European Journal of Plant Pathology **107:** 337–347, 2001.

Fitness of *Cercospora beticola* field isolates – resistant and – sensitive to demethylation inhibitor fungicides

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Accepted 30 January 2001

Key words: Cercospora leaf-spot, competitive ability, flutriafol, fungicide resistance, sugar beet, triazoles

Abstract

Isolates of *Cercospora beticola* resistant to fungicides that inhibit sterol demethylation (DMIs) were collected from sugar beet fields in Northern Greece. Fitness of these isolates was compared to that of DMI-sensitive isolates. The parameters measured were competitive ability both under growth chamber and field conditions, mycelial growth, spore germination, germ tube length, incubation period, virulence and spore production. The competitive ability under growth chamber conditions was measured for 4 pairs of one resistant and one sensitive isolate. Results showed that after 4 disease cycles, in 2 out of 4 pairs tested, the resistant isolates competed well with the sensitive isolates, but in the remaining two pairs the frequency of the resistant isolate decreased significantly. The competition experiment in the field was carried out by inoculating field plots with a conidial suspension consisting of a spore mixture from all the resistant and all the sensitive isolates used in this study. Results showed that at the end of the growing period the frequency of the resistant isolates had slightly decreased ($P < 0.05$). The measurements of fitness components of individual isolates showed that the resistant isolates had significantly lower ($P < 0.05$) virulence and spore production than the sensitive isolates, while no significant differences ($P > 0.05$) to the remaining 4 fitness components, were detected. With correlation analysis it was determined whether there is a relationship between values of each fitness component and the level of sensitivity to flutriafol of individual isolates. The correlation coefficients for virulence ($r = 0.45$) and spore production ($r = 0.41$) were significantly different from 0 ($P < 0.05$), indicating that resistance to DMIs affected, to some degree, the fitness of the resistant isolates.

Introduction

Sugar beet leaf-spot caused by *Cercospora beticola* is the most important foliar disease of sugar beet in warm and humid areas such as some places around the Mediterranean basin (Rossi et al., 1995), causing significant yield losses in the absence of control measures (Byford, 1996; Shane and Teng, 1992). Control of the disease is mainly achieved by fungicide spray applications. Demethylation inhibiting fungicides (DMIs) constitute one of the most important group of fungicides playing an important role in control strategies of the disease (Brown et al., 1986; Byford, 1996). In Greece they have been used since 1979, for controlling sugar beet leaf-spot, in mixture with a protectant fungicide, either maneb or chlorothalonil (Ioannidis, 1994). In order to determine the sensitivity of fungal populations to the triazole fungicides bitertanol and flutriafol, a monitoring programme was carried out during 1995. Results showed a shift toward decreased sensitivity in some areas of Northern Greece, but without significant effects on product performance (Karaoglanidis et al., 2000).

One of the most important factors affecting the evolution of resistance to fungicides is the parasitic fitness of the resistant isolates (Dekker, 1981; Skylakakis, 1987). The fitness of a pathogen strain is defined as its ability to survive, develop and reproduce

as compared to other strains under the same conditions (Anonymous, 1988). In a fungicide-treated environment, resistant strains are favoured by the selection pressure of the fungicide, potentially leading to an increase of the resistance frequency and probably to a decline of fungicide efficacy in the field. If the fitness of the resistant strains is less than that of the sensitive strains, the frequency of the resistant population may decline, in the absence of fungicide treatment. It has been frequently assumed that DMI-resistant fungal strains are less fit than the sensitive strains (Fuchs and de Waard, 1982; Köller and Scheinpflug, 1987). This assumption has contributed to DMIs being considered as of moderate risk for resistance development (Georgopoulos, 1985). However, the available scientific evidence on this subject is rather contradictory. In several pathogens there is a fitness cost associated with resistance development to DMIs (Fuchs et al., 1977; Henry and Trivellas, 1989; de Waard and van Nistelrooy, 1990; Al-Mughrabi and Gray, 1995; Holmes and Eckert, 1995; Cavelier et al., 1996), while in others there is no fitness reduction (Schepers, 1985; Kalamarakis et al., 1989; Porras et al., 1990; Peever and Milgroom, 1994). Only one study refers to *C. beticola*, showing that strains with laboratory-induced resistance to DMI-fungicides had lower virulence in comparison to sensitive strains (Henry and Trivellas, 1989). Previous studies on the fitness of *C. beticola* strains resistant to benzimidazole fungicides have shown that there was no fitness cost related to resistance development to benomyl (Dovas et al., 1976; Ruppel, 1975), while similar studies on the competitive ability of *C. beticola* strains that were resistant to fentin fungicides have shown that they were less competitive than the sensitive strains (Giannopolitis and Chrysayi-Tokousbalides, 1980).

The purpose of this study was to determine whether resistance development to DMIs is associated with fitness costs, both in terms of *predicted fitness* and *realized fitness* (Antonovics and Alexander, 1989). For *predicted fitness*, the following fitness components were estimated for each isolate: mycelial growth, spore germination, germ tube length, incubation period, virulence and spore production. For *realized fitness*, two competition experiments were carried out with mixed inocula of flutriafol-resistant and -sensitive isolates under both laboratory and field conditions and in the absence of DMIs treatment. Changes in isolates frequency were determined. A preliminary report on a part of this study has already been published (Karaoglanidis and Thanassoulopoulos, 1998).

Materials and methods

Fungal isolates

Nine flutriafol-resistant and twelve flutriafol-sensitive isolates of *C. beticola* were used for the measurement of fitness components and the competition experiment in the field. For the competition experiment in the growth chamber, 4 resistant and 4 sensitive isolates were used. All the isolates were collected from sugar beet fields in October 1996. All the isolates were collected as single-lesion isolates and were transformed to single-spore isolates using the dilution technique (Tuite, 1969). The sensitivity of each isolate to flutriafol was tested as for the bulk samples. The methodology of isolation and sensitivity determination has previously been described (Karaoglanidis et al., 2000). Details on the sensitivity to flutriafol of isolates and the location of isolation are given (Table 1).

Plant material

All the experiments were conducted on *Beta vulgaris* L. cv 'Rizor' plants, a cultivar which is very susceptible to Cercospora leaf-spot disease. Plants used for fitness assessment and in the competition experiments in growth chamber, were grown in the greenhouse (18–26 °C), in plastic pots (18 cm diameter) containing a 2 : 1 mixture of peat and perlite. Plants were fertilized once per week with 1%N : P : K (20 : 20 : 20) solution. Each pot contained three plants. Plants used for inoculations were 5–6 week-old, at the stage of 4–5 fully expanded leaves. For the competition experiment in the field, plants were sown on 31 March 1997 and 18 March 1998.

Inoculum preparation

Isolates for inoculum production were grown on V-8 juice agar medium (Tuite, 1969). After 10 days of incubation under fluorescent light at 25 °C sporulating colonies were rinsed with 15 ml distilled water and the conidial suspension was filtered through a double-layered sterile cheesecloth. To measure fitness components, suspensions were adjusted to 8×10^3 conidia per ml. For the competition experiments in the

Table 1. Level of sensitivity to flutriafol and the location of the isolation of *Cercospora beticola* used in this study

Resistant isolates				Sensitive isolates			
Isolate	EC_{50}[1]	RF[2]	Location	Isolate	EC_{50}	RF	Location
R152[3]	15.6	156	Serres	S70	0.17	1	Amyndeon
R193	8.8	88	Serres	S17[3]	0.22	2	Imathia
R61	3.2	32	Serres	S56	0.33	3	Amyndeon
R65[3]	5.8	58	Imathia	S60A	0.34	3	Amyndeon
R116[3]	6.4	64	Serres	S160	0.34	3	Amyndeon
R171	5.3	53	Serres	S60H[3]	0.24	2	Imathia
R215[3]	8.0	80	Serres	S162H	0.32	3	Imathia
R79	2.7	27	Imathia	S165	0.28	2	Amyndeon
R51	2.9	29	Imathia	S179[3]	0.12	1	Amyndeon
				S62	0.26	2	Amyndeon
				S75	0.28	2	Amyndeon
				S162A[3]	0.10	1	Amyndeon

[1] EC_{50} $\mu g\ ml^{-1}$ flutriafol, based on inhibition of mycelial growth.
[2] EC_{50} of the isolate divided by the lowest EC_{50} of the sensitive isolate S162A.
[3] Isolates R215, R65, R152, R116 and S60H, S17, S179, S162A were used in the competition experiment under growth chamber conditions designated as R1, R2, R3, R4 and S1, S2, S3, S4, respectively.

growth chamber, mixed-isolate inocula were prepared by mixing of appropriate volumes of conidial suspensions of R1 with S1, R2 with S2, R3 with S3 and R4 with S4 isolates, so as to produce suspensions containing 70%R : 30%S, 50%R : 50%S and 30%R : 70%S conidia. All suspensions contained a final concentration of 8×10^3 conidia per ml. Before inoculation one droplet of 0.1% Tween 20 was added to each suspension.

Inoculum used in the field experiments was produced on sugar beet seedlings inoculated with a mixture of conidia and hyphae produced on V-8 juice agar. After inoculation potted plants were put under plastic bags for 24 h and transferred to the greenhouse. After 24 h bags were removed and plants were incubated in the greenhouse for 15 days. At the time of symptom appearance, plants were covered again with plastic bags for 48 h, in order to induce abundant sporulation. Then, leaves with sporulating lesions were flooded in 1 l tap-water and gently shaken to dislodge the conidia. The resulting suspension was filtered through a single-layered cheesecloth to remove the plant debris and then a mixed-isolate inoculum was prepared, by mixing conidia of sensitive and resistant isolates in the appropriate volumes to produce suspensions containing 70%R : 30%S, 50%R : 50%S and 30%R : 70%S conidia. The final suspensions contained 5×10^2 conidia per ml. For each ratio a total volume of 30 l conidial suspension were used for the inoculation. Before inoculation, 0.3 ml l^{-1} of Agral 90 (Zeneca Hellas SA, Athens, Greece) was added to the inoculum suspension.

Competition experiments in growth chamber

Conidial suspensions of R1/S1, R2/S2, R3/S3 and R4/S4 with three different ratios of resistant and sensitive conidia (70%R : 30%S, 50%R : 50%S, 30%R : 70%S), were applied to whole plants. For each mixture of isolates and conidial ratio 12 plants (3 plants per pot) were inoculated with 30 ml of the aqueous conidial suspension. The inoculation was carried out by a spray atomiser and plants were sprayed until run off, on both leaf surfaces. After inoculation the plants were transferred into the growth chamber and kept at 25 °C and 99% RH, with a 16-h photoperiod. After the appearance of lesions, plants were removed from the growth chamber, covered with a plastic bag to promote fungal sporulation and transferred to the greenhouse for 48 h. Infected leaves were cut off to determine the frequency of resistant and sensitive spores and to prepare the inoculum for the next disease cycle. For this purpose, all the infected leaves of each treatment were flooded into tap-water and conidia were collected by gently shaking the leaves. The resulting suspension was filtered through a single-layered cheesecloth to remove plant debris and then used to inoculate a new set of plants. In this way, a new disease cycle was started. The experiment was terminated after 4 disease cycles and repeated three times.

Competition experiments in the field

The competition experiments in the field were conducted during the summers of 1997 and 1998, in the

area of Imathia in Northern Greece. A major concern was the possible risk of inter-plot interference such as the migration of conidia between plots. To minimize this risk as much as possible, large plots of 30 × 12 m were used and each plot was separated by a 4 m buffer zone which was free of sugar beet plants. The experiment was a completely randomized block design with 3 treatments (the three different ratios of resistant and sensitive conidia) and three replications per treatment. Plants were artificially inoculated after 'closing' of the rows on 20 June 1997 and 15 June 1998. The inoculation of the field was carried out in the evening, a few minutes after sprinkler-irrigation of the plots. Inoculum was applied uniformily with the aid of a tie-back sprayer. Fifteen days after inoculation the first symptoms appeared and the epidemic rapidly developed.

Determination of resistance frequency in competition experiments

The determination of the percentage of flutriafol-resistant conidia in a mixed population was carried out using single-lesion isolations of the pathogen. In the growth chamber experiment 10 single-lesion isolates per plant were obtained from the removed diseased leaves after the end of each disease cycle. Isolations were carried out on Potato Dextrose Agar (PDA, Oxoid), acidified with lactic acid to suppress bacterial growth. After the appearance of the colonies, they were transferred to new PDA Petri dishes amended with 4.0 μg ml^{-1} flutriafol. Technical material of futriafol (Zeneca Hellas SA, Athens, Greece), dissolved in methanol (Riedel-de Haën AG, Seelze, Germany) was used. The concentration of 4.0 μg ml^{-1} flutriafol was completely inhibitory for mycelial growth of sensitive isolates while allowing the growth of resistant isolates. The discrimination between sensitive and resistant isolates was carried out after 3 days of incubation at 25 °C in the dark. Totally 120 isolates were examined per treatment. This procedure was repeated in all successive disease cycles. The same procedure was used to determine the percentage of resistant conidia in the field experiment. During the summer, there were 4 sampling dates with 3–4 week intervals. Samples were taken only from young leaves, 3–4 days after a sprinkler-irrigation of the plots, in order to get newly formed conidia. Only one lesion per leaf was used for isolation. At each sampling date 100 single-lesion isolates per experimental plot were obtained. Totally 300 isolates per treatment were examined.

Fitness components

The following fitness components were estimated for flutriafol-resistant and -sensitive isolates of *C. beticola*: (1) mycelial growth in liquid media; (2) spore germination; (3) length of germ tube; (4) incubation period; (5) sporulation capacity and (6) virulence. Three experiments were carried out and each was replicated three times. The same 9 resistant and 12 sensitive isolates were used in all experiments.

Mycelial growth. Mycelia were grown in liquid malt medium consisting of 1.5% malt extract (Oxoid), 0.3% peptone (Merck, Darmstadt, Germany) and 3% glucose (Riedel-de Haën). Cultures were shaken at 150 rpm in the dark for 10 days. Cultures (125-ml flasks containing 50 ml of medium) were inoculated with five plugs, taken, with a 5-mm cork borer, from the margins of a fungal colony growing on PDA. The mycelium was harvested by filtration through filter paper (Whatman paper No. 3, Whatman, Maidstone, Kent, UK) in a Büchner funnel. For growth determination 4 separate cultures were filtered individually onto filter paper, dried in an oven at 105 °C for 24 h and weighed. Dry-weight values are means of 12 cultures per isolate.

Germination of conidia and germ tube length. For the measurement of conidial germination and the length of germ tube, conidia produced on V-8 agar medium were used. Two 10-μl droplets of aqueous conidial suspension (10^4 conidia per ml) were placed on Van-Tieghem glass slides. The slides were incubated for 24 h at 25 °C in the dark. The percentage of spore germination was determined by scoring 100 conidia per isolate. A conidium was considered as germinated, if the germ tube length was at least twice the length of the conidium. The length of the germ tube was determined by measuring 20 germ tubes of 20 randomly selected germinated conidia (only the longest germ tube was measured for each germinated conidium). Percentage of conidial germination are means of 300 conidia and length of germ tubes means of 60 conidia per isolate.

For the measurement of incubation period, virulence and sporulation capacity, 12 plants per isolate were artificially inoculated. Inoculation was carried out by spraying the conidial suspension until run off, with a spray atomiser. Both leaf surfaces were sprayed and the potted plants were transferred to the growth chamber and kept under the same conditions as described above for the competition experiment.

Incubation period. After inoculation, the plants were examined daily in order to determine the time period between inoculation and appearance of first lesions (incubation period). Incubation periods are the means of 36 values per isolate.

Virulence. When new lesions were no longer produced (about 15 days after inoculation), the plants were removed from the growth chamber and the disease severity was recorded, on 4 leaves per plant, according to the 9-category scale disease index of Kleinwanzlebener Saatzucht (KWS): 1 = healthy leaf, and 9 = leaf and leaf stalk dead and dried up (Shane and Teng, 1992). This scale was adopted because it was suitable for scoring the disease severity on individual leaves. Virulence values are means of disease scorings on 144 leaves per isolate.

Sporulation. After scoring the disease severity, pots were covered by a plastic bag and were transferred again to the growth chamber for an additional incubation of 48 h in order to induce abundant sporulation. After 48 h the plants were removed from the growth chamber and the leaves with sporulating lesions were cut off from the plants. Leaf segments (about 20 mm^2) with sporulating lesions were removed with the aid of a 5-mm diameter cork borer. Three segments were removed from a leaf and 3 leaves were selected from each plant. Twelve plants were tested per isolate. The leaf segments were placed in 3 ml distilled water in Eppendorf tubes. The tubes were vortexed to detach the conidia from the lesions and then the leaf segments were removed. Droplets (2 µl) of the conidial suspension were placed on the haemocytometer and the conidia counted. Four replicate droplets were counted for each tube. The number was expressed as number of conidia per mm^2 of diseased leaf surface.

Data analyses

The data on frequency (%) of the resistant isolates were expressed graphically. The pairwise *t*-test was used to compare the observed frequency with the initial frequency of resistant isolates. All data for each fitness component were subjected to an analysis of variance. Means of each isolate within sensitivity groups and means among sensitivity groups were compared using Duncan's Multiple Range Test. Correlation coefficients between fitness components and sensitivity to flutriafol (in terms of log EC_{50} values) were estimated using the mean values for each variable per individual isolates. All the statistical analyses were performed on Mstat-C statistical programme (Mstat-C, version 2.10, Michigan State University).

Results

Competition between paired isolates in Growth Chamber

The changes in the frequency of the resistant isolates were largely dependent on the competing isolates. In one out of four pairs used in this study, the R1/S1 pair, the frequency of the resistant isolate increased rapidly, in all the three tested ratios (Figure 1A). Starting with a 50R : 50S ratio the final proportion of the resistant isolate (after 4 disease cycles) was 84%, while in the 70R : 30S and 30R : 70S ratios the final proportion was 80 and 77%, respectively. All final frequencies were significantly different ($P < 0.05$) from the initial ratios.

In the second pair (R2/S2), the competing isolates expressed about equal competitive ability. The changes in frequency of the resistant isolate were extremely small ($P > 0.05$), in all the three tested ratios (Figure 1B). In the 70R : 30S ratio the proportion of the resistant isolate remained stable after the fourth disease cycle in comparison to the initial proportion, (68%) ($P > 0.05$), while in the 50R : 50S and 30R : 70S ratios a slight increase and a slight decline of the proportion of the resistant isolate was observed (58 and 23%, respectively). Both values were not significantly different from the initial proportion ($P > 0.05$).

For the remaining two pairs (R3/S3 and R4/S4) a rapid decline in the frequency of the resistant isolates was observed, in all three ratios after 4 disease cycles, indicating that the resistant isolates R3 and R4 were weak competitors in comparison to their partners, S3 and S4. After 4 disease cycles, in the R3/S3 pair, the proportion of the resistant isolate was 8, 22 and 11% for the 70R : 30S, 50R : 50S and 30R : 70S ratios, respectively ($P < 0.05$) (Figure 1C), while for the R4/S4 pair the respective values were 8, 10 and 4% ($P < 0.05$) (Figure 1D).

Competition experiments in the field

During both years, the changes in the frequency of the resistant isolates were slight. In the 1997 experiment the frequency in the 50R : 50S ratio remained almost

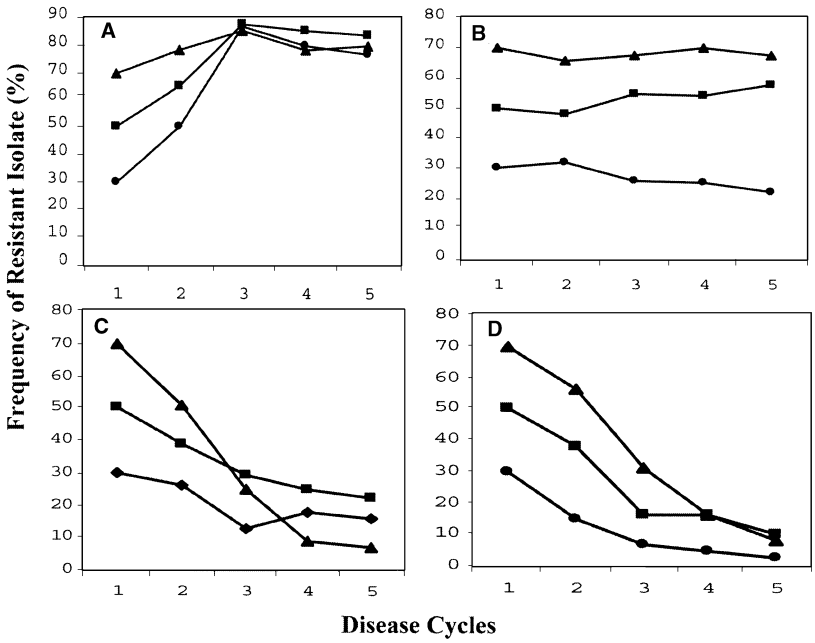

Figure 1. Competition between 4 pairs of flutriafol-sensitive and flutriafol-resistant isolates of *Cercospora beticola*, coinoculated on untreated sugar beet plants, with three different inital ratios of conidial mixtures. A, R1/S1 pair; B, R2/S2 pair; C, R3/S3 pair and D, R4/S4 pair.

stable, having a value of 52% at the end of the summer (not significantly different from the initial 50%) (Figure 2). In the 70R : 30S ratio a significant decline ($P < 0.05$) of the resistant isolates frequency to 59% at the end of the summer was observed (Figure 2), while in the 30R : 70S ratio the resistant fraction of the population remained almost stable ($P > 0.05$), fluctuating around the initial 30% (26% at the end of the experiment), (Figure 2).

In the 1998 experiment, the changes were almost the same, except that a significant decline ($P < 0.05$) of the resistant isolates in the 50R : 50S ratio was observed. In this ratio the resistant fraction of the population gradually declined from the initial 50 to 40% (Figure 2). Similarly, in the 70R : 30S ratio the frequency of the resistant isolates decreased from 70 to 63% ($P < 0.05$), while in the 30R : 70S ratio a slight increase ($P > 0.05$) of the resistant fraction was observed, from the initial 30% to a final 35% (Figure 2).

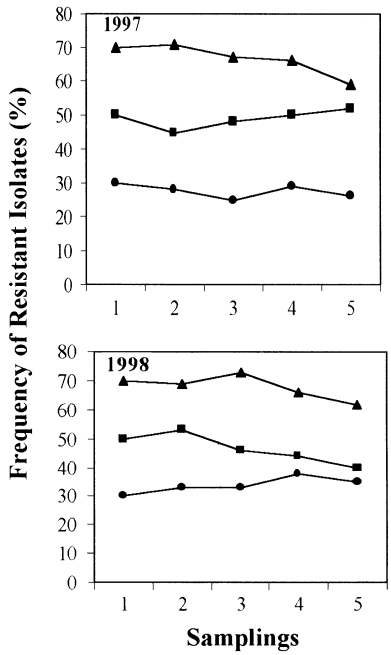

Figure 2. Competition between mixed flutriafol-sensitive and -resistant isolates of *Cercospora beticola*, coinoculated on untreated sugar beet crop with three different initial ratios of conidia.

Fitness components in single-isolate measurements

Six fitness components were measured for 21 isolates of *C. beticola* belonging to two different sensitivity groups. Great variability among the tested isolates

Table 2. Fitness components of flutriafol-resistant and -sensitive field isolates of *Cercospora beticola*

Isolate	Dry weight (g)	Spore germination (%)	Germ tube length (μm)	Incubation period (days)	Virulence[1]	Sporulation (spore number/ mm^2 of lesions)
Fitness components						
R152	0.37(0.05)[2]ab[3]	100(0.0)a	296(20)b	11.2(1.0)a	3.27(0.64)bcd	1.1×10^3(289)de
R193	0.33(0.16)bc	100(0.0)a	339(23)a	10.8(0.5)a	3.89(0.58)bc	2.4×10^3(390) c
R61	0.33(0.09)bc	100(0.5)a	249(36)c	9.7(0.6)b	4.95(0.94)a	1.2×10^3(295)de
R65	0.34(0.09)bcd	99(1.0)a	225(22)d	9.7(0.5)b	5.65(1.20)a	3.8×10^3(663)b
R116	0.20(0.08)e	100(0.0)a	193(15)e	11.0(0.5)a	3.26(0.46)cd	1.0×10^3(164)e
R171	0.25(0.08)cde	100(0.0)a	151(21)f	11.2(0.6)a	3.30(0.50)bcd	1.6×10^3(406)d
R215	0.46(0.13)a	100(0.0)a	282(28)b	9.6(0.8)b	3.97(0.67)b	1.3×10^3(298)de
R79	0.22(0.08)de	99(1.0)a	204(26)de	10.0(0.7)b	5.15(0.6)a	2.1×10^3(633)c
R51	0.40(0.11)ab	99(1.0)a	191(25)e	10.9(0.3)a	2.94(0.44)d	4.3×10^3(558)a
Mean	0.32(0.10)A[4]	100(0.25)A	237(59)A	10.4(0.6)A	4.04(0.71)B	2.1×10^3(441)B
S70	0.37(0.07)bcd	98(0.5)a	284(43)abcd	11.0(0.5)ab	4.86(0.6)bc	2.1×10^3(430)ef
S17	0.26(0.06)ef	99(1.0)a	310(47)a	11.2(0.6)a	3.86(0.39)e	2.2×10^3(435)ef
S56	0.31(0.03)de	100(0.0)a	240(25)ef	10.0(1.0)c	4.48(0.43)cd	2.2×10^3(263)ef
S60A	0.36(0.04)cd	98(1.0)a	258(33)def	10.5(0.5)abc	4.20(0.47)de	3.4×10^3(567)d
S160	0.37(0.08)bcd	98(0.5)a	263(37)def	11.0(0.7)ab	4.22(0.46)de	4.7×10^3(548)c
S60H	0.39(0.12)bc	100(0.0)a	265(34)bcde	11.2(0.6)a	4.66(0.75)bcd	1.6×10^3(403)g
S162H	0.25(0.04)f	100(0.0)a	276(36)bcd	10.3(1.1)bc	5.10(0.49)bc	2.5×10^3(612)e
S165	0.47(0.08)a	98(1.5)a	298(31)ab	10.0(0.8)c	4.85(0.52)bc	2.0×10^3(347)fg
S179	0.44(0.09)ab	100(0.0)a	263(35)cdef	10.5(1.0)abc	5.48(0.48)a	3.5×10^3(468)d
S62	0.33(0.06)cde	98(0.5)a	282(29)abcd	10.0(0.7)c	4.33(0.56)de	3.5×10^3(373)d
S75	0.37(0.08)bcd	99(1.0)a	292(27)abc	10.7(1.0)ab	4.61(0.74)bcd	9.5×10^3(732)a
S162A	0.35(0.07)cd	100(0.0)a	234(28)f	10.4(0.7)bc	4.67(0.45)bcd	5.4×10^3(384)b
Mean	0.35(0.07)A	99(0.9)A	272(22)A	10.5(0.7)A	4.61(0.54)A	3.6×10^3(480)A

[1]Measurements based on the nine-scale Disease Index of KWS.
[2]Standard Deviation
[3]Statistical analysis within groups. Means of individual isolates followed by the same letter are not different according to Duncan's Multiple Range Test at $P = 0.05$.
[4]Statistical analysis between groups. Mean values of groups followed by the same capital letter are not different according to Duncan's Multiple Range Test at $P = 0.05$.

existed, even within the same sensitivity group, for most of the fitness components measured.

Great variability ($P < 0.05$) existed in the mycelial growth of the isolates within both sensitivity groups, while means of each group were not significantly different ($P > 0.05$) (Table 2). In the resistant isolates the dry weight ranged from 0.20 to 0.46 g, with a mean of 0.32 g whereas in the group of sensitive isolates the dry weight ranged between 0.25 and 0.47 g, with a mean of 0.35 g.

For conidial germination no significant differences ($P > 0.05$) were detected both between individual isolates within each sensitivity group and the means of each group (Table 2). All isolates showed high percentages of conidial germination (98–100%). Measurements of germ tube length showed that significant differences ($P < 0.05$) existed between the tested isolates, while no significant differences ($P > 0.05$), were detected between the mean values of the groups (Table 2).

All isolates caused disease symptoms on the sugar beet cultivar selected for the experiment and produced spores on the lesions. For most of the tested isolates the first disease symptoms appeared approximately 10 days after inoculation. However, in some replicates the first lesions appeared on the eighth day while in some others appeared after 12 days. For both sensitivity groups the mean values of incubation period were almost the same ($P > 0.05$), (10.5 days for the sensitive and 10.4 days for the resistant isolates) (Table 2).

A large variation in virulence was found among tested isolates, even within the same sensitivity group. The mean disease index of individual isolates ranged

from 2.94 to 5.65. As a group, resistant isolates showed a significantly lower ($P < 0.05$) virulence (mean disease index of 4.04), while the sensitive isolates had a mean disease index of 4.61 (Table 2). However, within the resistant isolates there were two isolates (R65 and R79) with a disease index significantly higher compared to most sensitive isolates.

A large variation between isolates was also found for their sporulation capacity. For the sensitive isolates a mean spore production of 3.6×10^3 conidia per mm^2 diseased leaf surface was observed, while the resistant isolates produced significant lower ($P < 0.05$) number of spores with a mean value of 2.1×10^3 mm^{-2} diseased leaf surface.

Correlation between fitness components and sensitivity to flutriafol

Correlation coefficients between values of fitness components and sensitivity to flutriafol expressed as log EC_{50}, were estimated for all the 21 isolates. For 4 out of 6 fitness components including mycelial growth, germination of conidia, germ tube length and incubation period, the correlation coefficient was not significantly different from 0 ($P > 0.05$) (Figure 3A–D), indicating that there was no correlation between level of sensitivity to flutriafol and the respective fitness components. However, in the cases of virulence and spore production correlation coefficients were significantly

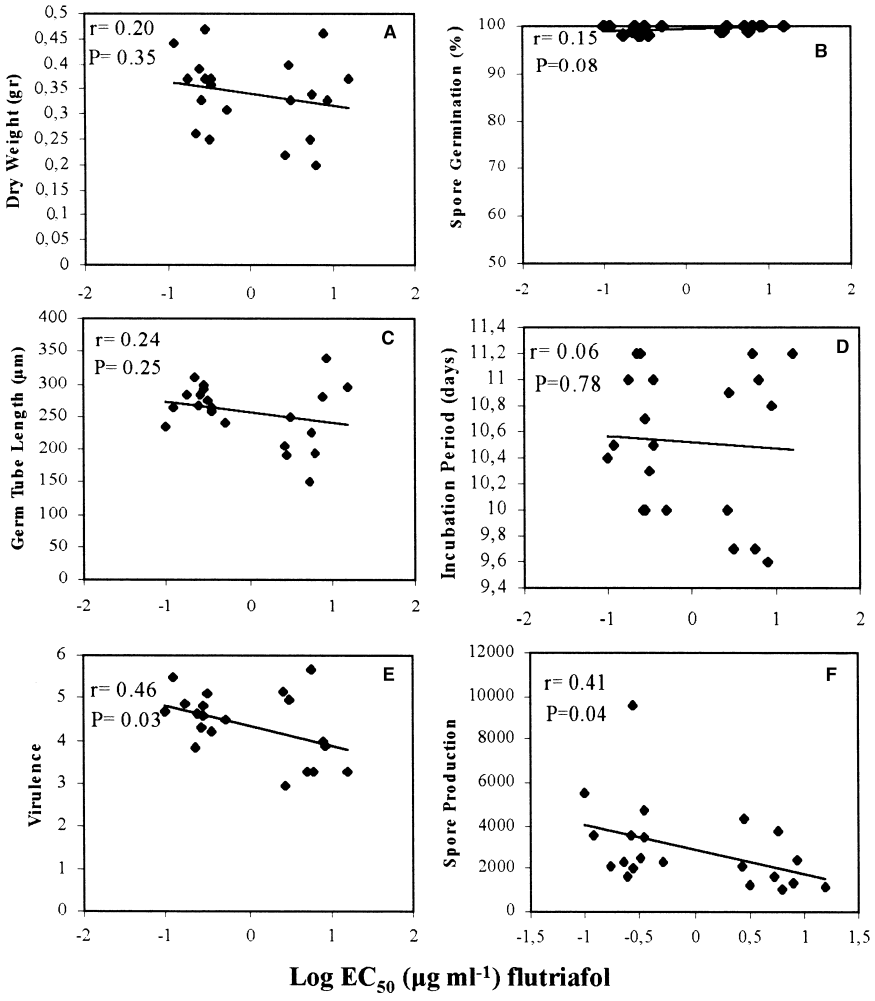

Figure 3. Correlation between level of sensitivity to flutriafol and dry weight (A), spore germination (B), length of germ tube (C), incubation period (D), virulence (E) and spore production (F) of *Cercospora beticola* isolates.

different from 0 ($P < 0.05$), indicating that both the lower virulence and spore production of the resistant isolates was related with resistance to flutriafol (Figure 3E,F).

Discussion

All isolates were obtained from the field, whereas many previous fitness studies involving fungicide-resistant strains were selected in the laboratory (de Waard et al., 1982; Henry and Trivellas, 1989; Nuninger-Ney et al., 1989). The determination of fitness parameters in fungicide-resistant strains produced in laboratory may have little relevance for field isolates. This is mainly because fungal mutants selected in the laboratory represent only a small subset of strains present in the field and probably such mutants cannot survive in the field (Koenraadt et al., 1992). In addition, laboratory- and field-selected resistant strains may be different in their fitness-modifying genes which can increase the fitness of field strains to a level similar to sensitive strains (Uyenoyama, 1986).

When the fitness components of 9 resistant and 12 sensitive isolates were determined, the resistant isolates appeared to have a lower fitness than the sensitive isolates. The results presented in this study showed that resistant isolates, as a group, had a lower virulence and spore production than the sensitive isolates, while no differences were observed in the other 4 fitness components measured. Similar results estimating virulence of *C. beticola* strains have previously been reported for laboratory-induced resistance to azole fungicides (Henry and Trivellas, 1989). The correlation coefficients between resistance, expressed as log EC_{50} of the individual isolates and the values of virulence and spore production, indicated a positive correlation. However, in all but the spore germination, fitness components varied within the two groups of isolates, reflecting probably a different genetic background of the isolates. Moreover, virulence and spore production of some resistant isolates were not lower than those of sensitive isolates, suggesting the possibility of selection of resistant isolates with high fitness, as has already, been stated (Uyenoyama, 1986). Similar results have previously been reported for *Penicillium digitatum* (Holmes and Eckert, 1995) and *Pseudocercosporella herpotrichoides* (Cavelier et al., 1996), while resistance development to DMIs was not associated with fitness cost for resistant strains of *Pyrenophora terres* (Peever and Milgroom, 1994), *Sphaerotheca fuliginea* (Schepers, 1985) and *Erysiphe gramminis* (Schulz and Scheinpflug, 1986).

Measuring fitness in the field is a very difficult task because of migration of conidia within and from outside the field and therefore such experiments are not done very often (Dovas et al., 1976). The competition experiments in the field were carried out during two successive years, 1997 and 1998. Instead of testing the competitive ability of single pairs of resistant and sensitive isolates, we preferred to mix the inoculum derived from all available resistant and sensitive isolates. This approach was chosen because it was closely related to the situation in nature where there is always a mixture of strains with diverse genetic backgrounds competing for dominance. Moreover, the need of large plots with adequate numbers of replications would not allow us to test a large number of treatments in the experimental design. In the absence of DMIs treatments, the frequency of the resistant strains was reduced, by the end of the growing period, in two out of three tested ratios, while in the third ratio it remained stable. If the proportions of resistant strains in mixed population are high we suggest that fungicide applications may select *C. beticola* strains that are, at the same time, resistant to DMIs and higher in fitness.

The presented results can be compared to other data on sensitivity of *C. beticola* populations to DMIs. From 1996 to 1999, an extensive monitoring programme was carried out to measure changes in sensitivity of the pathogen populations to DMIs in N. Greece. Fields were sampled twice per year, prior and after the DMIs spray applications. The results showed that the frequency of resistant isolates in the population was significantly lower at the first sampling of the year than after the end of the spraying period of the previous year (G.S. Karaoglanidis, unpubl.), indicating that resistant isolates either cannot compete well with the sensitive strains in the absence of DMIs treatment or they cannot survive well during the winter overseasoning period. However, the saprophytic ability to survive was not measured in this study. This fitness component may reveal significant correlation with resistance to DMIs, as it has been demonstrated for metalaxyl-resistant *Phytophthora infestans* isolates (Kadish and Cohen, 1992).

The results of this study encourage the evaluation of a resistance-management programme involving alternation of DMIs with other non-cross resistant fungicides. The exclusive use of DMIs for *C. beticola* control would probably lead to the selection of resistant isolates with higher fitness through selection by

fitness-modifying genes. Thus, the adoption of a spray programme involving alternation of different fungicides could probably delay the evolution of resistance to DMIs because strains resistant to DMIs, would be reduced when the sugar beet crop is treated with the alternate fungicide.

Acknowledgements

Critical reading of the manuscript by Dr Derek Hollomon is gratefully acknowledged. We also thank Zeneca Hellas S.A. for providing the pure technical grade of flutriafol, Stefanos Daucopoulos and George Akrivos for maintaining the field experiments and Lazaros Vekiaridis for his assistance in the artificial inoculations of field plots. The first of the authors was financially supported by the Greek Scholarship Foundation.

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