

## Sensitivity of *Cercospora beticola* populations in Turkey to flutriafol, mancozeb, and fentin acetate

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**Abstract:** The sensitivity of sugar beet leaf spot disease caused by *Cercospora beticola* to fungicides used in Turkey was determined using a mycelial growth assay in petri dishes with 100 fungal isolates. The isolates were obtained from samples collected from 6 sugar beet production areas in 2005 and 2006. Flutriafol, mancozeb, and fentin acetate were added to *Aspergillus* complete medium (ACM) at the rates of 0.0, 0.05, 0.1, 0.5, 1.0, 5.0, and 10.0  $\mu\text{g mL}^{-1}$ , and then mycelial growth was measured. ED<sub>50</sub> values of flutriafol for all the isolates from the sugar beet production areas ranged from 0.04 to >10.0  $\mu\text{g mL}^{-1}$ . Tolerance to this fungicide was very high in 2 regions (Adapazarı and Çarşamba) where the disease is very prevalent due to climatic conditions. In these 2 areas 74.1%, 55.5%, 81.2%, and 68.7% of the isolates grew with 5 and 10  $\mu\text{g mL}^{-1}$  of flutriafol, respectively (1  $\mu\text{g mL}^{-1}$  flutriafol is considered inhibitory to the growth of the fungus). In the other 4 regions included in the study, tolerance was not as high. Tolerance to mancozeb developed in all the regions; however, the percentage of tolerant isolates was lowest in Çarşamba (6%) and highest in Susurluk (47.3%). Tolerance to fentin acetate varied according to the criteria used for evaluation.

**Key words:** *Cercospora beticola*, fentin acetate, flutriafol, fungicide resistance, mancozeb

### Türkiye’de *Cercospora beticola* popülasyonlarının flutriafol, mancozeb ve fentin acetate’a duyarlılıkları

**Özet:** *Cercospora beticola*’nın neden olduğu Şeker Pancarı Yaprak Lekesi hastalığının Türkiye’de kullanılan fungusitlere karşı dayanıklılığı petri kaplarında mycelial gelişme deneyleri ile 2005-2006 yıllarında 6 şeker pancarı fabrikası üretim alanından elde edilen 100 fungus izolatu kullanılarak araştırılmıştır. Bunun için flutriafol, mancozeb ve fentin asetat *Aspergillus* Complete Medium (ACM)’a 0.0, 0.05, 0.1, 0.5, 1.0, 5.0, 10.0  $\mu\text{g mL}^{-1}$  dozlarda katılmış ve miselyal gelişme ölçülmüştür. Şeker pancarı üretim alanlarından elde edilen tüm izolatların flutriafol için ED50 değerleri 0.04 ile > 10.0  $\mu\text{g mL}^{-1}$  arasında değişmiştir. Bu funguside karşı tolerans, bu hastalığın gelişmesi için uygun iklim koşullarını içeren ve hastalığın şiddetli olduğu iki bölgede, Adapazarı ve Çarşamba, belirgin derecede yüksek bulunmuştur. Bu iki bölgede; izolatların sırasıyla %74.1, %55.5, %81.2 ve %68.7’i, fungusun gelişmesini engellediği kabul edilen 5 ve 10  $\mu\text{g mL}^{-1}$  flutriafol dozunda gelişmiştir. Diğer 4 bölgede, bu fungusite tolerans bu kadar yüksek oranlarda değildi. Mancozeb’ e tolerans tüm bölgelerde görülmüş ve tolerant izolatların yüzdesi Çarşamba’da en düşük (%6) ve Susurluk bölgesinde en yüksek (%47.3) olmuştur. Fentin asetat’ a karşı tolerans değerlendirilme kriterine bağlı olarak değişiklik göstermiştir.

**Anahtar sözcükler:** *Cercospora beticola*, fentin-asetat, flutriafol, fungusit dayanıklılığı, mancozeb

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## Introduction

Sugar beet is grown on 315,000 ha in 30 factory regions in Turkey and 20% of this area is infected by sugar beet leaf spot (SBLs) disease caused by *Cercospora beticola* (Özgür 2003). These areas are situated in the Alpullu, Susurluk, Adapazarı, Kastamonu, Çarşamba, Turhal, and Amasya sugar production regions.

Worldwide, SBLs is the most important foliar disease of sugar beet in warm and humid areas, including Turkey (Holtschulte 2000; Özgür 2003). It can cause serious yield losses in the absence of treatment, ranging from 10% to 50% (Shane and Teng 1992; Mohamed et al. 2005). Control of SBLs in Turkey and many other countries has relied on an integrated approach involving the use of tolerant cultivars, crop rotation, and fungicide application (Karaoglanidis et al. 2003a; Özgür 2003). Despite such efforts, the disease causes 6%-35% of sugar beet losses and 1%-26% of sugar losses in Turkey (Özgür 2003).

To date, various fungicides have been used against SBLs, beginning with the protectant fungicide copper oxychloride in the 1900s, which was followed by a great number of other fungicides until the recent development of strobilurin fungicides (Meriggi et al. 2000; Karadimosa et al. 2005; Mohamed et al. 2005). The primary reason for this shift in fungicides used to control SBLs is the rapid development of tolerance. In Greece, it is reported that use of benomyl began in 1971 with excellent results, but within 2 years the fungal populations developed tolerance and the fungicide's efficacy was completely lost (Karaoglanidis et al. 2003b). This has also been reported in other countries (Weiland and Halloin 2001). In Poland *C. beticola* populations gained resistance to thiophanate-methyl, and then carbendazim, while the rate of resistance to azole fungicides remained low (Piszczek and Czekalska 2006). In Greece, fentin derivatives were the only available fungicides that provided satisfactory control of *C. beticola* after the emergence of benomyl resistance. However, during the 1976 and 1977 growing seasons control of *Cercospora* leaf spot disease with fentin fungicides was unsatisfactory and laboratory tests showed that resistance had developed. Since then, use of fentin derivatives in most areas of sugar beet cultivation has been restricted to 2 or 3 applications in combination with maneb early in the

season (Giannopolitis 1978; Karaoglanidis et al. 2003b). Tolerance of *C. beticola* to fentin derivatives has also been reported in the USA (Bugbee 1995; Briere et al. 2001).

Triazoles, used at full rate, were reported to be very effective against SBLs; however, because of the specific site of action they were potentially able to select resistant strains of the fungus. Thus, in the 1980s it was recommended that all the new fungicides that belonged to this chemical group be used by alternating with other fungicides that have a non-specific site of action, including organotin compounds, copper salts, maneb, and chlorothalonil (Meriggi et al. 2000).

A similar regimen was also followed in Turkey, and for the last 10 years a new fungicide application program has been used (Özgür 2003); fentin acetate is applied at the beginning of the season, after mancozeb has been replaced, and finally flutriafol is applied as a systemically acting curative 2-3 times, depending on disease severity and durability (Özgür 2003).

In a previous study of ours carried out at 2 localities in the Susurluk sugar production region, flutriafol provided 64% and 34% control when applied at the registered rate (0.075 kg a.i.ha<sup>-1</sup>); the fungicide was applied 3 times, beginning with observation of disease onset (unpublished data). Additionally, farmers in the region have reported that flutriafol application is ineffective. Thus, the present study investigated the sensitivity of fungicides used against SBLs in Turkey based on in vitro laboratory tests, and fungicide tolerance was also assessed.

## Materials and methods

### Sampling sites

In all, 100 diseased leaf samples were collected during the 2005 growing season from 25 locations in 6 sugar production regions where SBLs is widely observed: Adapazarı, Amasya, Alpullu, Susurluk, Kastamonu, and Çarşamba. From these locations, 100 isolates of *C. beticola* were obtained (24, 18, 8, 19, 15, and 16 isolates, respectively). The 25 locations and number of the isolates were proportional to the acreage, and 1 sample represented a 600-ha area. The same fungicide regimen (fentin acetate and mancozeb

as preventive fungicides, and flutriafol as a systemically acting curative fungicide) was used at all of the locations for the last 10 years, but the number of flutriafol applications ranged between 1 and 3, based on the disease severity at each location. The highest number of flutriafol applications occurred at Adapazarı and Çarşamba.

### Isolation of *Cercospora beticola*

Sugar beet leaves with distinct sporulating lesions were selected for isolation of the pathogen. Single spore isolates of *C. beticola* were obtained by selecting undisturbed, clean sporulating lesions under a stereomicroscope, dissecting a 2 × 2-mm leaf disk, and placing it in 0.5 mL of distilled sterile water (with a drop of Tween 20 in 250 mL of water) in a PCR tube. Then 100 µL of spore suspension was streaked onto sugar beet leaf extract agar (SBLEA), as described by Calpouzou and Stallknecht (1966). After 2-5 days of incubation at 25 ± 1 °C in a chamber illuminated with NUV light tubes for 12 h, mycelial tips from the edges of the colonies growing from 1 spore were removed and transferred onto SBLEA and Difco potato dextrose agar (PDA) media in tubes. Duplicate cultures of the isolates were maintained in screw capped tubes with SBLEA and PDA in a refrigerator; the isolates were also stored at -80 °C in 15% glycerin.

### Sensitivity tests

The fungicides used in this study were commercial formulations of flutriafol (Future 125 SC, Pulcu Agrochemicals, Turkey), mancozeb (Fumazin 80 WP, Hektaş, Turkey), and fentin acetate (Safestan Conc, Safa, Turkey). Autoclaved *Aspergillus* complete medium (ACM) composed of 15 g of agar, 10 g of dextrose, 1 g of yeast extract, and 1000 mL of distilled water (acidified with a drop of lactic acid in order to suppress bacterial growth), was cooled to 50 °C and amended with aqueous fungicide solution at concentrations of 0.05, 0.1, 0.5, 1.0, 5.0, and 10.0 µg mL<sup>-1</sup>. Control petri dishes were not amended with fungicide. Tests for each isolate were replicated 3 times per each concentration of each fungicide. Mycelial plugs 5 mm in diameter were removed from the margins of colonies grown on ACM, placed upside down on the fungicide-amended and fungicide-free ACM in petri dishes, and incubated at 25 °C in the dark. After 7 days the colony diameter of each isolate

was measured (minus the diameter of the inoculation plug) and the percent inhibition (PI) values of each of the fungicide rates were calculated using the formula below:

$$\text{percent inhibition} = (a - b) / a \times 100$$

where a is the colony diameter of the control plate and b is the colony diameter of the fungicide-amended plate.

### Data analysis

PI values were subjected to regression analysis against the logarithmic values of the fungicide rates using the StatGraphics Centurion computer program and one-factor simple regression. The fungicides' ED<sub>50</sub> values for the isolates were calculated using the obtained regression equation. Differences between the isolates, both in and between regions, were determined by analyzing the PI values at all the rates using SPSS. PI values were subjected to general linear model variation (univariate) analysis and the significance of the difference between the treatments was determined using Duncan's multiple range analysis.

### Results

Fungicide tolerance in *C. beticola* has been evaluated in different ways by various researchers; therefore, the levels of sensitivity to flutriafol, mancozeb, and fentin acetate were considered separately.

#### Sensitivity to flutriafol

The sensitivity of the *C. beticola* populations to flutriafol differed significantly. Based on the mean PI values, 3 groups were formed: Adapazarı and Çarşamba isolates was the least affected group, Amasya and Susurluk isolates was the moderately affected group, and Alpullu and Kastamonu isolates was the most affected group (Table 1). All the isolates from Adapazarı, Çarşamba, and Susurluk grew at 1 mg mL<sup>-1</sup> of flutriafol, which is considered inhibitive for *C. beticola*. The majority of Adapazarı and Çarşamba isolates (74% and 55%, and 81% and 68%, respectively) grew at 5 and 10 µg mL<sup>-1</sup> of flutriafol, versus 44% and 11%, and 63% and 47% for the Amasya and Susurluk isolates. No isolates from Alpullu or Kastamonu grew at 10 µg mL<sup>-1</sup> of flutriafol

Table 1. Percentages of the isolates having growth at various rates of flutriafol and percentages of the isolates at 2 ED<sub>50</sub> values in 6 sugar beet factory areas and their significance.

Factory regions (No. of isolates)	Percentages of the isolates having growth at 3 rates ( $\mu\text{g mL}^{-1}$ ) <sup>1</sup>			Percentage of isolates at 2 ED <sub>50</sub> values ( $\mu\text{g mL}^{-1}$ )		Sig. <sup>2</sup>
	1	5	10	$\leq 0.34$	$\geq 9.4$	
Adapazarı (24) A <sup>3</sup>	100	74	55	0	20.8	S
Alpullu (8) C	50	12	0	100	0.0	NS
Amasya (18) B	83	44	11	22	0.0	NS
Çarşamba (16) A	100	81	68	0	37.5	S
Kastamonu (15) C	46	13	0	93	0.0	NS
Susurluk (19) B	100	63	47	26	5.2	S

<sup>1</sup> Isolates having growth at 1, 5, and 10  $\mu\text{g mL}^{-1}$  flutriafol are considered resistant by Karaoglanidis et al. (2003b).

<sup>2</sup> Sig. = Significance, S = the difference in the site (line) is statistically significant ( $P = 0.05$ ), NS = not significant.

<sup>3</sup> The capital letters show the differences in the mean PI values between the regions (columns). Regions having the same letter are not significantly different according to Duncan's multiple range test ( $P = 0.05$ ).

and the percentage of isolates that grew in these 2 regions at 5  $\mu\text{g mL}^{-1}$  of flutriafol was very low (12% and 13%, respectively).

ED<sub>50</sub> values of *C. beticola* isolates for flutriafol varied from 0.04 to >10  $\mu\text{g mL}^{-1}$  and none of the isolates from Adapazarı or Çarşamba had an ED<sub>50</sub> value of 0.34  $\mu\text{g mL}^{-1}$  or lower, which is considered indicative of sensitivity to this fungicide by some authors (Karaoglanidis et al. 2001). In total, 100% and 93% of the Alpullu and Kastamonu populations, respectively, had lower ED<sub>50</sub> values for the fungicide (lower than 0.34  $\mu\text{g mL}^{-1}$ ), whereas Amasya and Susurluk populations had slightly higher ED<sub>50</sub> values for flutriafol (Table 1).

#### Sensitivity to mancozeb

The mean PI values calculated for mancozeb for each of the regions did not differ significantly, but the difference between the isolates in 4 of the regions (Adapazarı, Amasya, Kastamonu, and Susurluk) was significant. Growth of the isolates with 5  $\mu\text{g mL}^{-1}$  of mancozeb, which indicates tolerance, varied in the regions. The highest rate of growth at this concentration was observed in Susurluk, where 47% of the isolates grew in the mancozeb-amended ACM medium (Table 2).

#### Sensitivity to fentin acetate

The sensitivity of the *C. beticola* populations to fentin acetate was characterized based on the 3 categories given by Giannopolitis (1978), Karaoglanidis et al. (2003b), and Weiland (2001): ED<sub>50</sub> value, growth at 0.25  $\mu\text{g mL}^{-1}$ , and growth at 1.0  $\mu\text{g mL}^{-1}$  (Table 3). In 2 of the sugar production areas (Adapazarı and Çarşamba) isolates had similar PI values for this fungicide, whereas in the other regions mean PI values of the isolates were significantly different. When the sensitivity of the isolates was evaluated according to the ED<sub>50</sub> parameter, as suggested by Giannopolitis (1978), the majority of the isolates from Adapazarı and Çarşamba were classified as susceptible (S) and moderately susceptible (MS). The highest rates of tolerant isolates were observed in Susurluk (Table 3).

The susceptible, moderately resistant, and resistant categories showed great variation between the 2 criteria taken into consideration. When the resistant categories in the 2 systems were compared congruity was not observed. For instance, 83.3% of the Adapazarı isolates grew at 1.0  $\mu\text{g mL}^{-1}$  of fentin acetate, while 31.2% of the Çarşamba isolates grew at that rate, and the percentages of total MR and R

Table 2. Percentages of the isolates having growth at 5 µg mL<sup>-1</sup> mancozeb, range of ED50 values, and percent inhibition of the isolates less than 80% at 5 µg mL<sup>-1</sup> mancozeb in 6 regions and their significance.

Factory regions (No. of isolates)	Percentages of the isolates having growth 5 µg mL <sup>-1</sup>	ED50 values (µg mL <sup>-1</sup> )		Percentages of the isolates having less than 80% PI at 5 (µg mL <sup>-1</sup> ) <sup>2</sup>	Sig. <sup>3</sup>
		Min	Max		
Adapazarı (24) A1	33.3	0.05	0.66	0.0	S
Alpullu (8) A	25.0	0.21	0.91	12.5	NS
Amasya (18) A	33.3	0.08	1.15	22.2	S
Çarşamba (16) A	6.2	0.25	0.69	0.0	NS
Kastamonu (15) A	33.3	0.03	0.95	13.3	S
Susurluk (19) A	47.3	0.13	1.09	26.3	S

<sup>1</sup>The capital letters in the first column show the differences in the mean PI values between the regions (P = 0.05)

<sup>2</sup>Percentages of the isolates having less than 80% PI at 5 µg mL<sup>-1</sup> mancozeb

<sup>3</sup> Sig. = Significance, S = the difference in the site (line) is statistically significant (P = 0.05), NS = not significant.

Table 3. Percentages of the isolates in 4 categories based on the ED50 values adapted from Giannopolitis (1978), percentages of the isolates in 3 categories based on the growth at 0.25 µg mL<sup>-1</sup>, percentages of the isolates having growth at 1.0 µg mL<sup>-1</sup> fentin acetate in 6 regions, and their significance.

Factory regions (No. of isolates)		Percentages of the isolates in 4 categories based on the ED50 values <sup>2</sup>				Percentages of the isolates in 3 categories based on the growth at 0.25 (µg mL <sup>-1</sup> ) <sup>3</sup>			Percentages of the tolerant <sup>4</sup> isolates having growth at 1.0 µg mL <sup>-1</sup>	Sig. <sup>5</sup>
		S	MS	MR	R	S	MR	R		
		Adapazarı (24) A <sup>1</sup>		58.3	25.0	12.5	4.2	0.0	87.5	12.5
Alpullu (8) B		37.5	25.0	12.5	25.0	4.1	91.8	4.1	25.0	S
Amasya (18) C		16.7	33.3	22.2	27.8	5.5	94.5	0.0	22.2	NS
Çarşamba (16) A		75.0	0.0	12.5	12.5	6.2	81.2	12.6	31.2	S
Kastamonu (15) D		0.0	46.7	26.7	26.6	20.0	80.0	0.0	0.0	S
Susurluk (19) BC		31.6	0.0	31.6	36.8	5.3	89.4	5.3	31.5	S

<sup>1</sup> The letters show the differences in the mean PI values between the regions (columns) (P = 0.05)

<sup>2</sup> Percentages of the isolates at 4 categories of ED50 values, based on the adaptation of ED50 values from Giannopolitis (1978). S = susceptible (0.0-0.5), MS = medium susceptible (0.5-2.0) MR = medium resistant (2.0-5.0), R = resistant (5.0-9.5)

<sup>3</sup> Percentages of the isolates at three categories of daily growth, S = susceptible (no growth), MR = medium resistant (<2 mm growth per day), R = resistant (>2 mm growth per day) based on the evaluation by Karaoglanidis et al. (2003b).

<sup>4</sup> Tolerance is based on having growth at 1.0 µg mL<sup>-1</sup> as given by Weiland (2001).

<sup>5</sup> Sig. = Significance, S = the difference in PI values in a site (line) is statistically significant (p = 0.05), NS = not significant.

categories based on growth at  $0.25 \mu\text{g mL}^{-1}$  were not in accordance with the values of growth at  $1.0 \mu\text{g mL}^{-1}$  in these 2 regions.

## Discussion

Fungicide resistance in *Cercospora beticola* is one of the most important factors limiting the chemical control of *Cercospora* leaf spot disease in many countries (Ioannidis et al. 2002). Growth of *C. beticola* isolates at  $1 \mu\text{g mL}^{-1}$  of flutriafol is considered a good indicator of sensitivity, as this rate was reported as almost equal to the mean  $ED_{50}$  values of sensitive isolates (Karaoglanidis et al. 2003b). When the isolates in the present study were rated based on this parameter, all grew at  $1 \mu\text{g mL}^{-1}$  of flutriafol in 3 of the regions (Adapazarı, Çarşamba, and Susurluk). These isolates apparently had low sensitivity to flutriafol, which is considered a moderate-risk fungicide. A high number of the isolates from Adapazarı, Çarşamba, and Susurluk also showed growth at 5 and  $10 \mu\text{g mL}^{-1}$  of flutriafol, indicating that the fungus acquired resistance in those locations.

Farmers' observations, especially in Çarşamba, and the results of previous experiments we conducted at 2 locations (unpublished data) clearly support the development of resistance to flutriafol.  $ED_{50}$  values of isolates for flutriafol are sometimes used to determine fungicide sensitivity, and isolates with  $ED_{50}$  values of  $9.4\text{-}13.9 \mu\text{g mL}^{-1}$  are rated as resistant and those with  $ED_{50}$  values of  $0.10\text{-}0.16 \mu\text{g mL}^{-1}$  are rated sensitive (Karaoglanidis et al. (2001). In another study  $ED_{50}$  values of 0.34 and lower were considered sensitive (Karaoglanidis et al. 2003a). The evaluation of fungicide sensitivity to flutriafol based on  $ED_{50}$  values was quite complicated, as our values exceeded the limits reported by Karaoglanidis et al. (2001). These findings show that there is not a clear-cut parameter for the evaluation of tolerance to flutriafol based on  $ED_{50}$  values. Nonetheless, a decrease in the sensitivity to flutriafol was observed.

Sensitivity to mancozeb is not high; however, tolerance may develop. Weiland (2001) considered isolates that grew with  $5 \mu\text{g mL}^{-1}$  of mancozeb as tolerant, and this limit was accepted by us in the present study. Based on this concentration, tolerance to this fungicide developed in all the areas studied, although there were significant differences between the isolates in 4 of the 6 study areas (Table 2). Even

though some of the isolates in the study areas grew, their growth was inhibited by more than 80%. In another words, mancozeb tolerance did not develop in Adapazarı or Çarşamba, where high sensitivity to flutriafol was observed.

Fungicide sensitivity to fentin acetate in the present study was determined according to Giannopolitis (1978), Karaoglanidis et al. (2003b), and Weiland (2001) (Table 3). Giannopolitis (1978) grouped *C. beticola* isolates into 3 categories based on  $ED_{50}$  values. We adapted this grouping system and added a fourth group with  $ED_{50}$  values of 0.5-2.0, as MS. Based on this evaluation, tolerance to fentin acetate was highest (36.8% of the isolates) in Susurluk, followed by Amasya (27.8%) and Kastamonu (26.6%). The same trend was noted when MR categories were added to the R category.

When tolerance to fentin acetate was assessed according to Karaoglanidis et al. (2003b) a very different composition appeared. Percentages of tolerant isolates were the highest in Adapazarı and Çarşamba, in contrast to the assessment based on  $ED_{50}$  values. Most of the isolates of *C. beticola* were in the moderately resistant group, which ranged between 80% and 94.5%. When the isolates were categorized according to growth at  $1.0 \mu\text{g mL}^{-1}$ , as proposed by Weiland (2001), tolerance to fentin acetate was highest in Çarşamba (75%) and lowest in Adapazarı (4.1%), which was not in accordance with the results published by Karaoglanidis et al. (2003b). This finding shows that tolerance to fentin acetate should be evaluated using different standards. Mean PI values differed significantly in and between the study regions. The present study's results concerning fentin acetate show that there was tolerance to this fungicide at every location and that for effective control of SBL disease in Turkey a change in fungicide should be considered.

## Conclusion

Tolerance of the causal agent of sugar beet leaf spot (*Cercospora beticola*) to fungicides is a well-known phenomenon that has been reported in many of the world's sugar beet producing regions. Different criteria have been used to evaluate fungicide sensitivity and they are not in accord with the criteria used universally. As a result, when various parameters are used it can be

concluded that resistance of *C. beticola* populations to flutriafol obviously occurred in the Adapazarı and Çarşamba sugar producing regions, and that alternative fungicides should be used in these areas. Resistance to this sterol-inhibiting systemic fungicide might also occur in the other sugar beet production areas of Turkey. Tolerance to mancozeb and fentin acetate was also clearly demonstrated and their use should also be reconsidered.

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