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Cross-resistance patterns among sterol biosynthesis inhibiting fungicides (SBIs) in *Cercospora beticola*

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Abstract

Thirty single-spore isolates of *Cercospora beticola*, collected from several fields in northern Greece, representing a broad spectrum sensitivity to the sterol demethylation-inhibiting (DMIs) fungicide flutriafol, were tested for sensitivity to eleven other sterol biosynthesis-inhibiting (SBI) fungicides and to the guanidine fungicide dodine. Sensitivity was measured as EC_{50} values for each fungicide and log-transformed EC_{50} values to each fungicide were pairwise correlated and the correlation coefficient estimated. These pairwise comparisons showed high correlation coefficients between the DMIs suggesting a cross-resistance relationship between these fungicides. However, the degree of cross-resistance between DMIs varied greatly. Conversely, low correlation coefficients were obtained for the pair-wise comparisons with the morpholine fungicide fenpropimorph suggesting a lack of cross-resistance between morpholines and DMIs in *C. beticola*. Similarly, there was no correlation between the sensitivity (EC_{50} values) to dodine and all the other fungicides tested, indicating that there was no negative cross-resistance relationship between dodine and SBIs in *C. beticola*. Based on these results, combinations or alternations of fungicides which show no cross-resistance relationship should be used to control the disease in areas where reduced sensitivity to DMIs has been already observed.

Introduction

Cercospora leaf-spot caused by *Cercospora beticola* is the most important foliar disease of sugar beet in the Mediterranean basin (Rossi et al., 1995). Control of the disease is mainly achieved by using fungicides, primarily of the sterol-demethylation-inhibitors (DMIs) class (Byford, 1996). In Greece, DMIs have been used since the late 1970s (Ioannidis, 1994). However, the extensive and prolonged use of DMIs has lead to a reduction of fungal population sensitivity, detected in some areas of Northern Greece for first time in 1995 (Karaoglanidis et al., 2000). Despite this sensitivity shift, fungicide field performance was not significantly affected until 1998. However, during 1999 the first signs of reduced field performance were detected in an area of northern Greece (Karaoglanidis et al., 2002). Field experimental data showed that DMI-resistant strains were not efficiently controlled by the recommended application doses of DMIs (Karaoglanidis et al., 2003).

Fungicides of the sterol biosynthesis inhibitors (SBIs) group constitute an important group of fungicides used in agriculture. SBIs are divided in two subgroups, the DMIs and the morpholines, on the basis of their specific mode of action. DMIs inhibit the demethylation of eburicol at the C-14 position while morpholines inhibit Δ^{14} reduction and $\Delta^{8}\rightarrow\Delta^{7}$ isomerization (Berg et al., 1984).

Cross-resistance has been defined as resistance to two or more fungicides conferred by the same genetic factor (Georgopoulos, 1977). Despite the existence, as a general rule, of cross-resistance among the several DMI fungicides it is also well established that

variability exists in the cross-resistance patterns among certain fungicide pairs (Kendall, 1986; Hildebrand et al., 1988; Köller and Wubben, 1989; Kendall et al., 1993; Gisi and Hermann, 1994).

The object of this study was to define the cross-resistance patterns between several SBI fungicides available for control of *C. beticola*. Dodine, a non-SBI fungicide was also included in the study to test the existence of negative cross-resistance between this fungicide and the SBIs.

Materials and methods

Pathogen isolates

Thirty single-spore isolates of *C. beticola* were obtained from sugar beet fields of northern Greece. The isolates were collected during a routine monitoring program carried out to determine the fungal population sensitivity to the triazole fungicides flutriafol and difenoconazole. Techniques for the isolation and assay of *C. beticola* have been described (Karaoglanidis et al., 2002). The isolates selected, represented the complete range of sensitivities to flutriafol. Isolates were classified based on their EC_{50} values and resistance factors (RF) relative to flutriafol. Isolates with RF values greater than 10 were resistant (R); those with RFs between 2 and 9 were moderately resistent (MR); all other isolates were sensitive (S). In total, 9 isolates were resistant, 9 moderately resistant and 12 sensitive.

Fungicides

Fungicides used were chosen to represent the several chemical classes of the SBIs group. At least one fungicide from each class was chosen and included flutriafol, difenoconazole, flusilazole, propiconazole, bitertanol, myclobutanil and triadimefon that belong in the triazole subgroup, the pyridine pyrifenox, the pyrimidine fenarimol, the imidazole imazalil, the piperazine triforine, the morpholine fenpropimorph and the guanidine fungicide dodine. Triadimefon, imazalil, triforine and dodine are not registered for Cercospora leaf-spot control in Greece. Where available, fungicides were used as technical grades, otherwise commercial formulations were used. Fungicide stock solutions were prepared by dissolving the fungicides in methanol or in water if commercial formulations were used. Autoclaved *Aspergillus* complete medium (ACM) was amended with several doses of each fungicide by adding appropriate volumes of the fungicide stock solutions into the medium after autoclaving. Control medium was not amended with fungicide. In all cases, the final methanol concentration was not greater than 0.1% (v/v). Tests for each isolate were replicated three times per concentration of each fungicide.

Sensitivity tests

Sensitivity was determined as EC_{50} values (effective concentration that causes 50% inhibition of mycelial growth). Mycelial plugs (5 mm diameter) were removed from colony margins, placed upside down on the fungicide-amended and unamended culture medium and incubated at 25 °C in the dark for 7 days. The mean colony diameter was measured (minus the diameter of the inoculation plug) and expressed as percentage of the mean diameter of the untreated control. The EC_{50} value of each isolate was calculated by regressing the relative growth (RG: colony diameter on fungicide-amended medium divided by the colony diameter on unamended medium $\times 100$) against the Log_{10} fungicide concentration. The resistance factor (RF) of each isolate for each fungicide was calculated by dividing the EC_{50} value of the isolate by the mean EC_{50} value of the group of sensitive isolates.

To measure cross-resistance between pairs of the fungicides tested, the Log EC_{50} values were correlated and the correlation coefficient was estimated for each pair. Statistical analyses were performed using the Mstat-C statistical program (MStat-C, version 2.10, Michigan State University, USA).

Results

Intrinsic activities

The ranges of EC_{50} values and the respective RF within the groups of isolates tested, to all fungicides, are summarized in Table 1. Results showed great variation regarding to the intrinsic activity of the fungicides tested. The ranking of the intrinsic activities against *C. beticola*, as indicated by the mean EC_{50} value of the group of the sensitive isolates was: difenoconazole > pyrifenox > propiconazole > flusilazole > flutriafol = bitertanol = fenarimol = myclobutanil > fenpropimorph > imazalil > triadimefon > dodine > triforine. Difenoconazole and pyrifenox were 25 and 8 times more active than

Table 1. Sensitivity *of C. beticola* isolates to several fungicides, in terms of EC_{50} and resistance factor (RF) values

Fungicide	Resistant isolates		Moderately resistant isolates		Sensitive isolates	
	EC_{50}^{1}	RF[2]	EC_{50}^{1}	RF[2]	EC_{50}^{1}	RF[2]
Flutriafol	2.7–15.6	11–62	0.50–0.84	2–3	0.10–0.34	1
Difenoconazole	0.15–0.76	15–76	0.005–0.05	1–5	0.008–0.02	1–2
Bitertanol	0.63–6.80	3–27	0.26–1.02	1–4	0.10–0.44	1–2
Propiconazole	0.49–2.66	12–67	0.04–0.11	1–3	0.01–0.08	1–2
Flusilazole	0.71–2.55	14–56	0.05–0.15	2–3	0.03–0.08	1–2
Myclobutanil	2.78–15.9	10–55	0.21–0.78	1–3	0.15–0.58	1–2
Triadimefon	51.7–>100	6–>11	13.9–35.8	1–4	3.9–15.5	1–2
Pyrifenox	0.05–0.39	2–13	0.02–0.05	1–2	0.02–0.05	1–2
Fenarimol	2.5–15.2	9–52	0.31–0.93	1–3	0.20–0.72	1–3
Imazalil	2.24–8.00	3–10	0.80–2.27	1–3	0.41–1.14	1
Triforine	43.1–>100	2–>4	27.2–>100	1–>4	10.5–62.1	1–2
Fenpropimorph	0.23–0.53	1–1	0.15–0.72	1–1	0.16–0.59	1–2
Dodine	18.8–42.9	1–2	21.3–42.0	1–2	12.3–45.4	1–2

[1]EC_{50} in $\mu g\,ml^{-1}$.

[2]Resistance Factor: EC_{50} of the isolate divided by the mean EC_{50} value of the group of sensitive isolates (flutriafol $= 0.25\,\mu g\,ml^{-1}$, difenoconazole $= 0.01\,\mu g\,ml^{-1}$, bitertanol $= 0.25\,\mu g\,ml^{-1}$, propiconazole $= 0.04\,\mu g\,ml^{-1}$, flusilazole $= 0.05\,\mu g\,ml^{-1}$, myclobutanil $= 0.29\,\mu g\,ml^{-1}$, triadimefon $= 9.37\,\mu g\,ml^{-1}$, pyrifenox $= 0.03\,\mu g\,ml^{-1}$, fenarimol $= 0.29\,\mu g\,ml^{-1}$, imazalil $= 0.80\,\mu g\,ml^{-1}$, triforine $= 28.4\,\mu g\,ml^{-1}$, fenpropimorph $= 0.45\,\mu g\,ml^{-1}$ and dodine $= 26.5\,\mu g\,ml^{-1}$).

flutriafol, respectively, while propiconazole and flusilazole were 5–6 times more active than flutriafol. The fungicides bitertanol, fenarimol and myclobutanil showed intrinsic activity similar to that of flutriafol. The morpholine fungicide fenpropimorph was 2 times less active than flutriafol, while the remaining fungicides triadimefon, dodine and triforine were even less active.

Correlation coefficients

The sensitivity to the fungicides was plotted against the sensitivity of the other fungicides, and the log-transformed EC_{50} values were analysed by establishing correlations and linear regressions. Data on the correlation coefficients between all the fungicide pairs are summarized on Table 2. Correlation coefficients between pairs of DMI fungicides were always significant ($P < 0.05$), indicating a positive cross-resistance pattern between these fungicides. However, the levels of cross-resistance between them varied, but could be distinguished into 3 groups. The first group consists of the triazole fungicides flutriafol, bitertanol, propiconazole, flusilazole and myclobutanil, the pyridine pyrifenox, the pyrimidine fenarimol and the imidazole imazalil. For these fungicides, all pairwise combinations, except the pair flusilazole–imazalil, generate correlation coefficient values greater than 0.76, and for some pairs greater than 0.90. Into the second group were the triazole fungicides difenoconazole and triadimefon. In pairwise comparisons of these two fungicides with the remaining DMI fungicides the correlation coefficient values ranged from 0.53 to 0.78. Despite the fact that both difenoconazole and triadimefon are triazoles they showed lower levels of cross-resistance to the remaining triazoles tested. These results suggest that cross-resistance 'subgroups' do not always correspond to chemical structures. Only the piperazine fungicide triforine belongs to the third group, which showed relatively low (although significantly different from 0 ($P < 0.05$)) correlation coefficients with the rest of the DMIs. Such differences in the level of cross-resistance between DMI fungicides have previously been shown for several other pathogens (Kendall, 1986; Hildebrand et al., 1988; Peever and Milgroom, 1993, Hsiang et al., 1997; Robbertse et al., 2001). These differences in cross-resistance levels among DMIs may reflect differences in the genetic control of resistance since different genes may control resistance. Differences in the level of cross resistance may also reflect to differences in the mechanism by which DMIs interfere with demethylation process or to differences in the resistance mechanism (Hildebrand et al., 1988; Kendall et al., 1993).

Table 2. Patterns of cross-resistance between several fungicides in *C. beticola* isolates

Fungicide	Correlation coefficient											
	Difenoconazole	Bitertanol	Propiconazole	Flusilazole	Myclobutanil	Triadimefon	Pyrifenox	Fenarimol	Imazalil	Triforine	Fenpropimorph	Dodine
Flutriafol	0.70	0.90	0.91	0.78	0.92	0.68	0.90	0.94	0.83	0.43	0.14 *	0.10*
Difenoconazole	—	0.70	0.70	0.53	0.65	0.54	0.78	0.75	0.64	0.26	0.10*	0.17*
Bitertanol	—	—	0.85	0.78	0.90	0.76	0.88	0.94	0.76	0.41	0.07*	0.08*
Propiconazole	—	—	—	0.83	0.92	0.56	0.89	0.93	0.87	0.37	0.07*	0.16*
Flusilazole	—	—	—	—	0.80	0.61	0.76	0.85	0.67	0.36	0.20*	0.10*
Myclobutanil	—	—	—	—	—	0.64	0.91	0.95	0.84	0.36	0.10*	0.10*
Triadimefon	—	—	—	—	—	—	0.65	0.70	0.55	0.42	0.02*	0.07*
Pyrifenox	—	—	—	—	—	—	—	0.92	0.84	0.36	0.10*	0.03*
Fenarimol	—	—	—	—	—	—	—	—	0.84	0.38	0.07*	0.10*
Imazalil	—	—	—	—	—	—	—	—	—	0.30	0.01*	0.11*
Triforine	—	—	—	—	—	—	—	—	—	—	0.05*	0.05*
Fenpropimorph	—	—	—	—	—	—	—	—	—	—	—	0.14*

*Correlation coefficients not significant at $P = 0.05$.

Correlation coefficients between the morpholine fungicide fenpropimorph and the DMI fungicides were never significant ($P > 0.05$, Table 2). Such low correlation coefficients indicates that no cross-resistance exists between morpholine and DMI fungicides in *C. beticola*, suggesting that different genes may control development of resistance to DMIs and morpholine fungicides. Similar results have been reported for *Erysiphe graminis* (de Waard et al., 1986; Girling et al., 1988), *Venturia inaequalis* (Thind et al., 1986), *Rhyncosporium secalis* (Girling et al., 1988), *Monilinia fructicola* (Nuninger-Ney et al., 1989), *Pyrenophora teres* (Peever and Milgroom, 1993) and *Ustilago maydis* (de Waard and van Nistelrooy, 1990; Markoglou and Ziogas, 1999).

The guanidine fungicide dodine was included in the study, despite the fact that it is not an SBI fungicide, since there was a report of negative cross-resistance with the pyrimidine fungicide fenarimol in various fungi (de Waard and van Nistelrooy, 1983). However, our results indicate that the DMIs-resistant/sensitive strains of *C. beticola* were equally sensitive to dodine. This suggests that the introduction of dodine into the spray programs applied to control sugar beet leaf-spot would not provide any particular benefit towards the reduction of DMIs-resistance frequency within the *C. beticola* populations by eliminating selectively the resistant strains. A similar lack of correlation between DMIs and dodine sensitivity has previously been reported in *Venturia inaequalis* (Stanis and Jones, 1985).

Discussion

The considerable differences in the level of cross-resistance between the fungicides tested have both theoretical and practical implications for management of resistance. Maintenance of the field performance of DMIs is important for sugar beet leaf-spot control since alternative fungicides are unsatisfactory, particularly under climatic conditions favourable for the development of the disease, like those prevailing in most areas of Mediterranean basin. In the current study, high positive correlation coefficients were measured among most DMI–DMI pairs tested, and this precludes replacing one DMI by another. Despite the fact that an extensive monitoring program carried out during a 4-year period (1996–1999) showed that the fungal populations sensitivity to difenoconazole increased when only flutriafol used in spray programs while sensitivity to flutriafol also increased when only difenoconazole was used in the spray programs (Karaoglanidis et al., 2002), such alternations must be used with caution in resistance management schemes since strains with extremely high levels of resistance to both fungicides can be selected. Relatively low correlation coefficients were measured for the pairs of triadimefon and triforine; however, these two DMI fungicides showed very low intrinsic activity against *C. beticola* and are unsuitable for successful control of the disease.

A lack of any cross-resistance between DMIs and fenpropimorph suggests that different genes control resistance to each fungicide group. Combining or alternating these fungicides could be useful in controlling the disease and managing resistance development to DMIs. Field experiments carried out to determine the effect of several fungicide spray programs on the pathogen's sensitivity to DMIs showed that applications of flutriafol–fenpropimorph mixtures combined both excellent disease control and lower resistance frequency compared to successive treatments with flutriafol either at full or reduced rate (Karaoglanidis et al., 2001). Such an approach has been adopted in Greece by using mixtures of DMIs with fenpropimorph into the spray programs applied to control sugar beet leaf-spot.

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