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Sensitivity of South African *Ramulispora herpotrichoides* isolates to carbendazim and ergosterol biosynthesis inhibitors

B. ROBBERTSE, G. HOLZ *and* P. W. CROUS
Department of Plant Pathology, University of Stellenbosch, Stellenbosch 7600, South Africa

In a survey conducted during 1991–1992, single-spored isolates of the eyespot fungus from the Swartland area were characterized and tested for sensitivity to carbendazim and ergosterol inhibiting fungicides. The 100 isolates tested were all fast growing, even marginate, and designated as *Ramulispora herpotrichoides*. Fungal growth was completely inhibit on PDA amended with carbendazim (1 μg/ml), indicating that the local population of the fungus is still at baseline sensitivity to benzimidazoles. The mean concentration of prochloraz calculated to inhibited growth by 50% (IC_{50} value) was $0{\cdot}043 \pm 0{\cdot}029$ μg/ml, which is comparable with the baseline sensitivity reported for European isolates. Of the 36 representative isolates screened against 2 μg/ml triadimenol, 44% were sensitive, while 36% were resistant. The triadimenol-resistant isolates were sensitive to propiconazole and flusilazole. However, four of the triadimenol-resistant isolates were also resistant to tebuconazole. These results indicate that South African isolates of *R. herpotrichoides* are sensitive towards carbendazim, prochloraz, propiconazole and flusilazole. They were found to differ, however, in sensitivity towards triadimenol and tebuconazole, where some isolates had an IC_{50} value greater than 2 μg/ml.

INTRODUCTION

Eyespot, caused by *Ramulispora herpotrichoides* (Fron) von Arx (= *Pseudocercosporella herpotrichoides* var. *herpotrichoides* [Fron] Deighton), is a common disease of wheat in several countries (Fitt *et al.*, 1988), including the Swartland and Koeberg areas of the Western Cape Province of South Africa (Welgemoed, 1987; Lochner, 1989; Scott, 1990; Robbertse *et al.*, 1994). *R. herpotrichoides* var. *herpotrichoides* and *R. herpotrichoides* var. *acuformis* (Nirenberg) Boerema, Pieters and Hamers are regarded as comparable with the W- and R-type respectively (King & Griffin, 1985; Sanders *et al.*, 1986; Julian & Lucas, 1990). R-type isolates are equally virulent on wheat and rye, while W-type isolates are more virulent on wheat seedlings than R-types (Lange-de la Camp, 1966; Scott *et al.*, 1975; Hollins *et al.*, 1985). These varieties also differ in conidial and culture morphology, mating populations, infection processes, isozyme zymograms and DNA polymorphisms (Lange-de la Camp, 1966; Scott *et al.*, 1975; Moreau *et al.*, 1989; King, 1990; Julian & Lucas, 1990; Daniels *et al.*, 1991; Nicholson *et al.*, 1991). Recent results based on DNA relatedness have led Takeuchi & Kuninaga (1994) to conclude that the W- and R-type should be treated as two species, rather than varieties. In subsequent studies combining data from random amplified polymorphic DNA profiles and esterase zymograms (Campbell, 1995; Robbertse *et al.*, 1995), R-types were shown to have only 25–40% similarity with W-types. Based on the low percentage DNA similarity, as well as the differences in colony and spore morphology, infection processes, and distinct mating populations (Dyer *et al.*, 1994; Robbertse *et al.*, 1994), it was concluded that these two varieties should be treated as separate biological species, namely *R. herpotrichoides* and *R. acuformis* (Nirenberg) Crous.

In South Africa, local wheat cultivars are highly susceptible to the eyespot pathogen and the disease is primarily controlled by fungicide application (Trench *et al.*, 1992). In 1986, prochloraz, a demethyl inhibiting fungicide (DMI), and a combination of prochloraz and diclobutrazol, were the first fungicides recommended locally for eyespot control (Bot *et al.*, 1987). Other fungicides presently registered against eyespot mostly have triazole as sole active ingredient and are used in combination with either carbendazim or prochloraz (Nel *et al.*, 1993). DMI fungicides inhibit the C14

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demethylation step in the fungal ergosterol biosynthesis (Copping *et al.*, 1984), whereas benzimidazole fungicides (methyl-benzimidazole-carbamate-generating [MBC] fungicides) act by affecting tubulin synthesis (Davidse, 1973).

Following the development of eyespot resistance against MBC fungicides in Europe (Rashid & Schlösser, 1975, 1977; Bateman *et al.*, 1985; Hollins *et al.*, 1985; King & Griffen, 1985; Cavelier *et al.*, 1985), carbendazim was primarily replaced by prochloraz (Hoare *et al.*, 1986). It was generally believed that insensitivity or resistance to sterol inhibitors under field conditions was unlikely, because resistant strains in the laboratory tended to be less fit than the wild-type strains (Fuchs & Drandarevski, 1976). However, resistance to triazole fungicides has been recorded in *Pyrenophora teres* (Sheridan *et al.*, 1985), *Venturia inaequalis* and *Guignardia bidwellii* (Thind *et al.*, 1986). Furthermore, strains of *R. herpotrichoides* with reduced sensitivity to prochloraz have been isolated from cereals in the UK, Denmark and West Germany (Gallimore *et al.*, 1987). In 1990, several prochloraz-resistant strains of *R. acuformis* were isolated from northern France where wheat fields had previously been treated with prochloraz (Leroux & Marchegay, 1991). These resistant isolates tolerated prochloraz *in vitro* at levels of up to 10 μg/ml (Leroux & Marchegay, 1991).

Hollomon (1984) stated that baseline sensitivity studies provide a useful basis for comparison to determine whether resistance has developed among field isolates. Furthermore, the establishment of baseline sensitivity is important to monitor gradual shifts in sensitivity, so that a build up of resistance can be anticipated and alternative fungicide programmes installed before the commercial application of a fungicide becomes ineffective. In the light of the short history of fungicide usage in the Swartland area, information on baseline sensitivity among local isolates of the pathogen towards the different chemicals is required to formulate strategic fungicide programmes. The aim of the present study, therefore, was to determine the sensitivity of local isolates to carbendazim, prochloraz, triadimenol, propiconazole, tebuconazole and flusilazole under laboratory conditions.

MATERIALS AND METHODS

Pathogen isolation and identification

Wheat plants and stubble were collected randomly along the borders of wheat fields near Moorreesburg, Gouda and Philadelphia during the 1991 and 1992 season. Stem tissue bearing eyespot lesions were removed and surface sterilized in a 1% NaOCl solution for 2 min. Symptomatic tissue was rinsed twice in sterile distilled water, dried, placed in petri dishes with moistened filter paper, and the dishes sealed with Parafilm. The material was incubated for 14 days at 10°C under near-ultraviolet light to induce sporulation. After sporulation, conidia were suspended in water and dispersed on potato dextrose agar (PDA) (200 g potatoes, 20 g dextrose, 15 g Biolab agar) amended with streptomycin sulphate (PDAS) (0·05 g streptomycin sulphate/l). PDAS plates were left under a laminar flow hood to dry. Conidia were located under a stereomicroscope and single-conidial isolates established. These isolates were further categorized by their growth on PDA (Biolab) after 10 days at 20°C in the dark as either fast-growing (±12 mm in diameter) with smooth margins (*R. herpotrichoides*), or slow-growing (±5 mm in diameter) with feathery margins (*R. acuformis*) (Nirenberg, 1981).

Fungicide compounds and determination of fungicide sensitivity

Autoclaved PDA was cooled to 50°C and amended with an aqueous fungicide solution. DMI fungicides used in this study were prochloraz (450 g/l EC Sportak), triadimenol (250 g/l EC Bayfidan), flusilazole (250 g/l EC Capitan), tebuconazole (250 g/l EW Folicur) and propiconazole (250 g/l EC Tilt). Isolate sensitivity was tested on fungicide-amended PDA at concentrations of 0·005, 0·01, 0·05, 0·1, 0·5, 1, and 2 μg/ml, respectively. Control plates contained no fungicide. Isolates were also evaluated for sensitivity to the MBC-fungicide, carbendazim (500 g/l SC Bavistin) at a discriminatory concentration of 1 μg/ml.

The single-spored colonies were grown for 28 days on PDA at 20°C in the dark, after which mycelial plugs (3 mm diameter) were removed from the colony margins. Inoculum plugs were placed upside down on three replicate plates per fungicide concentration and inverted plates incubated at 20°C in the dark. Radial growth of each isolate was assessed after 10 days by calculating the mean of two perpendicular colony diameters of three separate colonies. The fungicide concentration which inhibited colony growth of isolates by 50% compared to

the control (IC_{50} value), was determined by a regression analysis of the log-inhibition. Ward's minimum variance cluster analysis (Ward, 1963; Milligan, 1981) was used to identify possible fungicide sensitivity shifts in the eyespot population. Ward (1963) developed a technique which minimizes the joint sum of the inner-cluster square at each level. With this technique, the merging of any two clusters is determined by the values of clusters that cause the least increase of the total inner-cluster square (Milligan, 1981).

One hundred *R. herpotrichoides* isolates were initially screened against carbendazim, of which a representative subsample of 67 was screened against prochloraz, and 36 prochloraz-sensitive isolates further screened against triadimenol, propiconazole and tebuconazole. The aim of this study was to determine whether representative isolates of the South African eyespot population were still sensitive towards the fungicides currently employed for disease control.

RESULTS AND DISCUSSION

All isolates were fast-growing, even marginate, and designated as *Ramulispora herpotrichoides*. The identity of isolates were further confirmed following isozyme and random amplified polymorphic DNA analysis (Campbell, 1995).

All isolates were sensitive to the discriminatory carbendazim concentration of 1 μg/ml, suggesting that the local population of *R. herpotrichoides* is still at baseline sensitivity to MBC-fungicides. The latter may be due to the fact that MBC-fungicides were never used as sole agents for eyespot control in fungicide formulations, but in combination with prochloraz and other triazoles (Bot *et al.*, 1988; Vermeulen *et al.*, 1990, 1992; Nel *et al.*, 1993).

Ward's minimum variance cluster analysis of prochloraz sensitivity data divided the local isolates into three clusters after the cut-off point was arbitrarily determined. The r^2-values of the cluster analysis were expressed in a dendogram as a percentage similarity with regard to the isolate's response to prochloraz (Fig. 1). Calculated IC_{50} values for clusters I, II and III were 0·0958–0·133 μg/ml, 0·0462–0·0719 μg/ml, and 0·01788–0·04451 μg/ml, respectively (Fig. 1). Selection for resistance against an imidazole fungicide such as prochloraz tends to be gradual,

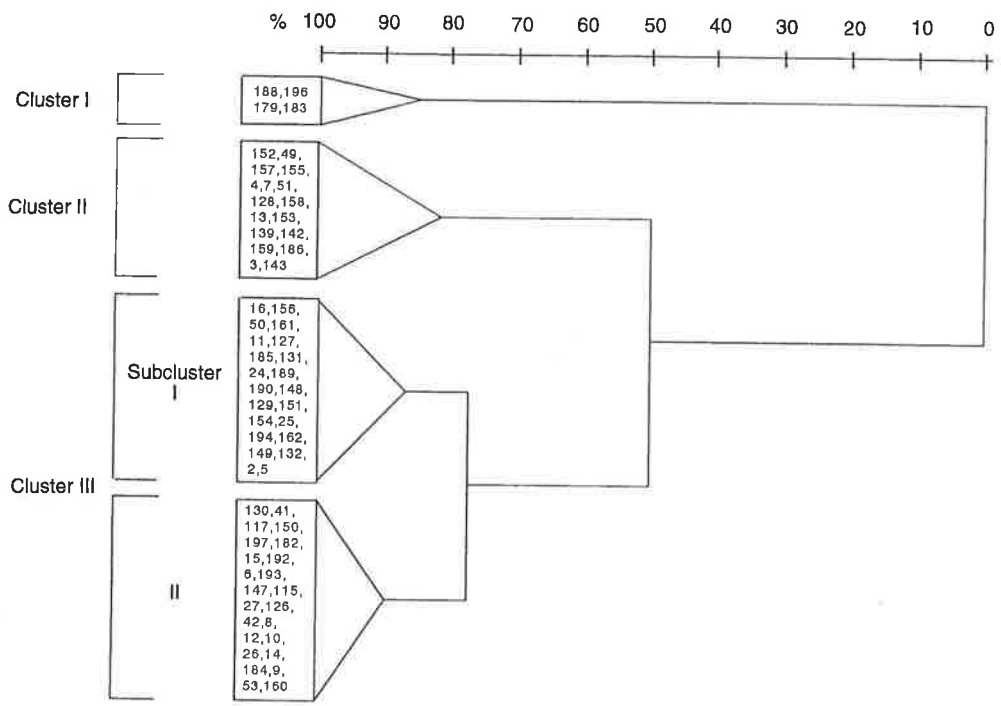

Fig. 1 A dendrogram of (%) similarity between *R. herpotrichoides* isolates (grouped by Ward's minimum variance cluster analysis) based on IC_{50} values of prochloraz sensitivity.

Table 1 The mean IC_{50} values of type Ia and Ib isolates of *Ramulispora herpotrichoides* to different triazole fungicides

Type	Mean IC_{50} value (± SE)		
	Flusilazole ($P = 0{\cdot}0001$)	Propiconazole ($P = 0{\cdot}0005$)	Tebuconazole ($P = 0{\cdot}0012$)
Ia	0·05 (0·007)[a]	0·15 (0·01)[a]	0·217 (0·015)[a]
Ib	0·139 (0·016)[a]	0·44 (0·07)[a]	1 (0·2)[a]
Ia and Ib	0·1 (0·07)	0·31 (0·24)	0·7 (0·8)

[a] Significant difference between type Ia and Ib isolates.

rather than disruptive as observed for MBC resistance (Schepers, 1985). Ward's minimum variance cluster analysis is therefore an ideal method for estimating shifts in the population. According to this analysis, isolates grouped in cluster I might have changed in sensitivity, as they were obtained from stubble on a farm where prochloraz was applied regularly since 1985. In contrast, only 10 of the 63 isolates in clusters II and III were obtained from fields with a prochloraz history. However, the variation in prochloraz sensitivity among local *R. herpotrichoides* isolates was comparable to that found in 1984 by Gallimore *et al.* (1987) for isolates of *R. herpotrichoides* and *R. acuformis* in Europe and the UK. The mean IC_{50} value for local isolates, which was calculated at 0·0434 μg/ml (SD = 0·029), is furthermore similar to the mean fungicide sensitivity values reported for *R. herpotrichoides* and *R. acuformis* (Gallimore *et al.*, 1987). Isolates used in the present study can therefore be regarded to also be at baseline sensitivity to prochloraz.

The mean IC_{50} values of the local isolates to tebuconazole, flusilazole and propiconazole (Table 1) were in the same category as that reported for *R. herpotrichoides* isolates collected in France on winter wheat (Leroux *et al.*, 1988). The latter authors reported variable sensitivity towards triadimenol in their field isolates and classified *R. herpotrichoides* isolates as either type Ia or Ib. Type Ia isolates were regarded as those

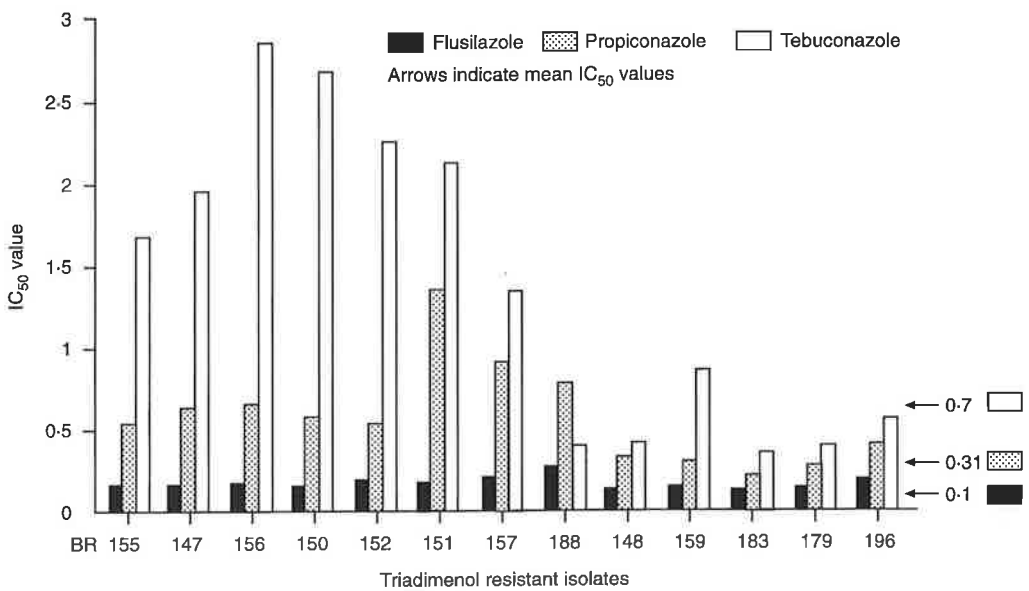

Fig. 2 Fungicide sensitivity of triadimenol resistant isolates towards flusilazole, propiconazole and tebuconazole.

with mycelial IC_{50} values (based on radial growth) below 2 mg/l, and type Ib isolates those that could tolerate greater concentrations of the fungicide (Leroux *et al.*, 1988). Of the 36 isolates evaluated for triadimenol sensitivity, 19 had IC_{50} values higher than 2 μg/ml and were resistant to the fungicide. All triadimenol-resistant isolates (type Ib) were sensitive to propiconazole. Four of the latter isolates (BR156, BR150, BR152, BR151), however, were resistant to tebuconazole (Fig. 2, Table 1). Furthermore, type Ib isolates had an IC_{50} value of 0·139 μg/ml towards flusilazole, which was significantly higher than the IC_{50} value of type Ia isolates towards flusilazole (Table 1).

The triadimenol resistant isolates were obtained from wheat fields that had previously been treated with DMI fungicides. In some of these fields, tebuconazole was applied for 5 years, whereas others were sprayed for seven consecutive years with prochloraz. Wild-type isolates collected from a field that had never been treated with fungicides were more sensitive to triadimenol, and had IC_{50} values of 0·3–1·5 μg/ml. This suggests that DMI fungicide application in wheat fields may have selected for isolates less sensitive to the triazoles.

Because of a lack of commercially suitable, resistant winter wheat cultivars in South Africa, it seems probable that eyespot disease will be exclusively controlled by fungicides for several years to come. Given the unique situation that only *R. herpotrichoides* has thus far been associated with eyespot symptoms in South Africa, it would seem that disease control in the local situation may be far simpler than under conditions encountered in Europe. However, a warning that continuous high selection pressure may lead to DMI resistance in wheat fields in the Swartland area seems appropriate. Screening for fungicide sensitivity against the commercially used fungicides should therefore be routinely conducted in future.

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