Effect of Selected Fungicides on *Fusarium* Growth and Toxins Production

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Abstract

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We examine the effects of selected fungicides on the *Fusarium* growth and toxins production especially of DON and T-2 toxins. Appropriate nutritive media were prepared containing different concentrations of the fungicides tested (Horizon 250 EW and Falcon 460 EC), inoculated with *Fusarium* moulds, and incubated under various conditions. The extraction of *Fusarium* toxins and their determination were carried out after the incubation using Agra Quant®Deoxynivalenol Test kit and Agra Quant® T-2 toxin Test kit. The results indicated a considerable variability of the individual strains in the formation of toxins. The strains of *Fusarium graminearum* CCM F-683 and *Fusarium* spp. (isolated from wheat) produced large quantities of both DON and T-2 toxins. *Fusarium poae* CCM F-584 and *Fusarium sporotrichioides* CCM F-352 always produced larger quantities of only T-2 toxin in the given nutrient media. The experiment showed that the use of lower concentrations of fungicides resulted in an increased production of the monitored mycotoxins. The presence of *Tri5* gene in the tested *Fusarium* strains, coding trichodiene synthase, was confirmed by polymerase chain reaction. The enzyme – trichodiene synthase – catalyses the isomerisation and cyclisation of farnesyl phosphate to trichodiene, the first step in the biosynthetic pathway of trichothecenes.

Keywords: genus *Fusarium*; mycotoxin; deoxynivalenol; T-2 toxin; fungicide; PCR

The fungi of *Fusarium* (*F.*) genus cause human and animal diseases. They are mostly isolated from agricultural crops and decaying plant materials and are producers of a wide range of mycotoxins, which are secondary metabolites of organic compounds produced by fungi. They are chemically diverse with a low molecular weight ranging from 200 to 500 g/mol (Nelson *et al.* 1993). Deoxynivalenol (DON), T-2 toxin (T-2) and zearalenon (ZEN) are among the most commonly obtained and food contaminating *Fusarium* toxins (Nedělník & Moravcová 2005). The exposition to trichothecene toxins can result in liver damage, damage to endocrine and nervous systems, nausea and vomiting. ZEN has been studied due to its estrogenic effects (Creppy 2002). From the toxicity point of view, group B type (DON, nivalenol fusarenon-X) is more severe than A type (ET-2 and T-2 toxin) (Weidenbörner 2001). Ear fusariosis has an adverse effect on the quality of the grain and causes great yield losses. It is mainly caused by *F. avenaceum*, *F. graminearum, F. culmorum* and *F. nivale*. *Fusarium* species infect a majority of the cultivated and wild plants (Chadová 2006). Strategies different, against these diseases are investigated related to the combination of the crops grown in the fertile soil with the use of resistant varieties (Haller *et al.* 2008). Rainy weather promotes fungal infec-

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tion of ears on which pink- or salmon-coloured mycelium can be seen. However, high doses of nitrogen fertilisers or fungicides applied onto the ears can cause slow ripening. After the rainy season the mycelium of pink or salmon colour can be seen on the ears.

The effectiveness of fungicides depends on many factors, e.g. the climatic conditions, type of the product and its active substance and time and method of application (Simpson *et al.* 2001; Heier *et al.* 2005). Their application can reduce the infection (visually evaluated), but may also increase the level of toxins or intervene in the biochemical evolution of the grain, which is reflected on the quantity and quality of the grain (Homdork *et al.* 2000; Havlová *et al*. 2006). Active substances, present in fungicides Eminent 40 EW (tetraconazole), Tiptor S (cyproconazole + prochloraz), Horizon 250 EW (tebuconazole), and Amistar 250 SC (azoxystrobin[b]), significantly reduce the occurrence and severity of cereals infection in comparison with the untreated control group (Haidukowski *et al.* 2005). However Malachová *et al.* (2010) stated in her study that the levels of the toxins observed were higher in chemically treated barley varieties than in those chemically untreated varieties. The European Commission adopted the Regulation (EC) No 1126/2007, which indicates the maximum number of different contaminants allowed in food. Maximum allowed levels are set only for DON, ZEN and fumonisins. For the detection of *Tri5* gene, encoding trichothecene mycotoxins production, the method of polymerase chain reaction is used (PCR). Edwards *et al.* (2001) developed a light PCR method for the quantification of trichothecene mycotoxins based on the primers derived from *Tri5* gene. Various analytical and immunochemical methods are commonly used for the quantitative determination of trichothecene mycotoxins, such as high performance liquid chromatography coupled with mass spectrometry (HPLC/MS), gas chromatography with mass spectrometry GC/MS and thin layer chromatography (TLC). From the immunochemical methods, ELISA (enzyme-linked immunosorbent assay), ICA (immunoaffinity column assays), and RIA (radio immuno assay) methods are often used. Another approach using biosensors has also been published (Pemberton *et al.* 2006).

The aim of this work was to examine the effects of two types of fungicides (Horizon 250 EW and Falcon 460 EC) on the growth of *Fusarium* and its toxins production, especially DON and T-2 toxins.

MATERIAL AND METHODS

Strains of fungi and nutrient media. For the isolation of *Fusarium* mycotoxins the following fungi were used: *Fusarium poae* CCM F-584, *Fusarium sporotrichioides* CCM F-352, *Fusarium graminearum* CCM F-683 (Czech Collection of Microorganisms, Brno, Czech Republic), *Fusarium* spp. isolated from wheat and, as a negative control, *Fusarium* spp. without *Tri5* gene (both from the Collection of Microorganisms of the Department of Biological and Biochemical Sciences, University of Pardubice, Czech Republic).

Malt Agar and Potato Carrot Agar – PCA (all from Himedia, Mumbai, India) were used for the isolation of *Fusarium* mycotoxins and for monitoring the inhibition of *Fusarium* growth by various fungicides with different concentrations.

Procedure. The fungicides Horizon 250 EW and Falcon 460 EC were used to monitor the production of *Fusarium* mycotoxins in the presence of antifungal substances as well as the inhibition of *Fusarium* growth. The monitoring of the *Fusarium* growth inhibition proceeded in the following way. The diameters of the colonies were measured and tha appearence of colonies was observed. The monitoring of the *Fusarium* mycotoxins production in the presence of fungicides was performed as follows: per 1 ml of fungicides solutions of different concentrations (of three concentrations were used both fungicides – H = concentration of the Horizon 250 EW, H c = 1000 ml/ha, H_{500} c = 500 ml/ha, H_{20} c = 20 ml/ha; Fa = concentration of the Falcon 460 EC, Fa c = 600 ml/ha, Fa_{300} c = 300 ml/ha, Fa_{20} c = 20 ml/ha) were added into the nutrient medium – the method of overflow – which was left to solidify. On the nutrient medium thus oreoared, the tested strains of *Fusarium* were inoculated with the respective spores of fungi stabbed three times and cultivated under different conditions (15°C and 25°C for 7 days). The content of the respective Petri dish after cultivation was agitated with 100 ml of acetonitrile/distilled water mixture (84/16) for one hour at room temperature using an agitator at 250 revolutions per minutes. After the filtration of the substrate thus obtained, 1 ml of the filtrate was taken and the solvent was completely removed in a vacuum evaporator. Prior to the actual analysis, the residue after evaporation was dissolved in 1 ml of acetonitrile/distilled water mixture (3:1). *Fusarium* mycotoxins were analysed according to the instructions in Agra

Quant® Deoxynivalenol Test kit (detection limit is 10 ng/g) and Agra Quant® T-2 toxin Test kit (detection limit is 7 ng/g). 200 µl of the conjugate was mixed with 100 µl of sample or 100 µl of standard in blue-coded dilution micro-wells. 100 µl of this mixture was pipetted into antibody-coated micro-wells and incubated (15 min, laboratory temperature). The micro-wells were washed with buffer solution after the incubation. Then, 100 µl of the substrate was added and which was followed by incubation (5 min, laboratory temperature). One hundred µl of the stop solution was added the incubation. The absorption in the micro-wells was measured with a Tecan Sunrice microwell reader using a 450 nm absorbance filter. This experiment was repeated twice.

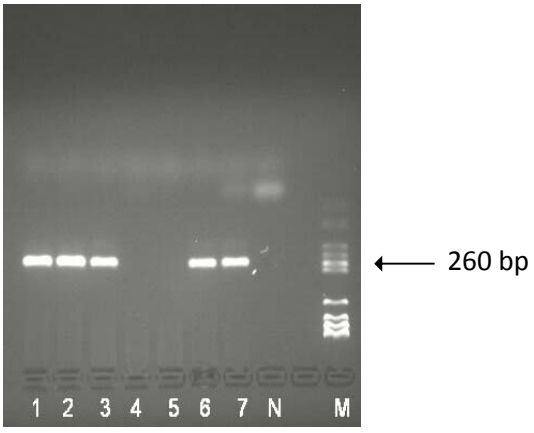
Extraction of DNA. Eppendorf tubes with 500 ml of the potato-glucose broth were inoculated with the selected strains of *Fusarium* fungi and incubated at 25°C for 5 days. After the removal of the broth supernatant (19 460 g, 5 min), the mycelium mass was transferred into a sterile test tube containing 100 µl of the extraction solution and was incubated at 95°C for 10 minutes. DNA was dissolved in 100 µl of the diluent solution and was extracted from the fungal cultures using a commercial kit intended for isolating DNA from plant leaves (REDExtract-N-AmpTMPlant PCR Kit; Sigma, **město?**, USA).

PCR reaction. The DNA segment (*Tri*5 gene) was determined using primers HA Tri/R (5'-GCA CAA GTG CCA CGT GAC-3') and HA Tri/F (5'-CAG ATG GAG AAC TGG ATG GT-3'). The final volume for PCR was 20 µl containing 4 µl of cell lysate, 10 µl of PCR reaction mixture (REDExtract-N-AmpTMPlant PCR Kit; Sigma, St. Louis, USA), 0.25 µl of each primers and 5.5 µl of destilled water. PCR was carried out in a thermal cycler with a temperature program consisting of the following steps. The first one was the initial denaturation (3 min 15 s at 94°C) followed by 35 repeated cycles (1 min at 94°C, 1 min at 61°C, 1 min 45 s at 72°C). The last step was the final extension, which ran for 4 min 15 s at 72°C (Brožková *et al.* 2007). The final product contained 260 bp fragments. DNA marker 155-970 bp (Top-Bio, Prague, Czech Republic) was used. Electrophoresis was performed at 100 V for 50 minutes.

RESULTS AND DISCUSSION

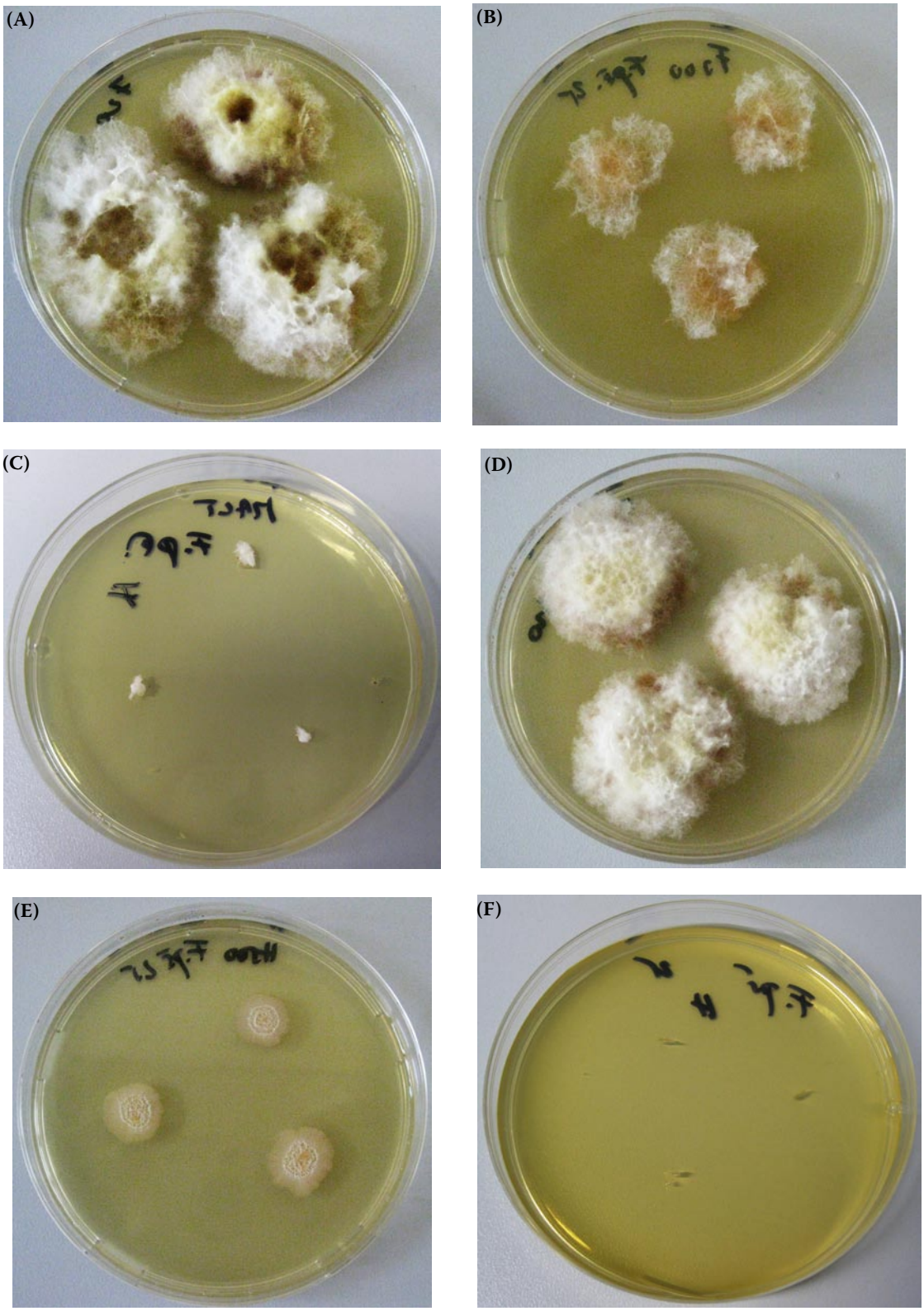
Strains of *Fusarium* in all tested, the presence of *Tri5* gene was confirmed using PCR method; the results are shown in Figure 1.

Production of mycotoxins in nutrient media without the addition of fungicides. On a MALT agar, *Fusarium poae* CCM F-584 and *Fusarium sporotrichioides* CCM F-352 produced the largest amounts of T-2 toxin in the range of 264–320.7 ng/g at both temperatures tested. *Fusarium graminearum* CCM F-683 produced the highest amount of T-2 (67 ng/g) toxin at 15°C on PCA. *Fusarium graminearum* CCM F-683 and *Fusarium* spp. – isolated from wheat – appeared to be the greatest producers of deoxynivalenol (DON) on PCA. *Fusarium graminearum* CCM F-683 produced 430.9 ng/g. *Fusarium* spp. isolated from wheat produced 95.2 ng/g. It was found that maximum quantity of *Fusarium* mycotoxins was produced at 25°C with the exception of *Fusarium graminearum* CCM F-683 with which the greatest production of *Fusarium* mycotoxins was recorded at 15°C. This is consistent with the findings of Weidenbörner (2001) reporting that the optimum temperature for the production of mycotoxins by psychrophilic species may be in the range of 8–15°C.



1 – *Fusarium poae* CCM F-584; 2 – *Fusarium sporotrichioides* CCM F-352; 3 – *Fusarium graminearum* CCM F-683; 4 and 5 – *Fusarium* spp. isolated from a smear of mat; 6 and 7 – *Fusarium* spp. isolated from wheat; N – negative control; M – DNA marker (155–970 bp)

Figure 1. Confirmation of *Tri5* gene in *Fusarium* fungi



Fusarium spp. – isolated from wheat cultivated on MALT agar with fungicide Falcon 460 EC (A) *c* = 20 ml/ha, (B) c = 300 ml/ha, (C) *c* = 600 ml/ha at 25°C and with fungicide Horizon 250 EW (D) *c* = 20 ml/ha, (E) *c* = 500 ml/ha, (F) *c* = 1000 ml /ha at 25°C

Figure 2. Inhibition of *Fusarium* growth by various fungicides with different concentrations

Fungal growth and production of mycotoxins in nutrient media with the addition of fungicides. The monitoring of the inhibition of *Fusarium* growth followed this procedure: the diameters of the colonies were measured and the appearance of colonies was observed. Figure 2 shows the effect of different concentrations of Falcon 460 EC and Horizon 250 EW on the growth of *Fusarium* spp. isolated from wheat and cultivated on MALT agar at 25°C. It was found that Horizon 250 EW had a greater inhibitory effect on the fungal growth.

In our study, we verified the effects of Horizon 250 EW and Falcon 460 EC. The fungicide Horizon 250 EW is specifically aimed at ear fusariosis, and obviously, this is the reason why it is better in preventing the trichothecene mycotoxins formation. Its active ingredient is Tebuconazole from the azole group with the concentration of 250 g/l. Falcon 460 EC is the fungicide with a variety of applications (Bayer CropScievce 2002). Its active ingredients are spiroxamine – from the spirokatelamin group with the concentration of 250 g/l, Tebuconazole – from the azole group (concentration of 167 g/l) and triadimenol – from the azole group (concentration of 43 g/l) (Bayer CropScience 2002). In Table 1, the results of the mycotoxins production by *Fusarium* spp. in the presence of fungicides are listed. The decrease in T-2 and DON toxins production was noticeable by using Horizon 250 EW on *c* = 500 ml/ha. This concentration is half that recommended by the manufacturer.

The fungicide Falcon 460 EC caused a decrease in production of T-2 toxin only by using the highest concentration tested (*c* = 600 ml/ha). To affect the DON production by *Fusarium* spp., it was necessary to use maximum concentration recommended (*c* = 00 ml/ha), while for *Fusarium graminearum* CCM F-683, the effective concentration was 300 ml/ha (this concentration is half that is recommended by the manufacturer).

The efficiency of fungicides depends on the climatic conditions, concentration of fungicides and their composition, the manner and duration of the application, and penetration of the active substance into the tissue (Havlová *et al.* 2006; Bittner 2009; Kostelanská *et al.* 2009). Our

Table 1. The production of *Fusarium* mycotoxins in the presence of fungicide on a MALT agar – sample *Fusarium* spp. isolated from wheat

Concentration*	Culture conditions (7 days)	Quantity of toxin in simple (ng/g)	
		DON	T-2
Positive control	25°C	78.2	9.3
Fa_{20}		363.6	44.6
Fa_{300}		415.7	72.4
Fa		71.6	<
Positive control	15°C	66.1	7.2
Fa_{20}		277.5	62.4
Fa_{300}		368.35	69.6
Fa		<	8.2
Positive control	25°C	78.2	9.3
H_{20}		49.4	71.0
H_{500}		<	60.0
H		<	<
Positive control	15°C	66.1	7.2
H_{20}		335.4	91.1
H_{500}		<	60.4
H		<	<

*Fa – concentration of fungicide Falcon 460 EC; Fa *c* = 600 ml/ha, Fa_{300} *c* = 300 ml/ha, Fa_{20} *c* = 20 ml/ha, H – concentration of fungicide Horizon 250 EW, H *c* = 1000 ml/ha, H_{500} *c* = 500 ml/ha, H_{20} *c* = 20 ml/ha, < less than limit of detection; positive control – *Fusarium* spp. isolated from wheat without fungicide

experiment showed that low concentrations of fungicides resulted in an increased production of mycotoxins (Table 1), which was also confirmed in other study (Heier *et al.* 2005).

CONCLUSION

The results indicate a considerable variability of the individual strains in the toxins formation. The strains of *Fusarium graminearum* CCM F-683 and *Fusarium* spp. (isolated from wheat) produced large quantities of both DON and T-2 toxins in all culture media, while *Fusarium poae* CCM F-584 and *Fusarium sporotrichioides* CCM F-352 always produced larger quantities of only one toxin in the given nutrient media.

By comparing various concentrations of Falcon 460 EC and Horizon 250 EW fungicides on the production of *Fusarium* mycotoxins, it was found that Horizon 250 EW had a greater inhibitory effect. Its active ingredient is tebuconazole from the azole group. As Edwards *et al.* (2001) stated, the fungicide most effective against trichothecene-producing *F. graminearum* was tebuconazole. The experiment showed that the use of lower concentrations of fungicides resulted in an increased production of the mycotoxins monitored, which was also confirmed in the study of Heier *et al.* (2005). It is necessary to use the highest fungicide concentration recommended for the treatment of wheat. The effect of fungicide could be reduced by air conditions (rain, humidity) resulting in increasing mycotoxins production. Further research will be focused on the monitoring of the production of DON by different strains of *Fusarium* isolated from grain and grain-based products, and on the possibilities of reducing the production of *Fusarium*.

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