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In vitro Sensitivity of *Fusarium graminearum* (Schwabe) to Difenoconazole, Prothioconazole and Thiophanate-Methyl

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SUMMARY

A survey of *in vitro* sensitivity of eight isolates of the *Fusarium graminearum* to the fungicides difenoconazole, prothioconazole and thiophanate-methyl, was undertaken. The isolates were isolated from infected wheat heads collected from 8 different localities in Serbia over the period 2005-2006. Among the tested isolates ZE (RF=11.34), RU (RF=5.98) and AR (RF=5.08) isolates showed moderate resistance to difenoconazole. The EC_{50} values of this isolates were 19.16, 10.09, and 8.59 mg l^{-1}, respectively. The isolates ZE (RF=5.38) and RU (RF=4.43) also showed the moderate resistance to prothoconazole. The EC_{50} values were 9.69 and 7.97 mg l^{-1}, respectively. All the isolates tested were the least sensitive to thiophanate-methyl. The EC_{50} values were in range of 12.1 to 64.03 mg l^{-1}. The highest resistance factor was found for the isolates ZE (RF=5.29).

Keywords: *Fusarium graminearum*; Antifungal activity; Difenoconazole; Prothioconazole; Thiophanate-methyl

INTRODUCTION

Fusarium graminearum Schwade [teleomorph: *Gibberella zeae* (Schweinitz) Petch] is one of the main pathogens causing Fusarium head blight (FHB) on wheat and other cereals. FHB is economically important worldwide, being responsible for extensive damage to wheat in humid and semi-humid regions of the world (Bai and Shaner, 1994; Chen and Zhou, 2009). In recent years the incidence and severity of the disease have increased in Serbia and can be explained mainly because by climatic conditions, i.e. high spring precipitation during the april and may at the blossom stage, growing the susceptible cultivars, and changing agriculture practices that favor retention of crop residues and consequently increase the survival of the FHB agents.

Fungicide sprays have been used to control FHB during the last few decades as only a few cultivars

with effective resistance to this disease are available (Chen and Zhou, 2009). After being first introduced in the late 1960s, the MBC fungcides (**M**ethyl **B**enzimidazole **C**arbamates) i.e. benomyl, carbendazim and thiophanate-methyl, provided excellent control of many phytopathogenic fungi, including FHB (Delp, 1987). After several years of intensive use it was found that a selection of resistant strains of target fungi reduced the usefulness of these compounds (Smith, 1988).

Fungicides containing triazoles as active ingredients are the most effective plant protection agents against Fusarium species at present (Mesterhazy et al., 2003). Difenoconazole and prothioconazole are the widely used fungicides from triazole group. Their mode of action is the inhibition of 14α demethylase, an enzyme that is essential for biosynthesis of ergosterol (Buchenauer, 1987). Fungicides inhibiting this target site are commonly denominated as demethylation inhibitors or DMIs (Kuck and Mehl, 2004).

Difenocozole was launched in 1989, whereas prothiconazole was introduced into the market in 2004. Difenoconazole, like the other members of DMI fungicides has a wide spectrum of activity for the control of plant pathogenic fungi, including different species of *Fusarium* (Suty-Heinze and Dutzmann, 2004). The disease spectrum controlled by prothioconazole in wheat covers leaf spot diseases such as a Septoria leaf spot (*Septoria tritici*), and tan spot (*Drechslera tritici-repentis*) as well as rust (*Puccinia triticina*) and powdery mildew (*Blumeria graminis* f.sp. *tritici*) which are also within the spectrum of activity of the product. Further, prothioconazole is one of the rare triazoles providing excellent protection against FHB (Krämer and Schirmer, 2007).

Resistance to DMI fungicides has been well characterized during the last 20 years. Problems with DMI performance typically became obvious only after several years of intensive use with efficacy degrading stepwise. The mechanism of resistance is mostly controlled by the accumulation of several independent mutations and is generally referred to as "continuous selection", "quantitative resistance" or "shifting" (Anonymous, 2010). In cross resistance studies prothioconazole revealed a unique profile as far as no positive cross resistance to either triazole-resistant or to prochloraz-resistant isolates could be detected (Kuck and Mehl, 2004).

The objectives of this study were to test the sensitivity of eight *Fusarium graminearum* isolates, collected in Serbia over the period 2005-2006, to the fungicides difenoconazole, prothioconazole and thiophanate-methyl.

MATERIAL AND METHODS

Fusarium isolates

Isolates of *F. graminearum* were isolated from infected wheat heads collected from eight different localities in Serbia in 2005 and 2006 (Table 1). Small fragments of diseased wheat heads were placed on wet sterilized filter paper in Petri dishes. After incubation (at 25±1°C for 7 days) mycelium was transferred to fresh PDA medium to obtain a pure culture. The identity of isolates as *F. graminearum* was confirmed by their morphological traits according to Christensen and Nelson (1971) and Levic (2008). The isolates are kept on PDA at 5°C in the culture collection of the Institute of Pesticides and Environmental Protection.

Fungicides

Commercial formulations of the three widely used fungicides for control of *F. graminearum* namely difenoconazole (Score 250-EC, 250 g l^{-1}, Syngenta-Agro), prothioconazole (Proline 250 EC, 250 g l^{-1}, Bayer CropScience), and thiophanate-methyl (Funomil, WP, 700 g kg^{-1}, Agromarket) were tested in this study. Freshly-made stock solutions were prepared to give specific concentrations of active ingredient in mg l^{-1}. Volumes of stock solution were added to molten (50°C) sterile culture media prior to pouring, producing active ingredient concentrations ranging from 1.5625 to 200 mg l^{-1} (Leroux and Gredt, 1972; Locher and Lorenz, 1991).

Table 1. *Fusarium graminearum* isolates

Code of isolate	Origin	Year of isolation
RŠ3	Rimski Šančevi (Novi Sad)	2006
ZE	Zeoke (Lazarevac)	2006
Pom	Požarevac	2005
AR	Arilje	2005
RU	Ruma	2006
BJM	Bački Jarak	2006
Č	Čačak	2005
TiM	Titel	2005

Fungicide sensitivity and EC_{50} assays

F. graminearum isolates grown on PDA medium amended with the fungicides: difenoconazole, prothioconazole and thiophanate-methyl, were used for sensitivity tests. Preliminary concentrations of all selected fungicides were: 1000, 100, 10, 1, 0.1, and 0.01 mg l^{-1}. Based on the results obtained, the selected concentrations of difenoconazole and prothioconazole for further study were: 1.5625, 3.125, 6.25, 12.5, 25 and 50 mg l^{-1}; thiophanate-methyl: 12.5, 25, 50, 100 and 200 mg l^{-1}. Each plate was inoculated with an inverted mycelium agar disc (10 mm), taken from the edge of seven day-old cultures of *F. graminearum* isolates, placed centrally onto the fungicide-amended and fungicide-free media and incubated at 20°C. Three replicates per treatment were used. Colony diameter was measured after seven days of cultivation. Mycelial growth on the fungicide-amended media was measured as a percentage against control. The EC_{50} (fungicide concentration which inhibits mycelial growth by 50%) was determined for each isolate and data on fungicide concentration and relative inhibition were analysed using probit analysis, according to Finney (1971).

The resistance factor (RF) was expressed as the ratio of the EC_{50} and the lowest EC_{50} of the isolates tested (Gouot, 1994). The level of resistance factor (RF) was expressed according to following scale (Gouot, 1994):

- RF < 3 – sensitive isolates;
- RF = 3 – 20 > – moderate resistant isolates;
- RF = 100 > – high resistant isolates.

RESULTS

Antifungal activity of difenoconazole, prothioconazole and thiophanate-methyl

Among eight field-collected *F. graminarum* isolates tested for sensitivity to the difenaconazole, the ZE (RF=11.34) (Figure 2), RU (RF=5.98) and AR (RF=5.08) isolates showed moderate resistance. The EC_{50} values of this isolates were 19.16, 10.09, and 8.59 mg l^{-1}, respectively. The RŠ3 isolate had the lowest EC_{50} value (1.69 mg l^{-1}) (Figure 1). The EC_{50} values of the Pom, BJM, Č and TiM isolates were between 2.43 and 4.46 mg l^{-1} (Table 2).

Table 2. *In vitro* sensitivity of *F. graminearum* isolates to difenoconazole

Code of isolate	Difenoconazole					
	EC_{50} (mg l^{-1})		b		H	RF
	Value	Range*	Value	Range*		
RŠ3	1.69	0.05-4.77	0.49	0.35-0.64	1.82	1
ZE	19.16	14.19-24.9	0.99	0.84-1.14	1.73	11.34
Pom	3.48	0.43-7.45	0.54	0.39-0.67	0.86	2.06
AR	8.59	5.68-11.44	1.44	1.29-1.59	1.47	5.08
RU	10.09	6.83-13.33	1.09	0.95-1.24	0.31	5.98
BJM	2.43	0.27-5.55	0.58	0.44-0.72	0.18	1.44
Č	4.46	1.81-7.28	0.85	0.71-0.99	0.46	2.64
TiM	3.05	0.82-5.73	0.75	0.54-0.97	0.42	1.81

EC_{50} – Fungicide concentration which inhibits mycelial growth by 50%; RF – The resistance factor was expressed as the ratio of the EC_{50} and the lowest EC_{50} for the isolates tested; b – Regression coefficient;
H – Heterogenity; *95% confidence interval (P=0.05)

The tested *F. graminarum* isolates were capable of good growth at 1.5625 mg l^{-1} prothioconazole concentration, but were severely inhibited at 6.25 mg l^{-1} and above. The isolates ZE (RF=5.38) (Figure 4) and RU (RF=4.43) showed the moderate resistance. The EC_{50} values were 9.69 and 7.97 mg l^{-1}, respectively. Among the tested isolates, the Pom isolate had the lowest value of EC_{50} (EC_{50}=1.8 mg l^{-1}) (Figure 3). The EC_{50} values of the other investigated isolates were betweeen 3.94 and 4.39 mg l^{-1} (Table 3).

Table 3. *In vitro* sensitivity of *F. graminearum* isolates to prothioconazole

Code of isolate	Prothioconazole					
	EC_{50} (mg l^{-1})		b		H	RF
	Value	Range*	Value	Range*		
RŠ3	4.39	3.4-5.31	1.81	1.59-2.03	0.77	2.44
ZE	9.69	8.29-11.23	1.94	1.78-2.09	2.16	5.38
Pom	1.8	1.09-2.5	1.07	0.92-1.22	1.21	1
AR	3.61	2.38-4.81	1.25	1.1-1.4	1.04	2
RU	7.97	6.58-9.46	1.66	1.5-1.82	0.96	4.43
BJM	3.94	3.3-4.62	1.81	1.65-1.98	0.28	2.19
Č	5.24	3.74-6.74	1.22	1.08-1.36	0.24	2.91
TiM	4.19	2.98-5.36	1.37	1.21-1.53	2.48	2.32

EC_{50} – Fungicide concentration which inhibits mycelial growth by 50%; RF – The resistance factor was expressed as the ratio of the EC_{50} and the lowest EC_{50} for the isolates tested; b – Regression coefficient;
H – Heterogenity; *95% confidence interval (P=0.05)

All investigated *F. graminearum* isolates demonstrated ability to tolerate thiophanate-methyl at higher concentrations compare to difenoconazole and prothioconazole. They were capable of growth at 25 mg l^{-1}, but were severely inhibited at 50 mg l^{-1} or above. The highest resistance factor was found for the isolates ZE (RF=5.29) (Figure 6). The isolates TiM (RF=5.2) and AR (RF=4.2) also showed moderate resistance. The EC_{50} values of this isolates were 64.03, 62.92, and 50.84 mg l^{-1}, respectively. The lowest EC_{50} value was found for the isolate Č (12.1 mg l^{-1}) (Figure 5). The EC_{50} of the other tested isolates were between 17.6 and 36.29 mg l^{-1}. (RF=1.91). All of these isolates were considered to be sensitive to thiophanate-methyl.

Table 4. *In vitro* sensitivity of *F. graminearum* isolates to thiophanate-methyl

Code of isolate	Thiophanate-methyl					
	EC_{50} (mg l^{-1})		b		H	RF
	Value	Range*	Value	Range*		
RŠ3	17.6	13.24-21.67	1.64	1.43-1.86	0.21	1.45
ZE	64.03	54.52-74.73	1.99	1.78-2.21	0.79	5.29
Pom	36.29	28.74-43.44	1.91	1.69-2.14	0.91	2.99
AR	50.84	39.36-62.69	1.42	1.22-1.62	1.02	4.2
RU	26.74	20.67-33.15	1.37	1.16-1.59	0.61	2.21
BJM	19.8	14.53-24.49	1.47	1.21-1.62	0.13	1.64
Č	12.1	8.07-15.5	1.64	1.42-1.85	0.21	1
TiM	62.92	53.89-72.96	2.09	1.88-2.31	0.27	5.2

EC_{50} – Fungicide concentration which inhibits mycelial growth by 50%; RF – The resistance factor was expressed as the ratio of the EC_{50} and the lowest EC_{50} for the isolates tested; b – Regression coefficient;
H – Heterogenity; *95% confidence interval (P=0.05)

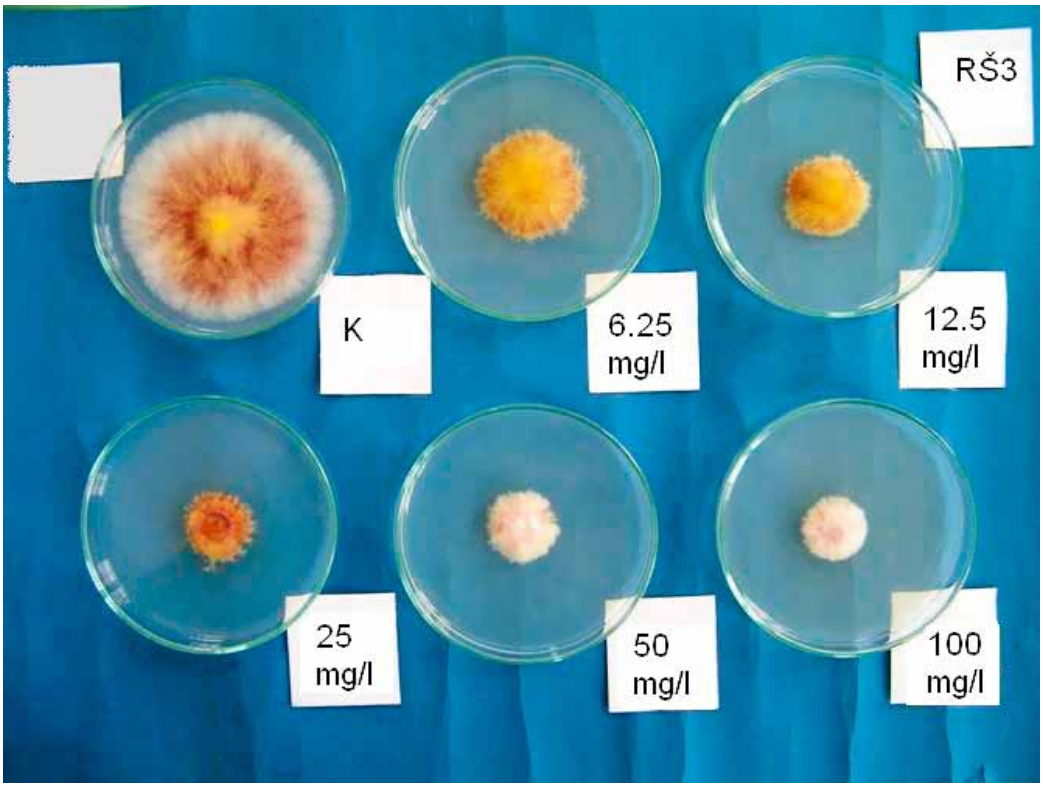

Figure 1. Growth of isolate RŠ3 on PDA amended with difenoconazole

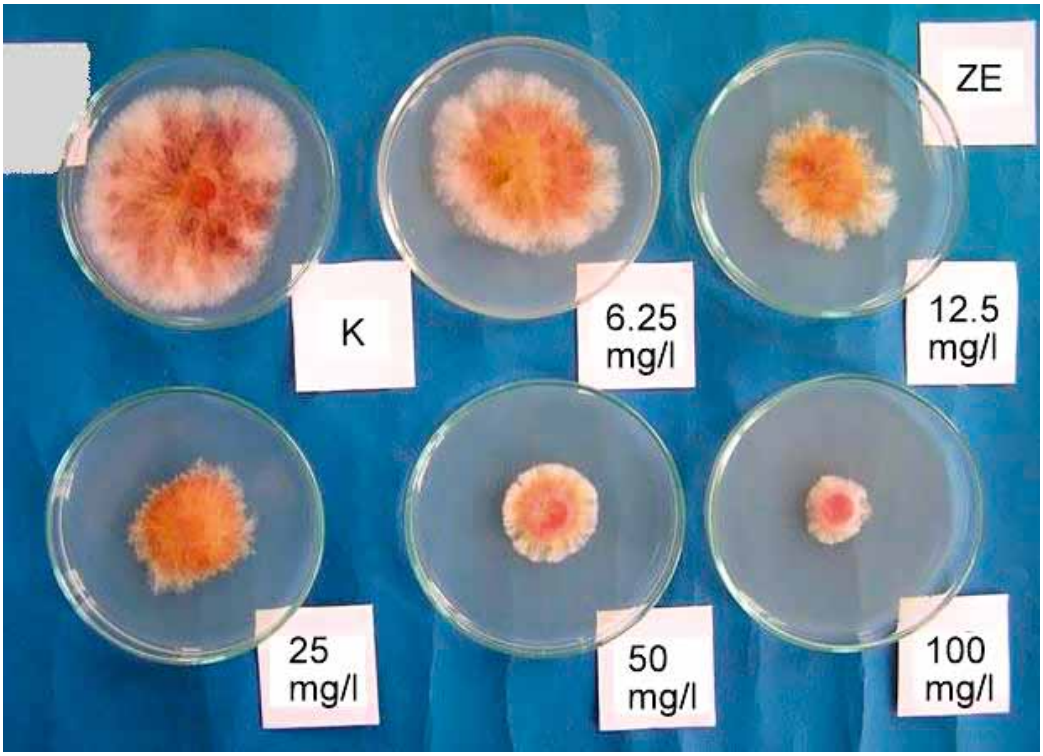

Figure 2. Growth of isolate ZE on PDA amended with difenoconazole

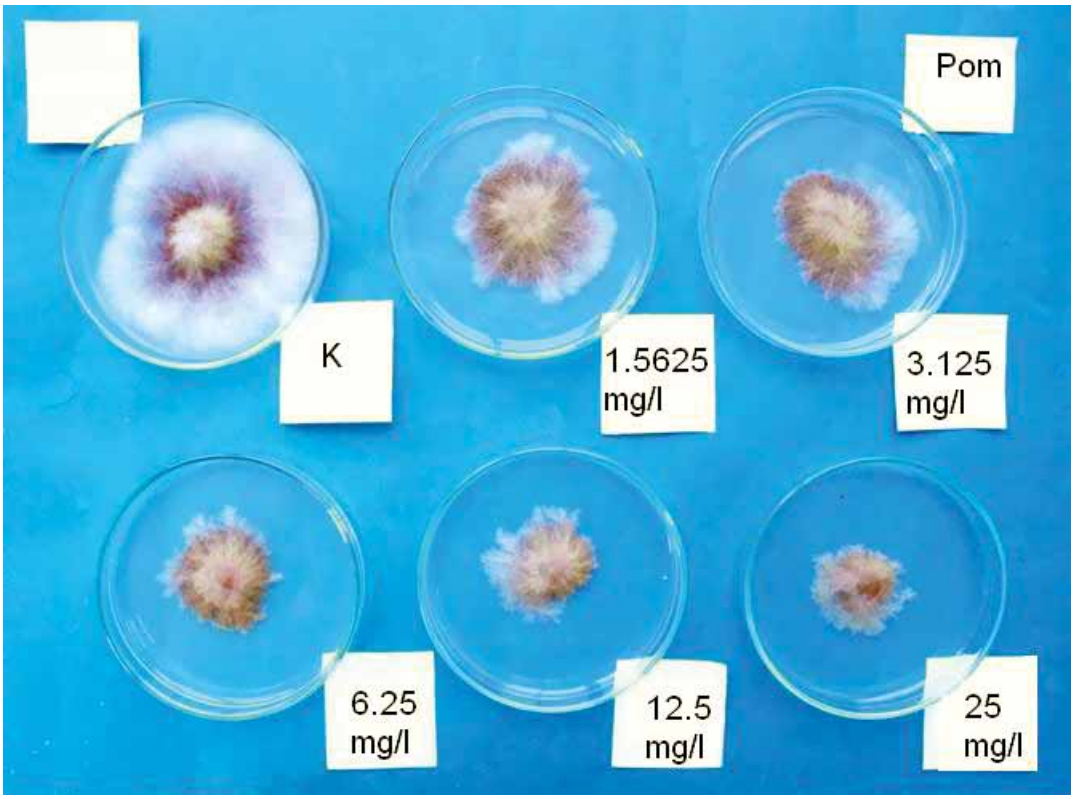

Figure 3. Growth of isolate Pom on PDA amended with prothioconazole

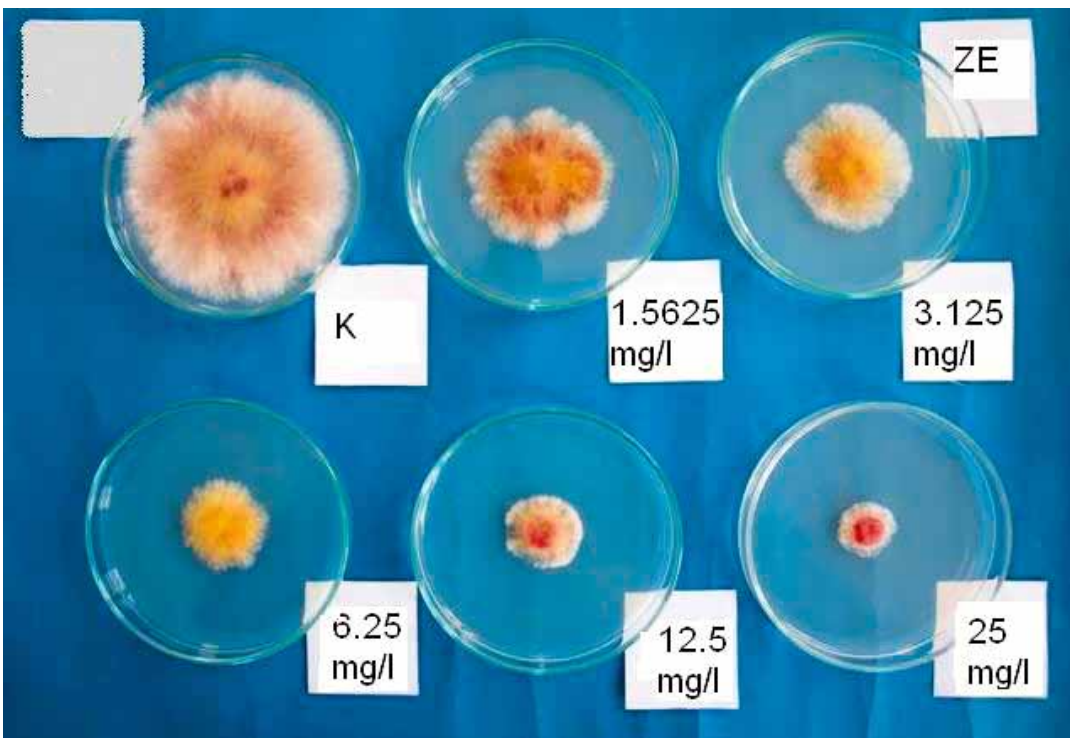

Figure 4. Growth of isolate ZE on PDA amended with prothioconazole

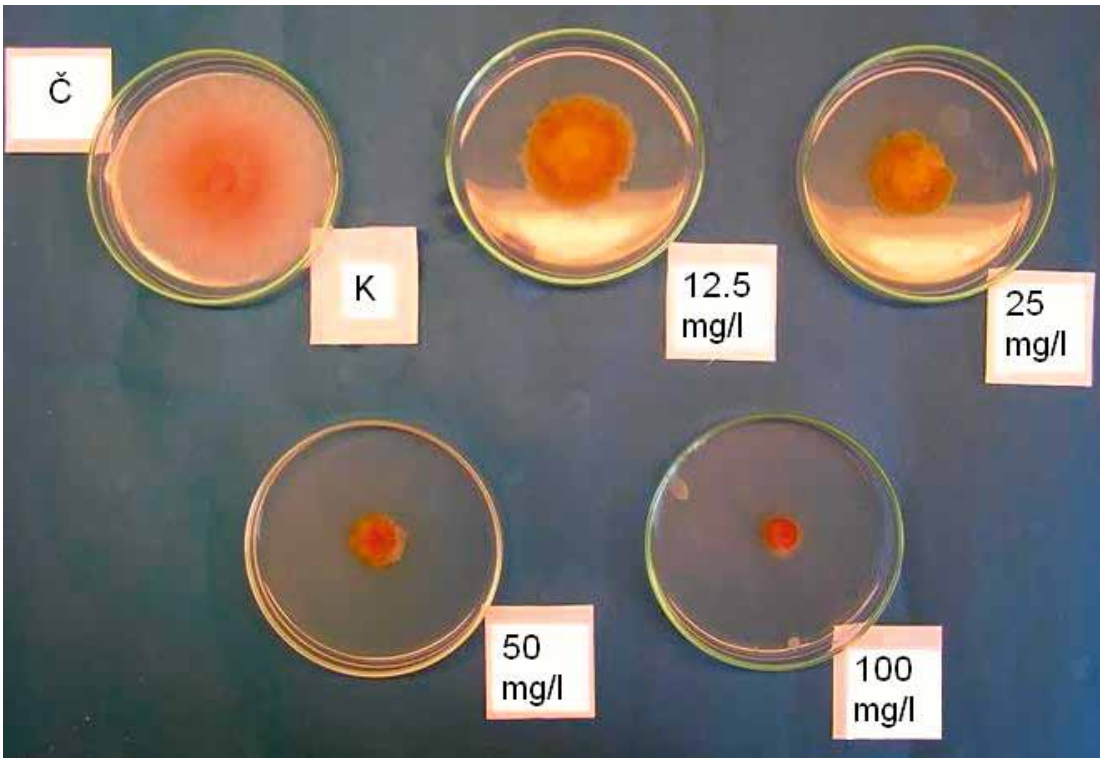

Figure 5. Growth of isolate Č on PDA amended with thiophanate-methyl

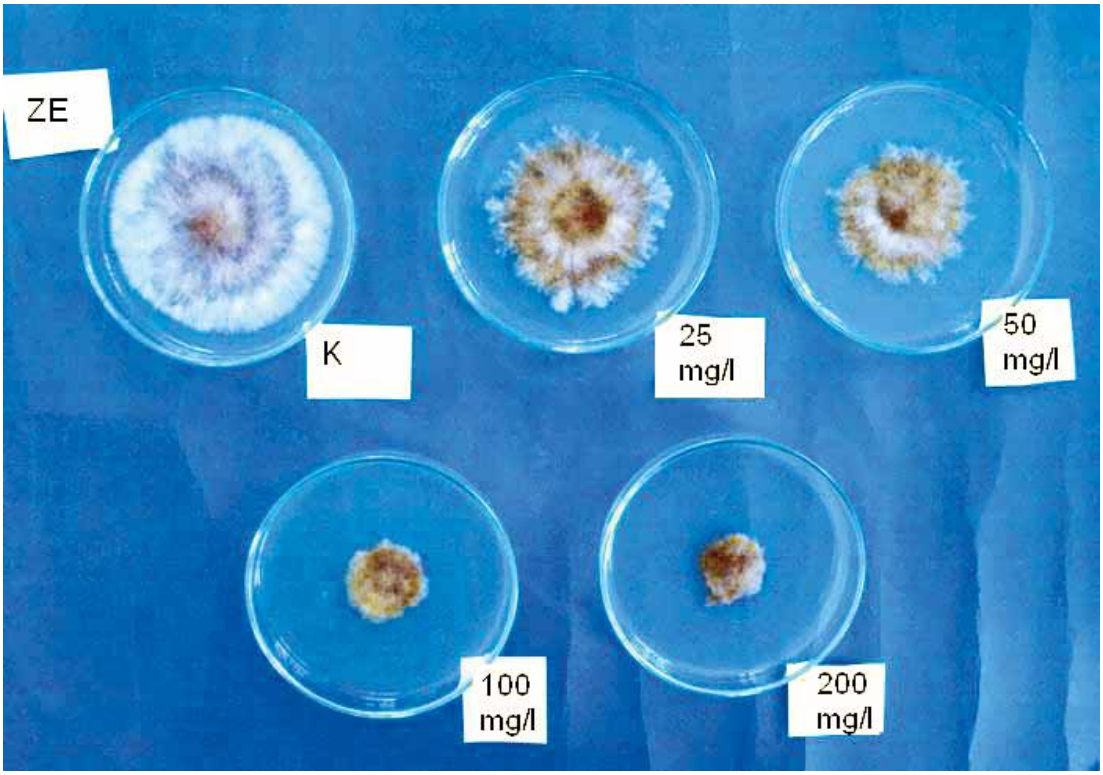

Figure 6. Growth of isolate ZE on PDA amended with thiophanate-methyl

DISCUSSION

Our results show that most *F. graminearum* isolates collected from eight different places in Serbia are sensitive to tested fungicdes. The isolates ZE, RU and AR were showed moderate resistance to DMI fungicides difenoconazole and prothioconazole (RF 4.43-11.34) according to criteria established by Gouot (1994). It does not necessarily imply that resistance will become a problem because the resistance against DMI fungicides is mostly based on the accumulation of several independent mutations (Kuck, 2002). Some resistance mechanisms are known as for example the target site mutation Y136F, amplification of ABC transporters and cell membrane alterations. The mutation of one single gene can lead to a disruptive selection type as it is known from benzimidazoles and strobilurins. If resistance occurs with DMI fungicides it is typically developing stepwise after several years of intensive use (Kuck, 2002; Kuck and Mehl, 2004). This situation is common in Serbia as a consequence of consecutively use of DMI fungicides without implementation of adequate resistance management strategies. In order to prevent resistance development to DMIs, SBI working group (Anonymous, 2010) suggest applying of the following measures: (*i*) limitation of the number of applications per season (using less than the registered number); (*ii*) using DMIs in mixture or alternation systems (or both) with fungicide groups showing no cross-resistance to DMIs; (*iii*) avoiding curative/eradicate use (especially if no warning systems are available); (*iv*) using the manufacturer's recommended dose rate; (*v*) lowering the disease pressure with all tools of integrated disease management.

The obtained values of RF indicate that most isolates obtained from infected wheat heads in Serbia are sensitive to thiophanate-methyl. However, isolates ZE (RF=5.29), TiM (RF=5.2) and AR (RF=4.2) were showed moderate resistance. Also, the obtained EC_{50} values were higher (12.1-64.03 mg l^{-1}) than those recorded for carbendazim (7.02-11.86 μg ml^{-1}) by Chen et al. (2007). Zhou and Wang (2000) were determined that the carbendazim resistance was developed in many fungi after the fungicide had been used for 2-3 years. Chen and Zhou (2008) recorded that the first carbendazim-resistant isolates of *F. graminearum* from the field were detected in the Zhejiang Province of China. The benzimidazole fungicide resistance mechanism has been shown to be a replacement of glutamic acid with either alanine or lysine at codon 198 in most phytopathogenic fungi. However, Chen et al. (2007) recorded that no mutation in beta-tubulin was found in *F. graminearum*.

There are no specific recommendations for MBC fungicides. Both mixtures and alternations are valid strategies to minimize the risk of resistance development. In case of tank-mixtures, the benzimidazole fungicide must be applied at its label dose together with the appropriate dose of an effective, non-cross-resistant partner fungicide (Anonymous, 2010).

The emergance of moderate resistance of *F.garaminearum* isolates from Serbia to thiophanate-methyl, difenoconazole and prothioconazole reduces the value of MBC and DMIs fungicdes in the control of FHB. Our work suggests that monitoring of *F. graminearum* field populations with respect to fungicide resistance is important.

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In vitro osetljivost *Fusarium graminearum* (Schwabe) na difenokonazol, protiokonazol i tiofanat-metil

REZIME

Ispitivana je osetljivost osam izolata *Fusarium graminearum* na difenokonazol, protiokonazol i tiofanat-metil. Izolati su dobijeni iz zaraženih klasova pšenica sakupljenih tokom 2005. i 2006. godine sa osam različitih lokaliteta iz Srbije. Izolati ZE (RF=11,34), RU (RF=5,98) i AR (RF=5,08) su ispoljili umerenu rezistentnost na difenokonazol. Dobijene EC_{50} vrednosti ovih izolata bile su 19,16, 10,09, i 8,59 mg l^{-1}. Izolati ZE (RF=5,38) i RU (RF=4,43) su ispoljili umerenu rezistentnost na protiokonazol. Dobijene EC_{50} vrednosti iznosile su 9,69 i 7,97 mg l^{-1}. Svi ispitivani izolati ispoljili su najmanju osetljivost na tiofanat-metil. Dobijene EC_{50} vrednosti su bile u opsegu od 12,1 do 64,03 mg l^{-1}. Najveći faktor rezistentnosti je utvrđen kod izolata ZE (RF=5,29).

Ključne reči: *Fusarium graminearum*; antifungalna aktivnost; difenokonazol; protiokonazol; tiofanat-metil