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# Occurrence of *Cercospora beticola* populations resistant to benzimidazoles and demethylation-inhibiting fungicides in Serbia and their impact on disease management

Nenad Trkulja [a, *], Anja Milosavljević [a], Rade Stanisavljević [a], Milana Mitrović [a], Jelena Jović [a], Ivo Toševski [a, b], Jelena Bošković [c]

[a] Institute for Plant Protection and Environment, Teodora Drajzera 9, 11000 Belgrade, Serbia
[b] CABI Europe – Switzerland, 1 Rue des Grillons, 2800 Delémont, Switzerland
[c] Faculty of Economics and Engineering Management in Novi Sad, University Business Academy in Novi Sad, Cvećarska 2, 21000 Novi Sad, Serbia

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ABSTRACT

The emergence of *Cercospora beticola* populations that are resistant to benzimidazoles (MBC) and demethylation-inhibiting fungicides (DMI) has been recently reported in Serbia and has resulted in a reduced efficacy of fungicides in controlling Cercospora leaf spot (CLS). Between 2008 and 2011, using a discriminatory concentration method in sugar beet fields in two separate regions of Serbia, we determined that 93.3%–98.6% of collected *C. beticola* isolates were resistant to MBCs, whereas 6.2%–42.4% were resistant to DMI fungicides. At the same localities, field trials were conducted to investigate the impact of resistant *C. beticola* populations on disease management. From the MBC group of fungicides, both thiophanate methyl and carbendazim failed to suppress the spread of CLS at both of the tested localities. Between 2008 and 2010, DMI fungicides expressed moderate efficacy at a South Banat locality (79.8%–84.6%) whether they were applied individually (flutriafol, epoxiconazole) or in combination with MBCs (epoxiconazole/carbendazim, thiophanate-methyl/epoxiconazole). The frequency of resistant isolates in these test trials ranged from 6.2% to 10.9%. In 2011, at the same locality, conditions were observed to change in favor of the occurrence of resistant populations, which comprised up to 18.7% of the population, at the expense of DMI efficacy in CLS management. At a Srem locality, the frequency of *C. beticola* isolates that were resistant to DMIs was high during all four years of field testing (30.5%–42.4%), and the efficacy of these fungicides ranged from 48.4% to 68.0%. A combination of DMI and a protective chlorothalonil had a stable, moderate impact on disease management regardless of the frequency of DMI resistance, whereas a combination of the cyproconazole DMI with trifloxystrobin from the strobilurin group of fungicides expressed the highest efficacy. High correlation coefficient values ($r = 0.87$) indicated how strongly the frequencies of resistant populations affected disease severity in the trial plots that were treated with carbendazim and thiophanate methyl, as well as in the plots that were treated with flutriafol and epoxiconazole ($r = 0.98$). In shift sensitivity trials, MBC-resistant *C. beticola* isolates were found at equally high frequencies both before and after the treatments, indicating a complete loss of efficacy in CLS control. This test revealed the significant impact of multiple DMI applications in terms of increasing the frequency of resistant *C. beticola* populations following treatments with flutriafol, epoxiconazole, epoxiconazole/carbendazim, thiophanate-methyl/epoxiconazole and flutriafol/chlorothalonil, except when used in combination with trifloxystrobin, in which case CLS suppression was substantially high.



## 1. Introduction

Cercospora leaf spot (CLS), which is caused by *Cercospora beticola* Sacc., is one of the most economically important foliar diseases of sugar beet (*Beta vulgaris* L. subsp. *vulgaris*) both worldwide

* Corresponding author.
*E-mail address:* trkulja_nenad@yahoo.com (N. Trkulja).

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(Holtschulte, 2000) and in Serbia (Trkulja et al., 2013). Severe epidemics of *C. beticola* are manifested by progressive destruction of leaves, followed by a continual replacement of leaves at the expense of stored reserves in the root and significant yield reduction (Shane and Teng, 1992).

Disease management relies on an integrated approach that involves crop rotation, the planting of tolerant cultivars and multiple treatments with fungicides. However, chemical control remains the most important method of managing CLS. Depending on regional and weather conditions, disease pressure varies over a season and directly influences the efficacy of chemical treatments. Historically, the two main groups of systemic fungicides that have been used to control CLS during sugar beet production were benzimidazoles (MBC) and demethylation-inhibiting fungicides (DMI), whereas in the last decade strobilurins or quinone outside inhibitors (QoIs) have also been introduced and applied (Secor et al., 2010). To avoid or delay the evolution of resistant populations, these fungicides are applied with additional protectants, such as chlorothalonil, fentin-acetate and fentin-hydroxide (Karaoglanidis and Ioannidis, 2010). The manner in which using multiple treatments of select MBCs and DMIs in the control of *C. beticola* gradually influenced the development of pathogen resistance has been reported by several authors in North Dakota and Minnesota, USA (Campbell et al., 1998; Weiland and Smith, 1999; Secor et al., 2010) and in Greece (Karaoglanidis et al., 2003).

Intensive use of MBCs in the management of *C. beticola* in Serbia began in the early 1970s. At the beginning of their application, MBCs represented a new era in the control of CLS because of their high efficacy at very small doses in the field. However, their intensive use led to the emergence of *C. beticola* populations exhibiting resistance after a relatively short period of time and consequently resulted in a significant decrease in their success in the field (Marić et al., 1976). Upon the appearance of resistant populations, the use of MBCs has been reduced to only one treatment per year. Nevertheless, nearly 20 years later, a high number of MBC-resistant strains of *C. beticola* were still reported in the field (Gavran, 1991). Over the last two decades, MBCs have been used in mixtures with DMIs rather than as single formulations. Studies conducted over the last few years in several locations in Serbia have discovered high frequencies of *C. beticola* populations that are resistant to MBC fungicides (Trkulja et al., 2009; Budakov et al., 2014).

The problems encountered in the past in using MBCs to control CLS were overcome with the development and use of DMI fungicides, which have a different mode of action. In contrast to MBCs, in which single point mutations can lead to high levels of resistance, resistance to DMIs has been reported to be under polygenic control and tends to emerge in a step-wise manner. DMIs replaced MBCs in the early 1980s and continue to represent the most important group of fungicides that are applied for *C. beticola* control in Serbia, whether used as single formulations or in combinations with protectants, such as MBCs or QoIs. However, populations of *C. beticola* that are resistant to DMIs have recently been reported in several localities in Serbia (Trkulja et al., 2009; Budakov et al., 2014). To develop efficient and environmentally safe resistance management strategies against MBC- and DMI-resistant isolates of *C. beticola*, we have conducted monitoring and field trials to (i) investigate the current state of efficacy in using MBC and DMI applications to control CLS in Serbia, (ii) identify the occurrence and frequencies of *C. beticola* isolates that are resistant to MBC and DMI fungicides and (iii) determine how increasing resistance affects CLS management status and perspectives.

## 2. Material and methods

### 2.1. Field-testing of fungicide efficacies in controlling CLS

Field experiments were conducted from 2008 to 2011 in Srem and South Banat, which are two main sugar beet growing regions of Serbia. Treatment plots were arranged in a randomized complete block design with four replicates. Experimental plots were 16.5 m × 6 m, and each consisted of 12 rows with one hundred sugar beet plants per row. To limit possible interplay between different fungicide border effects in treatments, experimental plots were kept at a distance of 1 m from each other.

The test included treatments with MBCs and DMIs as single or mixed formulations (Table 1). Additionally, the efficacies of DMIs were tested in two mixtures; one included the protective fungicide chlorothalonil and the other included trifloxystrobin, which is a member of the QoI group. The fungicides that were used in the field trials were delivered as water suspensions that included the recommended commercial doses (Table 1). Control fields remained untreated. To determine a threshold for the first treatment, rosettes along a diagonal path in the experimental field were inspected by evaluating one hundred leaves of their central regions, as has been previously described by Wolf and Vereett (2002). Screening began during the phenophase of canopy closure, when the leaves of 90% of the beet plants that were in adjacent rows had begun to touch or overlap. The first applications of the fungicides were initiated when the incidence of disease in sugar beet plants reached 5%. The following two applications were carried out in intervals of 14–18 days. Fungicides were applied with a T4 sprayer (Bellspray, Inc., USA) under a pressure of 4 atm. For each experimental field, we prepared the fungicide solution in a volume of three liters.

The infection rate was assessed using a rating scale that was created by Verreet et al. (1996). Disease severity was evaluated at intervals of 15–20 days by examining one hundred leaves that were sampled from four central rows in the experimental plots. Disease severity (DS) was expressed as an average percentage of infected leaf area, which was calculated for each plot by averaging the severity estimates of each leaf. As a disease incidence parameter, an Area Under Disease Progress Curve (AUDPC) was calculated for the assessment period according to Wolf and Verreet (2002) and the following equation: AUDPC = (DS x days)/100. Values of AUDPC for control and fungicide treatment plots were subjected to an analysis of variance, and differences among treatments were analyzed using a Duncan's multiple range test at $P \leq 0.01$ and by calculating a coefficient of variation (CV%). We used a correlation coefficient ($r$) to evaluate the relationship between frequency of resistance and disease severity.

The observed efficacies of the tested fungicides were expressed in percentages and were calculated with the following equation: (disease severity in control − disease severity in treated plot)/disease severity in control. To estimate the extent of the interactions between the two fungicides that were applied as a mixture, we compared observed versus expected efficacy, which was calculated as $\%C_{exp} = A + B - (AB/100)$, wherein A and B represent the control levels of individual fungicides. If the ratio between the observed and expected efficacy of the mixture was under 0.5, then antagonistic activity existed between the two fungicides. If the ratio was between 0.5 and 1.5, then the interaction had an additive effect. Finally, if the ratio was above 1.5, then synergistic activity was present (Gisi, 1996).

### 2.2. Sampling and pathogen isolation

Samples of sugar beet leaves with symptoms of CLS were collected to isolate *C. beticola*. When the first symptoms appeared,

**Table 1**
Field testing treatments.

| Commercial name | Active ingredient (group of fungicides[a]) | Manufacturer | Amount of active ingredient g/ha |
|---|---|---|---|
| Galofungin T | thiophanate-methyl (MBC) | Galenika phytopharmacy | 315 |
| Galofungin | carbendazim (MBC) | Galenika phytopharmacy | 250 |
| Impact 25 SC | flutriafol (DMI) | Cheminova | 62.5 |
| Rubric | epoxiconazole (DMI) | Cheminova | 93.75 |
| Duett | epoxiconazole/carbendazim (DMI + MBC) | BASF | 93.75/93.75 |
| Duett ultra | epoxiconazole/thiophanate-methyl (DMI + MBC) | BASF | 112.2/186 |
| Sphere | trifloxystrobin/cyproconazole (QoI + DMI) | Bayer Crop Science | 131.25/56 |
| Bravo | chlorothalonil (PRO) | Syngenta | 1440 |
| Impact 25 SC/Bravo | flutriafol/chlorothalonil (DMI + PRO) | Cheminova/Syngenta | 50/720 |
| Control | – | – | untreated |

[a] MBC: benzimidazoles; DMI: demethylation-inhibiting fungicides; QoI: strobilurin; PRO: protective.

leaves were collected from four central rows in each of the control plots to avoid the selection pressure that can be imposed by fungicide treatments. Leaves with visible spots were inspected with a stereoscope to confirm the presence of *C. beticola* conidia. To obtain the isolates, conidia were transferred from the spots into a water agar medium that was amended with antibiotics. Forty eight hours later, germinated conidia were transferred onto fresh potato-dextrose agar (PDA) and incubated for three to five days at 25 °C in the dark to establish the isolates. In total, 130 to 150 isolates were obtained from each control plot.

### 2.3. Testing *C. beticola* sensitivity to MBCs and DMIs

The fungicides that were used in the assessment of *C. beticola* sensitivity are commercial formulations of MBCs and DMIs. The discriminatory concentration for carbendazim (MBC) and for all of the DMIs was 1 μg $ml^{-1}$ (Karaoglanidis and Bardas, 2006), except for thiophanate-methyl (MBC), which is known to have a discriminatory concentration of 5 μg $ml^{-1}$ (Weiland and Halloin, 2001). Prior to setting up the sensitivity test, fungicides were dissolved in distilled water. Autoclaved PDA media was cooled to 40–50 °C and was amended with fungicides in solution. As a control, we used a PDA medium that was amended with sterile distilled water. The radial growth of each of the isolates was measured after seven days of incubation at 25 °C. Relative growth was calculated by dividing average mycelia growth on fungicide-treated PDA with average growth of the control. Fungal isolates were classified as resistant if colony growth at the discriminatory concentration was ≥50% compared to the control (Russell, 2004). Each isolate was tested in two trials with three replicates per trial.

### 2.4. Shifts in *C. beticola* sensitivity after the spraying period

To observe the sensitivity shift in the *C. beticola* population following the application of fungicides during the test season, we sampled approximately 130–150 isolates from the control and treated plots in 2010 and 2011. An initial sampling was conducted prior to the spraying, when spots first appeared on the leaves in the field. Two weeks after the last spraying, leaves were collected from plots that were treated with thiophanate methyl, thiophanate-methyl/epoxiconazole, carbendazim, flutriafol, flutriafol/chlorothalonil, epoxiconazole, epoxiconazole/carbendazim, or chlorothalonil, in addition to the control plot. Isolates of *C. beticola* could not be sampled from plots that were treated with trifloxystrobin/cyproconazole because the level of infection was very low, and there were no active leaf spots that were suitable for pathogen isolation. All of the sampled isolates were included in the sensitivity test using their corresponding discriminatory concentrations, as described in 2.3. We used a Chi-square test to determine the significance of changes in resistance frequencies (STATISTICA ver. 8.0).

## 3. Results

### 3.1. Efficacy of fungicides in control of CLS

During all four years of the study, environmental conditions were favorable for the development of CLS epidemics, and a threshold was reached at the beginning of July in each year. Analysis of variance indicated that significant differences were present between the efficacies of the treatments over the four-year study period ($F = 171.1$; $p < 0.0001$). Over the course of the study period, variations in disease severity in the experiments that used MBCs were not significant at either of the two localities and had coefficients of variation in a range of 2.51%–6.85%. According to the AUDPC, the disease incidence following treatments with thiophanate methyl (THM) and carbendazim (CAR) corresponded to the incidence that was reported for the untreated control (Table 2). The efficacies of THM and CAR ranged from 0.8 to 5% and 1.2 to 6%, respectively. Conversely, according to a post hoc Duncan's test, the occurrence of pathogens following treatments with DMIs, whether applied singly or in combination, and treatments with the protective fungicide chlorothalonil (CHL) varied substantially in comparison to the control plots, although not between treatment groups (Table 2). A moderate efficacy (73–84.6%) was detected in the South Banat locality between 2008 and 2011 in trials using flutriafol (FLU) and epoxiconazole (EPO), including when they were in mixtures with MBC fungicides, such as CARB/EPO and THM/EPO. At the Srem locality, the impact of the same treatments, whether applied singly or in combination, was significantly lower (E = 48.4–68.8%). Overall, the protective fungicide CHL, both when applied individually and in combination with FLU/CHL, exhibited continual moderate efficacy in a range of 74.5–82.6% at both of the inspected localities (Table 2). Moreover, at the Srem locality, these fungicides produced higher impact on CLS control compared to treatments with FLU, EPO, CAR/EPO and THM/EPO. The best control of leaf spot disease at both of the localities during the four-year study was obtained in the plots that were treated with a mixture of two fungicides from the DMI and QoI groups, TFC/CPC, which had an efficacy of 99.3–99.6% (Table 2).

An impact of the frequency of resistant isolates on disease management was observed in the field for both groups of fungicides. Analysis of the frequencies of *C. beticola* isolates resistant to MBCs and AUDPC values in the plots treated with CAR and THM showed significant correlation ($r = 0.87$, $p = 0.001$; Fig. 1). An even higher correlation was found between the frequency of isolates resistant to DMIs and AUDPC values in plots treated with FLU and EPO ($r = 0.98$, $p = 0.001$; Fig. 2).

In Table 3, we presented a ratio between the observed and expected efficacy of the mixtures CAR/EPO, THM/EPO and FLU/CHL. From 2008 to 2011, the ratio ranged from 0.75 to 1 in each of the compared treatments with the designated combinations of

**Table 2**
Disease severity in the treated and control plots and the efficacy of the fungicides over the period from 2008 to 2011.

| Active ingredient[a] | 2008 | | | | 2009 | | | | 2010 | | | | 2011 | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Srem | | South Banat | | Srem | | South Banat | | Srem | | South Banat | | Srem | | South Banat | |
| | AUDPC[b] | E%[c] | AUDPC | E% | AUDPC | E% | AUDPC | E% | AUDPC | E% | AUDPC | E% | AUDPC | E% | AUDPC | E% |
| THM | 55.6 a | 5.0 | 55.6 a[d] | 3.6 | 55.5 a | 4.5 | 55.2 a | 1.4 | 49.8 a | 2.0 | 53.0 a | 4.7 | 50.2 a | 0.8 | 53.5 a | 0.9 |
| CAR | 57.3 a | 2.0 | 57.0 a | 1.2 | 56.7 a | 2.4 | 53.7 a | 4.1 | 50.1 a | 1.4 | 52.0 a | 6.5 | 49.8 a | 1.6 | 52.4 a | 3.0 |
| FLU | 19.0 b | 67.5 | 9.8 b | 83.0 | 21.3 b | 63.3 | 10.7 b | 80.9 | 26.2 b | 48.4 | 9.9 b | 82.2 | 23.6 b | 53.4 | 16.2 b | 70.0 |
| EPO | 20.2 b | 65.5 | 9.1 b | 84.2 | 20.3 b | 65.1 | 11.2 b | 80.0 | 25.4 b | 50.0 | 10.1 b | 81.8 | 23.7 b | 53.2 | 15.6 b | 71.1 |
| CAR/EPO | 18.7 b | 68.0 | 9.4 b | 83.7 | 21.2 b | 63.5 | 10.8 b | 80.7 | 25.9 b | 49.0 | 10.0 b | 82.0 | 24.3 b | 52.0 | 15.0 b | 72.2 |
| THM/EPO | 20.0 b | 65.8 | 8.9 b | 84.6 | 20.6 b | 64.5 | 11.3 b | 79.8 | 25.2 b | 50.4 | 9.1 b | 83.6 | 24.0 b | 52.6 | 14.6 b | 73.0 |
| TFS/CPC | 0.4 c | 99.3 | 0.2 c | 99.6 | 0.4 c | 99.3 | 0.4 c | 99.3 | 0.3 c | 99.4 | 0.3 c | 99.5 | 0.3 c | 99.4 | 0.3 c | 99.4 |
| CHL | 13.2 b | 77.4 | 11.9 b | 79.4 | 13.3 b | 77.1 | 12.4 b | 76.2 | 12.1 b | 76.2 | 12.0 b | 78.4 | 12.9 b | 74.5 | 13.0 b | 75.9 |
| FLU/CHL | 12.9 b | 77.9 | 10.3 b | 82.1 | 10.1 b | 82.6 | 12.0 b | 78.6 | 10.6 b | 79.1 | 11.9 b | 78.6 | 12.1 b | 76.1 | 10.6 b | 80.4 |
| control | 58.5 a | – | 57.7 a | – | 58.1 a | – | 56.0 a | – | 50.8 a | – | 55.6 a | – | 50.6 a | – | 54.0 a | – |

[a] THM, thiophanate methyl; CAR, carbendazim; FLU, flutriafol; EPO, epoxiconazole; TFS, trifloxystrobin; CPC, cyproconazole; CHL, chlorothalonil.
[b] AUDPC values were calculated according to Wolf and Verreet (2002).
[c] Efficacy of fungicides expressed in percentages and calculated as (disease severity in control - disease severity in treated plot)/disease severity in control x 100.
[d] Means followed by the same letter do not significantly differ according to Duncan's multiple range test at P = 0.01.

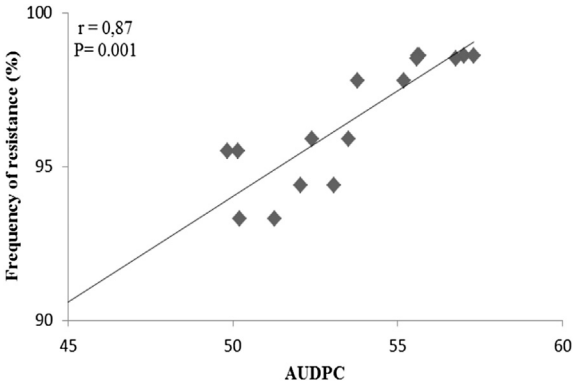


**Fig. 1.** Correlation analysis between the frequency of *C. beticola* isolates resistant to MBCs and AUDPC values in the treatment plot exposed to carbendazim and thiophanate methyl.

fungicides at both of the localities. Ratio values that are between 0.5 and 1.5 indicate that an additive interaction occurred between the components within all three of the mixtures.

### 3.2. Frequency of C. beticola resistance to MBC and DMI fungicides

The frequencies of isolates resistant to MBCs were extremely high during all four years of the study (93.3%–98.6%) and exhibited very limited annual variation ($CV_{Srem}$ = 2.41%; $CV_{South\ Banat}$ = 2.23%) (Fig. 3). At the South Banat locality, the frequencies of isolates resistant to DMI fungicides exhibited a growing trend that continually rose from 6.2% in 2008 up to 18.7% in 2011 ($CV_{South\ Banat}$ = 51.92%). The frequencies of DMI resistant isolates at the Srem locality were substantially higher than in South Banat, ranging from 30.5% to 42.4% with low annual variation ($CV_{Srem}$ = 15.32%) (Fig. 4).

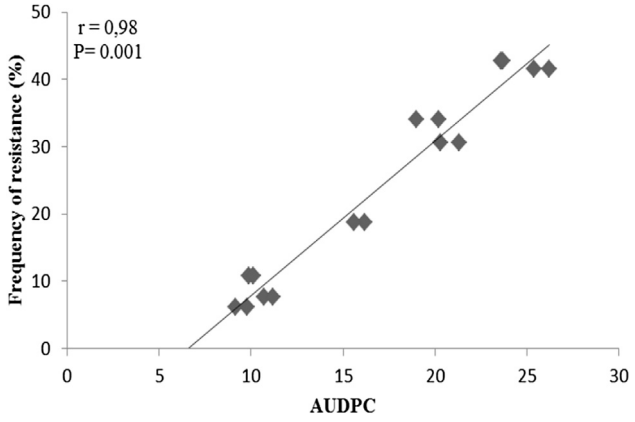


**Fig. 2.** Correlation analysis between the frequency of *C. beticola* isolates resistant to DMIs and AUDPC values in the treatment plot exposed to flutriafol and epoxiconazole.

### 3.3. Shift in C. beticola sensitivity to MBCs and DMIs

No significant differences were determined in the shift of *C. beticola* sensitivity to MBCs between 2010 and 2011 at either of the two localities ($\chi^2_{Srem}$ = 0.52; $\chi^2_{South\ Banat}$ = 0.36; p = 0.001). Overall, the frequency of resistant isolates in control plots was determined to range from 93.9 to 100% (Fig. 5). The frequencies in the treated plots prior to the application of fungicides (93.3–98.6%) were not significantly different from the control. After the treatments were applied, the sensitivity test indicated that the estimated frequencies of MBC-resistant isolates ranged from 94.1 to 100% at both of the inspected localities; however, no significant differences were observed regardless of whether the fungicides were applied individually or as mixtures (Fig. 5).

Unlike MBCs, a significant shift in *C. beticola* sensitivity to DMIs was reported between 2010 and 2011 ($\chi^2_{Srem}$ = 4.49; $\chi^2_{South\ Banat}$ = 20.97; p = 0.01). At the Srem locality, the frequency of resistant isolates did not vary significantly between control (40.8–44.1%) and test plots prior to spraying or between control plots and plots treated with MBCs and chlorothalonil as single formulations (37.5–42%) (Fig. 6). Conversely, the frequency of resistant isolates substantially increased after three consecutive treatments with FLU, EPO and a mixture of CAR/EPO, THM/EPO and FLU/CHL (72.6–88.8%) (Fig. 6). This same trend of increasing resistance was reported in plots treated with DMIs either individually or in mixtures with either MBC or chlorothalonil (48.6–62.5%) at the South Banat locality over both years.

## 4. Discussion

Considering that *C. beticola* spreads rapidly because it undergoes numerous cycles during the growing season, producers are forced to apply fungicides several times a year. The frequent use of fungicides has consequently increased the chances that resistant populations will develop, which led to an eventual reduction of their efficacy (Karaoglanidis et al., 2003). Moreover, farmers who are faced with problems arising from resistance tend to continue to

**Table 3**
Expected and observed efficacies of the fungicide mixtures.

| Active ingredient[a] | 2008 | | | | 2009 | | | | 2010 | | | | 2011 | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Srem | | South Banat | | Srem | | South Banat | | Srem | | South Banat | | Srem | | South Banat | |
| | $\%C_{exp}$[b] | Ratio[c] | $\%C_{exp}$ | Ratio | $\%C_{exp}$ | Ratio | $\%C_{exp}$ | Ratio | $\%C_{exp}$ | Ratio | $\%C_{exp}$ | Ratio | $\%C_{exp}$ | Ratio | $\%C_{exp}$ | Ratio |
| CAR/EPO | 66.2 | 1.0 | 84.4 | 0.99 | 65.9 | 0.96 | 80.8 | 1.0 | 50.7 | 0.97 | 83.0 | 0.99 | 53.9 | 0.96 | 72.0 | 1.0 |
| THM/EPO | 67.2 | 0.98 | 84.8 | 1.0 | 66.7 | 0.97 | 80.3 | 0.99 | 51.0 | 0.99 | 82.7 | 1.0 | 53.6 | 0.98 | 71.4 | 1.0 |
| FLU/CHL | 92.6 | 0.75 | 96.5 | 0.85 | 91.6 | 0.90 | 95.4 | 0.78 | 87.7 | 0.90 | 96.2 | 0.82 | 88.1 | 0.86 | 92.8 | 0.87 |

[a] THM, thiophanate methyl; CAR, carbendazim; FLU, flutriafol; EPO, epoxiconazole; TFS, trifloxystrobin; CHL, chlorothalonil.
[b] $\%C_{exp}$ expected efficacy of each mixture.
[c] Ratio between the experimentally observed efficacy and the expected efficacy of each mixture (if <0.5 antagonistic interaction; if 0.5–1.5 additive interaction; if >1.5 synergistic interaction).

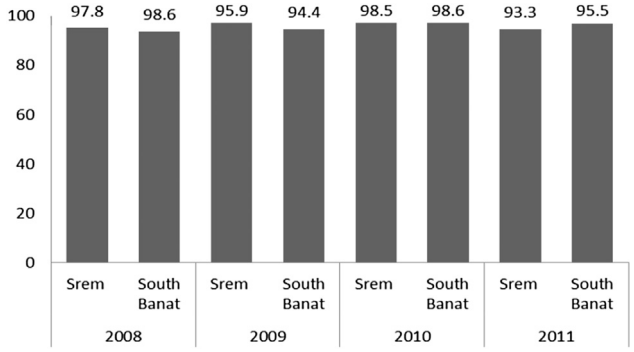


**Fig. 3.** Frequencies of *C. beticola* isolates resistant to carbendazim and thiophanate-methyl from 2008 to 2011.

use the same fungicides as previously, only in higher dosages or more frequently, which can negatively affect the success of disease management (Ishii, 2006). For this reason, monitoring *C. beticola* populations in terms of resistance to fungicides in the field is of vital importance for the development of a CLS management strategy.

MBCs were the first systemic fungicides that were available to control CLS in Serbia and their use intensified rather quickly due to their excellent protective and curative abilities; however, the first problems with resistance were reported several years later (Marić, 1976). A dramatic increase in *C. beticola* resistance to MBCs over a short period of time was found to be triggered by a single nucleotide mutation in the β-tubulin gene (Trkulja et al., 2013). Consequently, the use of MBCs as single formulations declined over the years, and attention instead focused on their application in mixtures with other fungicides, mainly DMIs. Four-year-long field tests in Serbia indicated that the efficacy of carbendazim and thiophanate methyl did not differ significantly from control plots at

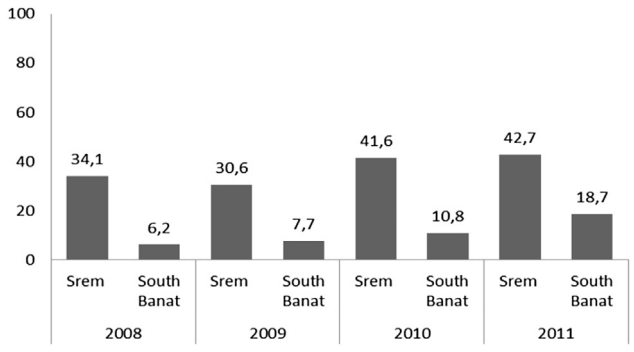


**Fig. 4.** Frequencies of *C. beticola* isolates resistant to flutriafol and epoxiconazole fungicides from 2008 to 2011.

either of the localities. These two fungicides possessed a much greater impact when in mixtures with epoxiconazole; the ratios between observed and expected efficacies for these mixtures suggests that an additive interaction exists between them. However, treatment with epoxiconazole alone was much more efficient, indicating that DMIs are responsible for the efficacies of mixtures of MBCs and DMIs.

According to van den Bosch and Gilligan (2008), resistant strains of fungi persist and will dominate fungal populations if the fitness cost of resistance to fungicides is smaller than fungicide efficacy and if the coexistence of sensitive and resistant strains is not possible. This study revealed that a very high frequency of *C. beticola* populations that are resistant to MBCs naturally persisted at both of the study localities, although MBCs have been rarely applied over the past two decades and are mainly used in mixtures with DMIs. A persistence of MBC resistance is typical for benzimidazoles, and this phenomenon is presumably influenced by high fitness and a competitive ability of resistant versus sensitive populations, which results in the predominance of resistant populations that persist even after MBCs are excluded from the control programs (Dovas et al., 1976).

According to van den Bosch et al. (2011), the development of fungicide resistance is a gradual process that occurs through the following phases: i) “emergence”, when the resistant strain arises through mutation and invasion; ii) “selection”, when the resistant strain is present in the pathogen population and the proportion of the pathogen population that is carrying resistance increases due to selective pressure imposed by fungicides; and iii) “adjustment”, when the resistant fraction of the pathogen population has become large enough to warrant the application of mixtures or high doses of fungicides to adjust for resistance. The very high frequency of MBC-resistant *C. beticola* populations in Serbia represents a practical example in which all three of the above phases occurred during the evolution of resistance. Consequently, the resistant populations completely dominate and persist in the field, rendering MBCs ineffective at controlling *C. beticola*. In addition, recent studies have shown a high frequency of resistant populations in beet root crops in Serbia that were never treated with MBCs and in fact were situated several hundred kilometers away from the nearest treated sugar beet fields (Trkulja et al., 2012), indicating the tendency of resistant populations to spread to surrounding sites when exposed to long-term selection pressure.

The frequency of *C. beticola* isolates that were resistant to DMI fungicides at the Srem locality was high during all four years of the study. Conversely, in South Banat, the frequency of resistance gradually increased, consequently reducing efficacy in plots treated with DMIs and with DMI mixtures with MBCs. This study did not find a significant effect in terms of slowing down the evolution of DMI resistance when DMIs were used in combinations with MBCs or chlorothalonil. At both of the localities, the highest impact in the test was reported for the mixture of DMI and QoI.


**Fig. 5.** Frequency of MBC fungicide resistance determined at the Srem and South Banat localities (2010–2011). The bars labeled as "before" correspond to samples that were taken prior to the spraying period. The "control" bars indicate that fungicides were not applied. The "carb" bars represent treatment with carbendazim; "thioph" represents thiophanate methyl; "flutr" represents flutriafol; "epo" represents epoxiconazole; "carb/epo" represents carbendazim/epoxiconazole; "thioph/epo" represents thiophanat/epoxiconazole; "chlot" represents chlorothalonil; and "flutr/chlot" represents flutriafol/chlorothalonil.

Over the past 30 years, the control of *C. beticola* in Serbia has been based on the use of DMI fungicides; up to four treatments can be applied per season, producing a continual selective pressure in terms of pathogen sensitivity. However, in South Banat, where DMIs have been applied for many years, the frequency of resistant populations was determined to range from 6.2% to 18.7%, suggesting their presumably low degree of fitness. In previous studies, it has been reported that *C. beticola* strains that are resistant to DMIs are less competitive than naturally susceptible isolates (Karaoglanidis et al., 2001; Moretti et al., 2003).

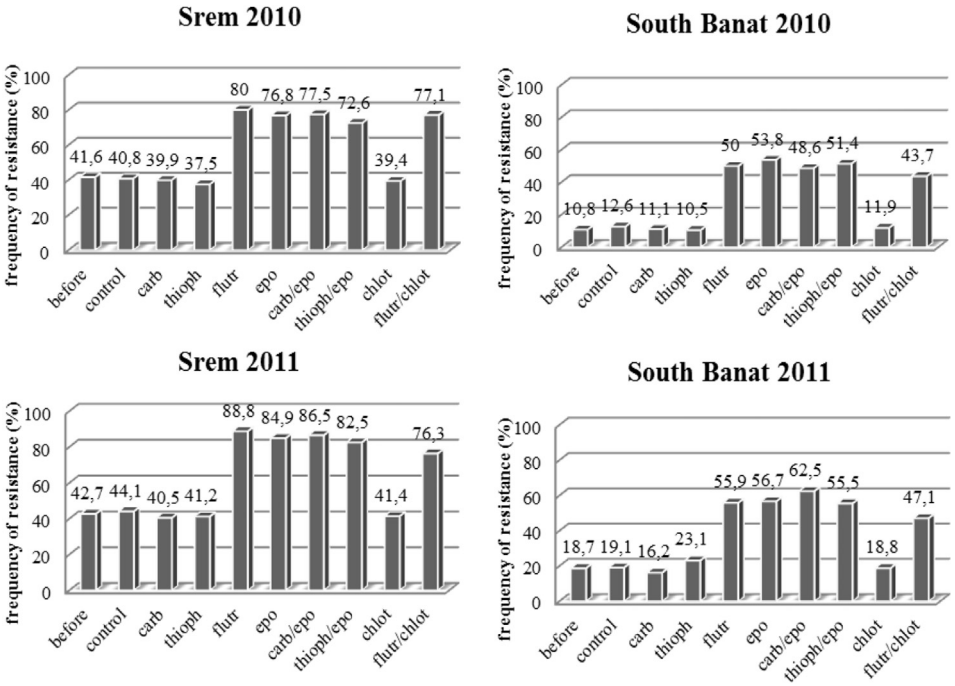


**Fig. 6.** Frequency of resistance to DMI fungicides determined on localities Srem and South Banat (2010–2011). Before – samples were taken before spraying period; control (without applying fungicides); carb– treated with carbendazim; thioph–thiophanate methyl; flutr–flutriafol; epo–epoxiconazole; carb/epo–carbendazim/epoxiconazole; thioph/epo–thiophanat/epoxiconazole; chlot– chlorothalonil; flutr/chlot–flutriafol/chlorothalonil.

DMIs belong to a group of fungicides that possess a medium risk of resistance (FRAC Code List, 2013), one of the reasons of which is the polygenic control of resistance and the low fitness of resistant populations in relation to sensitive populations. Under selective pressure, populations with reduced sensitivity to DMI fungicides will gradually enlarge, which can substantially reduce the control of leaf spot disease (Karaoglanidis and Ioannidis, 2010). In our study, sensitivity shift trials revealed that DMIs possess a very strong impact on the evolution of resistance in the field, which led to reductions in their efficacies. At both of the inspected localities, we observed a growing trend of resistant populations over the spraying period regardless of the initial frequency of resistant isolates in the field. Similar annual dynamics in resistance development corresponding to the field results that were obtained in Serbia have also been reported in Greece by Karaoglanidis et al. (2002). At the beginning of the vegetative season, they also reported a low density of resistant populations; however, by the end of the spraying season, which employed DMIs, resistant populations predominated the field. In the following seasons, the same authors reported a significant decline in the frequency of resistant populations in the field in spring, which they concluded was due to low fitness in resistant strains that rendered them unable to adapt to conditions during hibernation.

The development and frequency of resistance in the field imposes the need to choose proper fungicides to serve as suitable components in fungicide mixtures. The application of a systemic fungicide in combination with a partner fungicide that has a different mode of action has been suggested as a strategy for delaying the appearance of resistant strains (Edgington et al., 1980). DMIs are often used in mixtures with protective fungicides, such as maneb or chlorothalonil, to postpone or avoid the development of resistance. However, after years of treatments, these fungicides have reduced the frequency of sensitive populations in a number of fungal species, including *C. beticola* (Karaoglanidis et al., 2001). A mixture of DMI and QoI fungicides is very effective at controlling *C. beticola* and might serve as an exceptional solution at localities with high frequencies of resistant populations. Mikaberidze et al. (2013) proposed that if fitness costs are absent, then the use of high-risk fungicides in a mixture selects for resistance, and the fungicides eventually become non-functional. If there is a cost of resistance, then a mixture harboring an optimal ratio of fungicides can be developed, in which selection for resistance is expected to vanish and the level of disease control can be optimized.

The protective fungicides expressed limited efficacy in suppressing CLS during the tested seasons and in regions with favorable conditions for fungal development. However, the results of the field trials that were conducted in Serbia suggest that mixtures of DMIs and QoIs are able to provide a high level of crop protection. In addition, no *C. beticola* populations with dual resistance to DMIs and QoI fungicides have been reported to date. However, we must still take into consideration the assumptions made by Luo and Schnabel (2008) that selection for DMI resistance may simultaneously lead to increased selection for QoI resistance. Additionally, monitoring fungicide sensitivity in the case of *Monilinia fructicola* indicated that rotations of DMIs and QoIs did not reduce the frequency of resistant populations to either of the fungicide groups (Schnabel et al., 2012).

The occurrence of *C. beticola* populations that are resistant to MBCs in high densities and the presence of significant variations in resistance to DMIs clearly indicate the need for a continuous and extensive monitoring program of fungicide resistance in different regions with variable selective pressures. With accurate data on the presence and prevalence of resistant populations, chemical treatment programs can be adjusted to improve fungicide efficacy and to adequately apply anti-resistant strategies in the future.

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