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EFFECT OF FUNGICIDAL TREATMENT ON DIGESTIBILITY OF MYCOTOXINS IN VITRO

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ABSTRACT

In this experiment, the effect of fungicidal treatment on the release of various mycotoxins was tested in rumen fluid in vitro. The three groups of barley crop with different fungicide treatment were included in the experiment. The first group served as the control one without fungicide treatment. The second group of barley (variant A) was treated with Hutton (0.8 L/ha at BBCH 36) + Zantar (1.5 L/ha at BBCH 65). The third group of barley (variant B) was treated with the combination of Hutton (0.8 L/ha at BBCH 36) + Prosaro EC250 (0.75 L/ha at BBCH 65). In the original mass of barely, ten levels of mycotoxins were established. Subsequently, the samples were incubated in the machine Daisy II for 24 hours. The cellulase and pepsin enzymes were used in the incubation. Following mycotoxins were determined in the incubation fluid such as dexynivalenol, zearalenone, deoxynivalenol-3-glucoside and 3-acetyl-deoxynivalenol. In the variant A, the level of dexynivalenolu was higher by 36%, zearalenone by about 2%, deoxynivalenol-3-glucoside by 12%, and 3-acetyl-deoxynivalenol by 39%. Low levels of the mycotoxins were found out in the variant B. Deoxynivalenol level was lower by 19%, zearalenone by 30%, deoxynivalenol-3-glucoside by 37% ($p < 0.05$). The 3-acetyl-deoxynivalenol level was higher by 12% in a comparison with the control group. The obtained results showed that the fungicidal treatment and digestive enzymes could eliminate the transition of mycotoxins into incubative (rumen) liquid, and thereby to reduce the risk of the load of the organism by the mycotoxins. According to the results, it is obvious that low levels of various mycotoxins presented in the barley grains, as well as the transition of these mycotoxins in the incubation fluid were decreased. Some fungicides can play a significant role in the occurrence of mycotoxins barely grain.

Keywords: mycotoxins; barley; in vitro; digestibility

INTRODUCTION

Barley is classified as one of the most important cereals in the Czech Republic. It is used for livestock feed and food industry - especially malting **(Belakova et al., 2014, Horky et al., 2012a)**. Mycotoxins are fungal secondary metabolites having mutagenic, carcinogenic, and cytotoxic effects. They can often contaminate agricultural commodities in spite of the various protective measures **(Jancikova et al., 2012a)**. Recently, the attention has been focused on the so-called masked mycotoxins. The deoxynivalenol-3-glucoside and 3-acetyldeoxynivalenol metabolized from deoxynivalenol are the most common occurring. The presence of the masked mycotoxins presents the same hazard as the occurrence of classic mycotoxins. In the food productive process, the production of malt, beer, and bread are back metabolized to deoxynivalenol **(Horky et al., 2013**, **Zachariasova et al., 2012)**. Fusarium mycotoxins in foods are the most frequent type of contamination. Deoxynivalenon mycotoxins, zearalenone and T-toxin are responsible for the extensive damage to both feed and food. They directly threaten the health of consumers **(Horky, 2014a, Maul et al., 2014)**. Fungicides are pesticides that are used to eliminate harmful phytopathogenic fungi on crop plants and the substances of organic origin. Fungicides have the ability to eliminate the occurrence of fungal biomass in plants and thereby to reduce the risk of mycotoxin production **(Jancikova et al., 2012b, Schmidt-Heydt et al., 2013)**. After the fungicidal treatment, the development of mold is significantly reduced. Untreated plants can be characterized by higher levels of mold up to 260% **(Pirgozliev et al., 2012**). The similar effect as fungicides may have as well as the antioxidant enzymes **(Horky, 2014a, Horky et al., 2012b)**. The susceptibility of animals to mycotoxins is different. The least susceptible animals are ruminants due to their buffering ability of the rumen **(Horky, 2014b, Nevrkla et al., 2013)**. The aim of the experiment was to test the effect of fungicidal treatment of barley on the release of various mycotoxins in rumen fluid in vitro.

MATERIAL AND METHODOLOGY

Barley samples coming from Libcany area (the Czech Republic) were put in the experiment from the harvest in 2012. The barley was artificially treated with *Fusarium culmorum* (WGSm. Sacc. Strain KM16902; DON chemotype). The inoculation with a conidia suspension of the pathogenic isolate of *F. culmorum* (concentration 0.5 mil. conidia/1 mL of inoculum; spray dose of 200 $L.ha^{-1}$) was performed in the optimal vegetative phase according to the methodology of **Tvarůžek et al.** (**2012**). In the inoculation period, the vegetation was sprayed with clean water before the inoculation in dry and sunny weather. Subsequently, the chemical treatment with

fungicides was applied in the barley. The first group was untreated and served as the control one. The second group of barley (variant A) was treated with Hutton (0.8 L/ha at BBCH 36) + Zantar (1.5 L/ha, BBCH 65). The third group of barley (variant B) was treated with the combination of Hutton (0.8 L/ha, BBCH 36) + Prosaro EC250 (0.75 L/ha, BBCH 65).

Composition of Fungicides:

***Hutton* - active substance:**

Prothiokonazol 100 g/L 2-[2-(1-chlorcyklopropyl)-3-(2-chlorfenyl)-2-hydroxypropyl]-2,4-dihydro-1,2,4-triazol-3-thion.

Spiroxamin 250 g/L [(8-terc-butyl-1,4-dioxaspiro[4.5]dekan-2-yl)methyl]ethyl(propyl)amin.

Tebukonazol 100 g/L 1-p-chlorofenyl-4,4-dimethyl-3-(1H-1,2,4- triazol-1-ylmethyl)-pentan-3-ol

***Prosaro 250 EC* - active substance:**

Prothiokonazol 125 g/L 2-[2-(1-chlorcyklopropyl)-3-(2-chlorfenyl)-2-hydroxypropyl]-2,4- dihydro-1,2,4-triazol-3-thion.

Tebukonazol 125 g/L 1-p-chlorofenyl-4,4- dimethyl-3-(1H-1,2,4- triazol-1-ylmethyl)-pentan- 3-ol.

***Zantara* - active substance:**

Bixafen 50 g/L N-(3,4-dichloro-5-fluorobiphenyl-2-yl)-3-(difluoromethyl)-1-methylpyrazole-4-carboxamide.

Tebukonazol 166 g/L (3-1-(4-chlorfenyl)-4,4-dimethyl-3-[(1H-1,2,4-triazol-1-ylmethyl]pentan-3-ol.

Before the incubation (in Daisy II), all barley samples were analyzed for the content of individual mycotoxins. From each group, the three samples were collected and analyzed. The results of average concentrations of mycotoxins are listed in Table 1. The barley samples were grounded on the laboratory mill with mesh size 1 mm. The machine Daisy II Incubator - ANKOM Technology, New York was used for the incubation. A 4 g milled sample was taken for the incubation divided into the incubation bags - F57 (Ankom, Macedonia) in the amount of 0.25 g per incubation bag.

Preparation of Solutions

A 1.5 liter of solution was used for one incubation. For preparation of pepsin solution, 3 g of pepsin (*Pepsin from porcine gastric mucosa* 800 - 2500 units/mg protein - (Sigma-Aldrich, Germany)) dissolved in 1.5 liters of 0.1 M HCl then heated to 40 °C. Immediately, it was put in the incubation. Acetate buffer (pH 4.6): 10.2 g sodium acetate (3 H_2O) was dissolved in 1.5 l of distilled water. The pH value was modified using acetic acid or NaOH. In the preparation of cellulase solution, 1.5 g of cellulase was dissolved (*Cellulase Trichoderma viride*, 3-10 units/mg solid - Sigma Aldrich, Germany) in 1.5 liters of acetate buffer heated to 40 °C then the incubation could start. The incubation lasted for 24 hours at 37 °C. The obtained cultured fluids were analyzed on the concentration of mycotoxins.

Determination of Mycotoxins

Solid Samples - Extraction

A 2 g barley sample was weighed to PTFE centrifuge tubes (50 ml) followed by the addition of 10 ml of distilled water acidified (0.2% formic acid). Then the sample was shaked, closed and left for 30 minutes due to the wetting of the matrix. A 10 ml of acetonitrile was added in the sample with water followed by the extraction on the laboratory mixer for 30 minutes (240 RPM). The 4 g of $MgSO_4$ and 1 g of NaCl were put in the cuvette and shaken vigorously for 1 minute. The prepared sample was centrifuged for 5 minutes (10,000 RPM). After centrifuging, the sample was taken (approx. 1.5 ml) for purification using a microfilter with a porosity of 0.2 μm (centrifugation for 2 min, 5000 RPM). The sample was transferred to the vials and prepared for analysis. The samples were stored at -18 °C in glass vials before the analysis. For the identification and quantitative determination of the mycotoxins, Acquity UPLC® System (Waters, Milford, MS, USA) in a connection with tandem mass spectrometer QTRAP® (AB Sciex, Toronto, ON, Kanada) is used for the instrumentation of ultra-efficient liquid chromatograph Acquity UPLC ® System (Waters, Milford, MS, USA).

Table 1 Concentration of detected mycotoxins in control, A and B variant of the fungicidal treatment

Mycotoxins	Variant of fungicidal treatment		
	Control	A	B
Deoxynivalenol	12360.9 ±2045.5	19852.7 ±2173.8	11287.2 ±2718.8
deoxynivalenol-3-glukosid	6774.5 ±502.5	9042.7 ±678.3	5035.3 ±494.9
3-acetyl-deoxynivalenol	1449.4 ±219.4	1969.8 ±257.4	1135.8 ±230.3
Zearalenon	3737.5 ±880.3	4096.3 ±702.9	2947.2 ±704.0
Beta-zearalenol	89.9 ±18.2	107.3 ±16.5	58.8 ±8.1
Alternariol	56.3 ±12.1	13.5 ±5.2	12.1 ±4.2
Alternariol-methylether	2.3 ±0.9	2.6 ±0.9	2.4 ±0.1
Enniatin B	391.5 ±102.7	432.4 ±109.2	460.3 ±104.3
Enniatin A	4.5 ±2.8	4.7 ±3.3	5.5 ±3.0
Enniatin A1	25.8 ±12.4	27.7 ±18.3	32.4 ±13.0

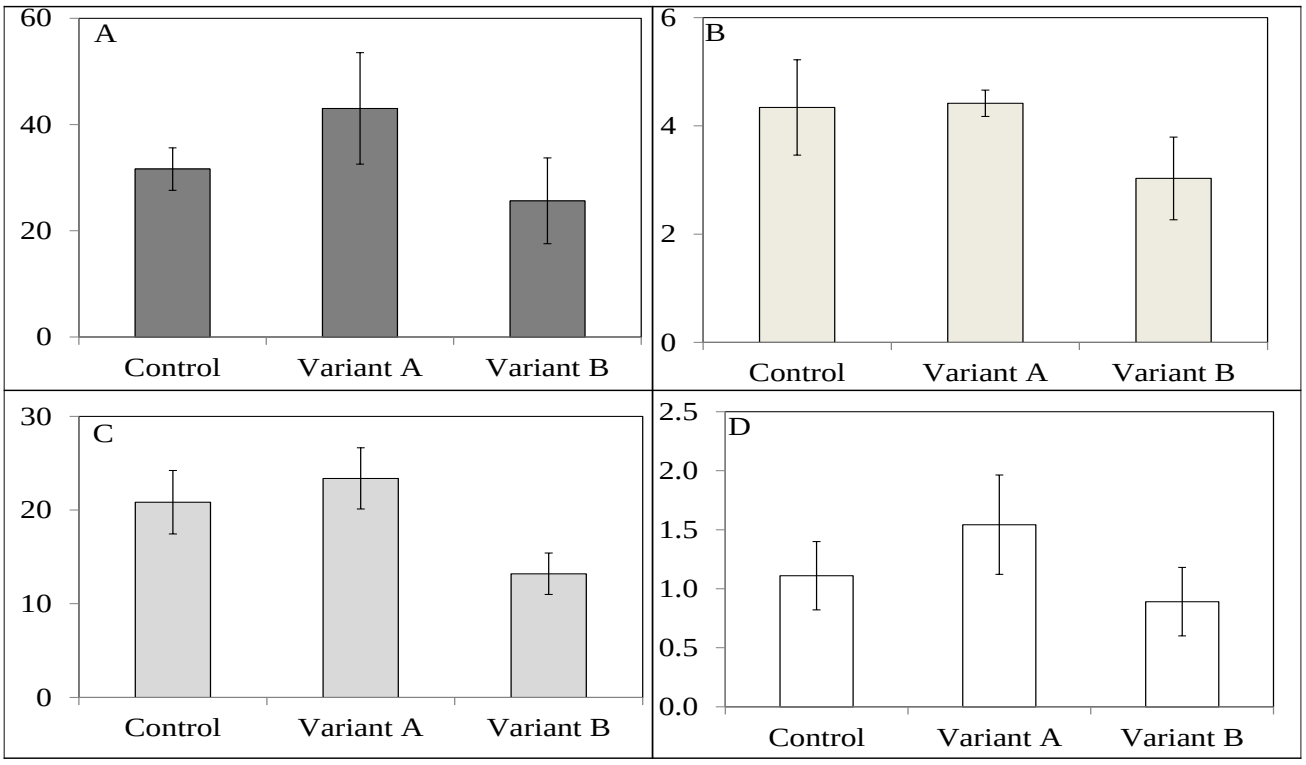

Figure 1 The concentration of mycotoxins detected in the incubation fluid (mg/kg): dexynivalenol (A); zearalenone (B); deoxynivalenol-3-glucoside (C); 3-acetyl-deoxynivalenol (D).

The program Analyst ® (Thermo Fisher Scientific) is used for data processing.

Liquid Samples

Liquid samples were purified using a microfilter with a porosity of 0.2 µm (centrifugation for 2 min, 5000 RPM) before the instrumental analysis. Furthermore, the solid samples were stored at -18 °C and measured using instrumentation consisting of ultra-efficient liquid chromatograph Acquity UPLC ® System (Waters, Milford, MS, USA) in connection with tandem mass spectrometer QTRAP ® (Applied Biosystems, Toronto, ON, Canada).

Determination of Mycotoxins

The total of 57 mycotoxins of microscopic filamentous fungi of the genus *Fusarium*, *Penicillium*, *Aspergillus*, *Alternaria*, *Claviceps* a *Stachybotrys* were set such as Fusarenon X, nivalenol, deoxynivalenol, alfa-zearalenol, beta-zearalenol, zearalenon, 3-acetyl-deoxynivalenol, patulin, alternariol, alternariol-methylether, deoxynivalenol-3-glukoside, enniatin B, enniatin B1, enniatin A, enniatin A1, ergokornin, ergokorninin, ergokristin, ergokristinin, ergokryptin, ergokryptinin, ergosin, ergosinin, ergometrin, ergotamin, ergotaminin, agroklavin, neosolaniol, diacetoxyscirpenol, fumonisin B1, fumonisin B2, fumonisin B3, 15-acetyl-deoxynivalenol, aflatoxin B1, aflatoxin B2, aflatoxin G2, aflatoxin G1, HT-2 toxin, T-2 toxin, sterigmatocystin, ochratoxin A, citrinin, beauvericin, cyklopiazon acid, mycophenolic acid, penicillin acid, rokfortin C, tentoxin, tenuazonic acid, verrucarol, verruculogen, penitrem A, stachybotrylaktam, phomopsin A, gliotoxin, meleagrin, paxillin.

Statistics

The data were processed statistically using STATISTICA.CZ, version 10.0 (the Czech Republic). The results were expressed as mean ± standard deviation (SD). Statistical significance was determined by examining the basic differences between groups ANOVA and Scheffé's test (one-way analysis). The differences with $p < 0.05$ were considered to be significant.

RESULTS

During the analyzing of the incubated fluid, the following mycotoxins such as deoxynivalenol, zearalenone, deoxynivalenol-3-glucoside, 3-acetyl-deoxynivalenol were detected. The mycotoxins that were analyzed in the original mass of barley such as beta-zearalenol, alternariol, alternariol-methylether, enniatin B, enniatin A, enniatin A1 were below the detection limit indicating the fact that the mycotoxins were largely eliminated by digestive enzymes. The highest concentrations were measured in deoxynivalenol fungicidal treatment of the variant A (by 36%) compared with the control group of barley. Conversely, the variant B had a lower concentration of deoxynivalenol about 19% (Figure 1A). The identical value of the mycotoxin was observed in the control and fungicide variants A during the assessing levels of zearalenone in the incubation fluid. The variant B had lower levels of zearalenone by up 30% compared with the control group in the incubation fluid (Figure 1B). The two masked mycotoxins deoxynivalenol-3-glucoside and 3-acetyl-deoxynivalenolwere were also detected in the incubation fluid. In the variant A, Deoxynivalenol-3-glucoside (Figure 1C) was increased by

12% compared with the control group. The variant B had significantly lower levels of mycotoxin by 37% ($p < 0.05$). High concentrations of 3-acetyl-deoxynivalenol was measured for variant A (39%). In the variant B, the quantities of the mycotoxin in the incubation fluid was increased by 12% compared with the control group (Figure 1D).

DISCUSSION

In our experiment, the effects of fungicidal treatment on digestibility of the individual mycotoxins were compared with the usage of Daisy II incubator. The following mycotoxins were detected in the incubation fluid such as dexynivalenol, zearalenone, deoxynivalenol-3-glucoside, 3-acetyl-deoxynivalenol. In the experiment, in which Fusarium toxins were added to the feed dose of ruminants in the amount of 60 and 30%, the reduction of synthesis of microbial protein was observed. In ruminal environment, the amount of Fusarium mycotoxins was significantly decreased **(Hildebrand et al., 2012)**. In our experiment, the similar effect was observed on the release of mycotoxins in the ruminal environment during the effect of enzymes (cellulase, pepsin). During the incubation of mold feed (for 24 hours), barley base, rapeseed cakes, alfalfa hay and barley straw (fungus 70%) was not affected by the degradation of dry matter of the individual components of the diet. The occurrence of mycotoxins was not monitored in the experiment. The mycotoxins such as aflatoxin 0-30%, deoxynivalenol 0-50%, T-2 toxin 0-70%, zearalenone 0-40%, deoxynivalenol 0-35%, ochratoxin A 50-100% are degraded in their derivatives with varying efficiency in the rumen. The susceptibility of animals to mycotoxins is different. The ruminants are the least susceptible category because of the buffering ability of the rumen. They are able to reduce and tolerate higher levels of mycotoxins **(Undi & Wittenberg, 1996)**. The rumen microorganisms apparently metabolize toxins into non-toxic metabolites. We can also agree with these findings in comparison with the results of our experiment. After the incubation in a mixture of enzymes (pepsin, cellulase), the mycotoxins such as beta-zearalenol, alternariol, alternariol-methylether, enniatin B, enniatin A, enniatin A1 were degraded with high efficiency. Dexynivalenol, zearalenone, deoxynivalenol-3-glucoside, 3-acetyl-deoxynivalenol were in the original mass of barley at very high concentrations. It was probably the reason why they were analyzed in the incubation fluid. From the findings of other studies, it could be suggested that the rumen environment completely eliminated the mycotoxin called ochratoxin A infected wheat straw **(Abdelhamid et al., 1992)**. Berthiller **(Belakova et al., 2014)** investigated the back hydrolysis of deoxynivalenol-3-glucoside to the original mycotoxin (deoxinivalenol) in the stomach monogastry (in vitro). Deoxynivalenol-3-glucoside was resistant to the acidic environment of the stomach incubated in 0.2 M hydrochloric acid for 24 hours at 37°C. Conversely, some of the lactic acid bacteria were able to hydrolyze deoxynivalenol-3-glucoside back to deoxinivalenol.

CONCLUSION

In the experiment, the effect of fungicidal treatment on the release of various mycotoxins was observed in rumen fluid in vitro. The experiment included the three groups of barley using different fungicide treatment. The first control group was without fungicidal treatment. The second group of barley (variant A) was treated with Hutton (0.8 L/ha at BBCH 36) + Zantar (1.5 L/ha at BBCH 65). The third group of barley (variant B) was treated with the combination of Hutton (0.8 L/ha at BBCH 36) + Prosaro EC250 (0.75 L/ha at BBCH 65). In the mass, the level of ten mycotoxins was measured. Then the samples of barley were incubated in the incubator Daisy II for 24 hours using the cellulase enzyme and pepsin. The dexynivalenol, zearalenone, deoxynivalenol-3-glucoside, 3-acetyl-deoxynivalenol were determined in the incubation fluid. In the variant A, dexynivalenol level was higher by 36%, zearalenone by about 2%, deoxynivalenol-3-glucoside by 12% and 3-acetyl-deoxynivalenol by 39%. Low levels of the mycotoxins were found out in the variant B, the level of deoxynivalenol was lower by 19%, zearalenone by 30%, deoxynivalenol-3-glucoside by 37% ($p < 0.05$), and 3-acetyl-deoxynivalenol by 12%. According to the results, it is obvious that low levels of various mycotoxins presented in the barley grains, as well as the transition of these mycotoxins in the incubation fluid were decreased. Some fungicides can play a significant role in the occurrence of mycotoxins barely grain.

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