom which produces elongated, light-brown lesions with dark-brown reticulations, and a spot-type symptom which produces dark-brown spots with distinct halos (Smedegard-Petersen 1971). *P. japonica* (anamorph *Drechslera tuberosa)* was originally described as the pathogen causing spot-type symptoms, whereas *P. teres* was associated with net-type lesions (Ito and Kuribayashi 1931). After successful mating between net- and spot-type isolates by Smedegard-Petersen (1971), it was concluded that the two types were formae of the same species, and were subsequently named *P. teres* f. *teres* (net-type isolates) and *P. teres* f. *maculata* (spot-type isolates). Both types occur within proximity of each other in the Western Cape Province of South Africa (Louw *et al*. 1995, 1996).

Fungicides are used routinely for the control of net blotch (Van den Berg and Rossnagel 1990; Scott *et al*. 1992; Toubia-Rahme *et al*. 1995). In South Africa, triadimenol has been used routinely since 1979 as a seed treatment to control various barley fungal diseases including net blotch. The foliar-applied fungicides, propiconazole, flusilazole and tebuconazole were introduced during 1984, 1988 and 1989, respectively. Although these fungicides have been used extensively on barley fields for almost 2 decades in South Africa, no information is available on the fungicide sensitivity of net blotch populations to these compounds.

Published data concerning fungicide application programs on barley in the Western Cape of South Africa is very limited. Information on fungicide sensitivity amongst

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local isolates of net blotch towards the different commercially used fungicides is urgently required, therefore, to assist in the formulation of strategic fungicide spray programs. The aim of the present study was to determine the sensitivity of local populations of *P. teres* to the five commonly used fungicides, triadimenol, bromuconazole, flusilazole, propiconazole and tebuconazole. A further aim was to determine if net- and spot-type isolates from fields with similar fungicide histories differed significantly in their sensitivity towards these fungicides.

Methods

Sampling

Leaves containing net- or spot-type symptoms of *P. teres* were collected from barley fields in the Western Cape, the major barley-producing region in South Africa. Leaves were sampled from four fields with comparable fungicide histories (triadimenol seed treatments since 1990, carbendazim/flusilazole or propiconazole spray applications since 1987). Two areas, each being approximately 30 ha in size and 15 km apart, were chosen for each symptom type. The four fields sampled were treated as replicates (Table 1). One diseased leaf per plant was sampled at 1 m intervals along a 25 m transect, giving 25 leaves per field.

Table 1. Isolates of *Pyrenophora teres* used in this study

Field	No. of isolates	Isolate type	Cultivar	Sampling year
A	21	Net	Stirling	1997
B	25	Net	Stirling	1997
C	24	Spot	Clipper	1997
D	19	Spot	Clipper	1996

Fungal isolation

Fungal isolates used in this study are listed in Table 1. Following sampling, the leaves were air-dried to reduce the probability of bacterial contamination during isolation. Symptomatic leaves were surface-sterilised by immersion for 30 s in 70% (v/v) ethanol, followed by 60 s in 2% (v/v) NaOCl and finally again for 30 s in 70% (v/v) ethanol. Air-dried leaf sections were mounted onto glass slides with petroleum jelly for adhesion, placed in moist chambers and incubated at 15°C under continuous near-ultraviolet light for 3–4 days to induce sporulation. Conidia were transferred onto 2% water agar (WA; Biolab, Merck, South Africa) plates and left to germinate. Single germinated conidia were transferred onto potato-dextrose agar (PDA; Biolab, Merck, South Africa) and incubated at 25°C. Isolate identity was confirmed as either net- or spot-type using the technique as explained in Campbell *et al*. (1999).

Fungicide sensitivity

The technique described by Robbertse *et al*. (1996) was followed. Mycelial plugs (3-mm-diameter) from 7-day-old cultures were placed at the centre of PDA plates amended with either triadimenol, bromuconazole, flusilazole, propiconazole or tebuconazole at concentrations of 0, 1, 10, 30 and 60 µg/mL for triadimenol and 0, 0.1, 0.3, 1, 3 and 10 µg/mL for the other fungicides. All isolates were tested in triplicate at each concentration for each fungicide. Stock solutions were prepared by dissolving the respective fungicides in 70% (v/v) ethanol. For each fungicide, control plates were amended to contain the same amount of solvent as plates containing the highest concentration of fungicide. Plates were incubated inverted at 25°C for 5 days. Colony diameters were determined by averaging two perpendicular measurements and subtracting the diameter of the agar plug. The degree of inhibition (per cent inhibition) was expressed as the proportion of radial growth on the fungicide-amended plates compared to growth on the control plates. After visual inspection a best curve was fitted to these data. The SAS/STAT software version 6.04 package was used to calculate the IC_{50} values for each isolate. Non-linear regression was used to calculate the IC_{50} for each isolate by regressing radial growth (as a proportion of the control) against log-transformed fungicide concentrations and using the fitted regression line to estimate IC_{50} values.

Analysis of variance

A two-way analysis of variance with symptom type (net- and spot-type) and fungicides as factors was performed on the data. Significant effects ($P \leq 0.05$) were examined with the Student's *t*-test.

Results

Fungicide sensitivity between net- and spot-type isolates

Differences in IC_{50} values were highly significant ($P = 0.0001$) with regard to fungicide sensitivities between net- and spot-type isolates (Fig. 1 and Tables 2 and 3). Spot-type isolates were less sensitive than net-type isolates towards all the fungicides screened.

Inhibiting capacities of different fungicides

Highly significant differences ($P = 0.0001$) were obtained for the different fungicides that were tested (Table 3). Using the triadimenol concentration of 10 µg/mL as a discriminating concentration between sensitive and insensitive (resistant) isolates to this fungicide (Peever and Milgroom 1992), it was found that 64% of the *P. teres* isolates tested in this study were resistant to this fungicide. The lowest sensitivity of both net- and spot-types occurred towards triadimenol and the greatest sensitivity occurred towards flusilazole (Fig. 2). The difference in fungal growth inhibitory capacities between the fungicides (indicated by IC_{50} values) was highly significant ($P = 0.0001$, Table 3), except between propiconazole and bromuconazole.

Discussion

In other barley-producing countries, different fungicides are continually being tested to determine their ability to inhibit the growth of *P. teres* (Sheridan *et al*. 1985; Martin and Sanderson 1988; Van den Berg and Rossnagel 1990; Scott *et al*. 1992). The majority of these studies have focussed on the two DMI fungicides, triadimenol as a seed treatment, and propiconazole as a foliar spray. However, there are several reports of resistance to triadimenol that was first detected in New Zealand (Sheridan and Grbavac 1985; Sheridan *et al*. 1985). These reports were later validated by Peever and Milgroom (1992, 1993), who proved that by mating triadimenol sensitive and insensitive isolates, progeny inherited triadimenol resistance from parental isolates at specific loci. Isolates of *P. teres* growing on

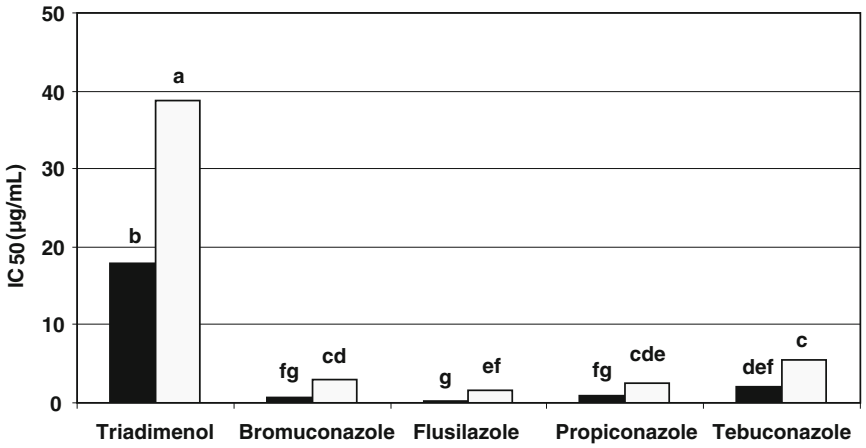

Fig. 1. IC_{50} values for *Pyrenophora teres* to DMI fungicides for net (closed bars)- and spot (open bars)-type isolates. Bars labelled with different letters are significantly different according to the Student *t*-LSD test ($P \leq 0.05$).

triadimenol-amended laboratory media at concentrations of 25–50 µg/mL have been reported (Sheridan and Grbavac 1985; Peever and Milgroom 1992). The results in the present study compare favourably with those obtained in other studies. That such high IC_{50} values were obtained in all the fields sampled can almost certainly be attributed to triadimenol being used extensively as a seed treatment in the Western Cape Province for the past 2 decades.

Propiconazole has been tested as a foliar spray for the control of net blotch on barley in various countries including South Africa (Martin and Sanderson 1988; Scott *et al*. 1992). However, there is no information regarding the sensitivities of *P. teres* towards the other commercially used DMI fungicides in South Africa. Most fungicide studies on barley have focussed on the barley scald pathogen, *Rhynchosporium secalis*, that in various countries has been shown to be a more serious pathogen than net blotch (Scott *et al*. 1992). Robberse *et al*. (2001) reported that *R. secalis* isolates also showed resistance towards triadimenol, but not to other DMI fungicides. The build-up of resistance by *P. teres* and *R. secalis* can be attributed to the use of triadimenol as a seed treatment since 1979. This treatment,

Table 2. Fungicide sensitivity ranges and mean IC_{50} values of *Pyrenophora teres* isolates from different fields to DMI fungicides

Field	Fungicide									
	Triadimenol		Bromuconazole		Flusilazole		Propiconazole		Tebuconazole	
	Range[A]	Mean[B]	Range	Mean	Range	Mean	Range	Mean	Range	Mean
A	0.307–67.060	20.409	0.020–3.957	0.932	0.025–1.030	0.314	0.095–7.693	1.262	0.012–11.098	2.351
B	0.264–57.739	15.765	0.172–1.711	0.671	0.002–0.972	0.192	0.004–0.702	0.415	0.080–6.893	2.008
C	3.625–55.360	43.335	0.186–5.579	3.406	0.178–3.905	1.791	0.138–5.772	3.056	0.022–24.046	6.447
D	4.920–42.907	28.751	0.276–5.429	2.276	0.055–3.699	1.029	0.219–2.714	1.444	0.165–7.634	3.574

[A,B] IC_{50} values in µg/mL.

Table 3. Analysis of variance of *in vitro* sensitivity of *Pyrenophora teres* isolates towards DMI fungicides

Source	df	Sum of squares	Mean squares	*F*	*P*
Fungicides (F)	4	300.50	75.13	69.48	0.0001
Symptom type (S)	1	60.10	60.10	55.59	0.0001
F × S	4	4.61	1.15	1.07	0.3778
Error (a)	88	95.14			
Error (b)	348	117.95			
Corrected total	445	578.31			

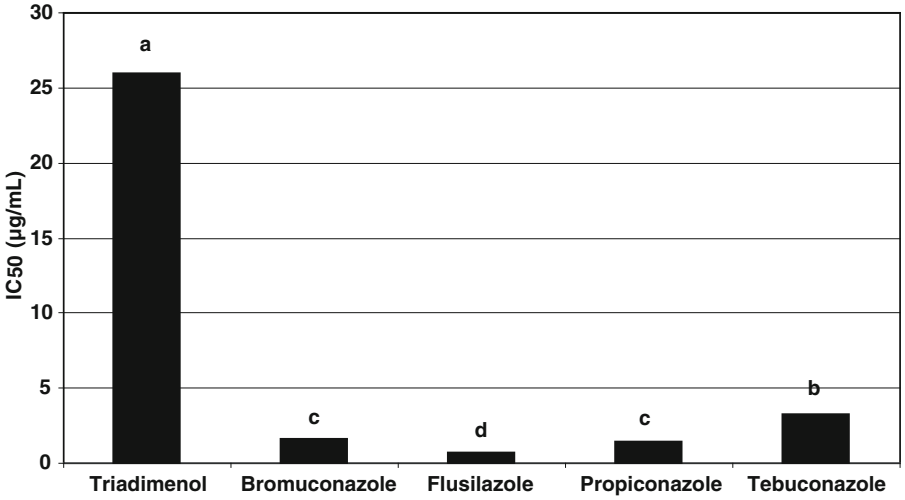

Fig. 2. IC_{50} values for *Pyrenophora teres* (net- and spot-types combined) for each DMI fungicide. Bars labelled with different letters are significantly different according to the Student *t*-LSD test ($P \leq 0.05$).

therefore, exerted selection pressure on these two pathogens. However, because a wide range of DMI fungicides was available for foliar application to control these pathogens, the specific DMI selection was limited. In the Western Cape of South Africa, net blotch and barley scald are treated with the same DMI fungicides, namely triadimenol, tebuconazole, propiconazole, bromuconazole and flusilazole.

In the present study, spot-type isolates of *P. teres* were found to be significantly more resistant than net-type isolates to all the fungicides tested. This could mean that spot-type isolates may have built up more resistance as a result of various evolutionary factors. First, Scott (1988) and Louw *et al*. (1996) indicated that until recently the spot-type of *P. teres* was the predominant type (83%) in the Western Cape Province. This was probably due to the introduction of the now dominant Australian cultivar Clipper that is susceptible to spot-type but resistant to net-type. A higher proportion of the spot-type net blotch populations in the Western Cape have thus been subjected to more intensive fungicide control programs and, as a result, have built up more resistance to them. Second, by crossing isolates of *P. teres*, Peever and Milgroom (1992) were able to show that triadimenol resistance in the progeny was controlled by a major gene as well as three to five other minor genes. Shaw (1989) reported that sexual recombination and quantitative inheritance of resistance could change the distribution of resistant phenotypes. This would mean that progeny with various degrees of resistance would be produced. Peever and Milgroom (1992) also stated that sexual reproduction occurring once a year in net blotch populations might be significant in increasing the genetic variation in resistance. This variation is then selected for in subsequent asexual generations. It is, therefore, feasible that sexual recombination increased the rate of resistance of spot-type isolates towards DMI fungicides, bearing in mind that the sexual stage of the spot-type has been collected from barley fields in the Western Cape (Louw *et al*. 1994). Third, various studies have indicated that the spot-type of *P. teres* is more difficult to control than the net-type. Scott *et al*. (1992) reported that two applications of propiconazole were required to reliably control spot-type, whereas only one application was required for net-type control (Scott *et al*. 1992).

In the Western Cape Province, it is not possible to set up a baseline sensitivity for *P. teres* populations towards DMI fungicides, as all barley production has been subjected to continual spray control programs for the past 2 decades. Baseline sensitivity could perhaps be determined only for bromuconazole, due to its recent introduction into South Africa in 1996. However, there are reports that *P. teres* displays cross-resistance for some DMIs (Peever and Milgroom 1993) and, therefore, genes resistant to other DMIs may mask the actual sensitivity of net blotch isolates towards bromuconazole.

In conclusion, the findings of this study clearly indicate that despite the recent introduction of bromuconazole into South Africa as a foliar spray for fungal pathogens of barley, the most effective fungicide against net blotch is still flusilazole. In addition, triadimenol is clearly not beneficial in terms of net blotch resistance programs, and an alternative seed treatment should be considered to combat the build-up of fungicide resistance.

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