The influence of *Fusarium culmorum* contamination level on deoxynivalenol content in wheat, malt and beer

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Abstract

The objectives of this study were to investigate the impact of the initial Fusarium culmorum contamination level on deoxynivalenol (DON) concentrations in wheat, green malt, dry malt, beer and its by-products and to determine the influence of applied unit operations during the wheat beer production process on DON concentrations. Wheat samples with different initial F. culmorum contamination levels were subjected to a micromalting procedure followed by microbiological and toxicological analyses of green malt (grain after steeping and germination), dry malt and beer in which the share of F. culmorum-contaminated grain and the DON concentration were determined. The results show that the strongest proliferation of fungus occurred during the steeping and germination phase, whereas the drying phase significantly reduced the contamination level. Mycotoxicological analysis showed that DON is stable during beer production, transfers from malt to wort, and is found in beer production by-products such as spent grain, spent yeast and germ/rootlets.

Key words: ELISA, Fusarium, wheat, beer, deoxynivalenol

Introduction

Wheat (*Triticum aestivum* L.) is a worldwide agricultural commodity used for human and animal consumption. In addition to its use in the cereal and baking industries, wheat has also been used for malt production and as a basic element in beer production. Wheat grains are nutritionally rich and therefore are a suitable substrate for microbial growth (1). Fungi of the genus *Fusarium* are important microorganisms in the malting and brewing industry. They proliferate during the malting process and affect the safety of malt by producing mycotoxins. Factors affecting *Fusarium* proliferation during malting include the initial contamination of the grain, interactions between species of the microbial population, nutritive characteristics of grain and most of all, process parameters (temperature, aeration, additives, and humidity) (2). Wheat is contaminated with *Fusarium* in the field from heading till grain maturity, but *Fusarium* can additionally be spread during transport and storage. The potential outcomes of cereal *Fusarium* contamination are decrease in yield, lower average seed dimensions, decrease in nutritive value, loss of color and changes in smell and taste (1; 2; 3). *Fusarium* head blight (FHB) is a severe disease of small grains (4), namely wheat, barley, and rye, but it can also result in *Gibberella* ear

rot in maize. There are a few ways to control FHB: crop rotation (the highest incidence of *Fusarium* is when wheat is grown after corn), tillage, use of fungicides and choice of wheat variety with Fusarium resistant genes. Moisture content (influenced by rain) has a huge impact on FHB development during wheat anthesis (5). Optimal conditions for infection are warm temperatures (20 - 25 °C) accompanied by high relative air humidity (> 80 %) (6; 7). In Europe, FHB can be caused by a complex of head blight pathogens, but according to KRSTANOVIĆ & al. (5) and SPANIC & al. (8), the most prevalent species on cereals in Eastern Croatia are Fusarium graminearum Schw. and F. culmorum (Wm. G. Sm.) Sacc. Recent research conducted by PLEADIN & al. (9), showed that deoxynivalenol (DON, vomitoxin) contaminates 68 % of oats, 59 % of barley, 57 % of wheat and 41 % of maize samples in Croatia. Fusarium contamination is directly connected with the accumulation of mycotoxins in the grain (10). Mycotoxins are secondary fungal metabolites that contaminate food and feed and have toxic effects on humans and animals (11). Because the most prevalent mycotoxin is DON, it has been identified as one of the most important indicators of quality and safety of barley and wheat used for malting (12). DON is a protein synthesis inhibitor in eukaryotic cells due to its ability to bind to ribosomes. Low to moderate concentrations of DON can significantly affect the health of animals by causing anorexia, whereas higher doses can cause vomiting (13; 14). Mycotoxins found in raw materials used for brewing, such as barley or wheat, transfer into malt and can consequently transfer to the final product – and these mycotoxins can be detrimental for human health (15). LANCOVA & al. (16) showed that 70 % of DON from barley naturally infected with Fusarium transfers to sweet wort, and in the case of artificially infected barley, 67 % of DON transfers to sweet wort. SCHWARZ & al. (17) reported that 80 - 93 % of DON from malt is found in beer, which, in the case of regular and heavy beer drinkers, means that the maximum tolerable limits for DON could easily be exceeded by an average individual drinking 1 L of beer contaminated with 89 μ gL⁻¹ (the most contaminated beer in their study) (18). According to the EUROPEAN COMMISSION REGULATION (19), the highest allowed DON concentration in non-processed food for humans is 1250 µg/kg. A research study conducted by NOOTS & al. (20) stated that 80 % of DON was synthesized after steeping. Germ and rootlets, malt byproducts, are used as animal feed and present a potential risk if they are contaminated with mycotoxins. WOLF-HALL (21), CAVAGLIERI & al. (22) and AWAD & al. (23) addressed this issue, but further studies should be conducted.

This paper examined the influence of different degrees of F. *culmorum* contamination on the mycotoxin content in starting wheat, green malt, malt and beer, spent grains, spent yeast and germ/rootlets.

Materials and methods

2.1 Inoculum production

To produce macroconidia of *F. culmorum*, a mixture of wheat and oat grains (3:1 by volume) was soaked in water overnight in 250 ml glass bottles and then the water was decanted and the seeds were autoclaved. After adding the *Fusarium* strain (isolate IFA 104), the seeds were kept for 2 weeks at 25° C in the dark. The concentration of the conidial suspension was 10×10^4 ml⁻¹ (24). The aggressiveness test was performed in Petri dishes as described by LEMMENS & al. (25) (data not shown). Tubes containing small aliquots of inoculum were frozen at -80 °C until use.

Field experiment and inoculation treatment

Research was conducted on samples grown in 2010 in Osijek, Croatia (45°27'N, 18°48'E) using winter wheat variety Nova Žitarka (a domestic variety from Agricultural Institute Osijek).

To control seed-borne diseases, the seed was treated with Vitavax 200 (Chemtura AgroSolutions) (thiram+carboxin) at a rate of 200 g 100 kg⁻¹. Genotype was sown in eight-row plots of 7 m length and 1.08 m wide in October at a sowing rate of 330 seeds m⁻². Wheat was grown according to standard agronomic practice. The plot area had sufficient nitrogen and other nutrients for normal crop growth. Spray inoculations were performed at flowering period (Zadok's scale 65) (26) using a tractor back-sprayer. Inoculations were performed in the late afternoon and repeated two days later. To maintain moisture of the ears, we sprayed water with a tractor back-sprayer on several occasions during the day. The control treatment consisted of three replications of plots left to natural infection. Symptoms of the disease were recorded 22 days after the first inoculation of artificial infection. Values for the entire area within the plot were visually rated using a linear scale from 0 (no infection) to 100 (100 % infection).

Microbiological analysis

Prior to further morphological analysis, the infected grains were analyzed according to NELSON et al. (27) and LESLIE & SUMMERELL (28). Synthetic Nutrient Agar (SNA) medium was used to examine conidial morphology and to detect the presence of chlamydospores. Potato dextrose agar (PDA) agar was used to assess colony characteristics, such as pigmentation and growth rate. To obtain six samples with diverse contamination levels (0 % - 50 %) the starting wheat sample was diluted in a way that contaminated and uncontaminated wheat were mixed in different scales. The degree of natural *F. culmorum* contamination of obtained wheat was 61 %. To provide surface disinfection of grains, 50 g of wheat was soaked in 1 % NaOCl for 10 minutes and then rinsed with sterile water. One hundred grains were carefully sorted onto manitol agar in Petri dishes and incubated in the dark at 30 °C for 3 days. After 3 days a white, cotton-like mycelium, confirmed to be *F. culmorum*, appeared around infected kernels on the agar. The sample means (% infected kernels) were calculated from three replicate analyses.

Micromalting

The micromalting procedure (29) was adjusted to wheat because wheat grain has no husk and, therefore, can adsorb water much quicker. As a result, the soaking time must be shortened and the relative humidity of the air must be decreased. Micromalting was performed according to standard procedure (29) in a laboratory incubator (ClimaCell, MMM Medcenter Einrichtungen, München, Germany). Five hundred grams of wheat was soaked in 500 mL of tap water according to the standard procedure described in **Table 1**. The *F. culmorum* contamination level in malt was also determined (29). The kilning of green malt was also performed according to the MEBAK (29) protocol. After drying, the malt was transferred into paper bags and kept at room temperature for three days for moisture equilibration.

1 st day	Immersion steeping for 5 h at 14.5 °C; Dry steeping for 19 h at 14.5 °C, relative air humidity 85 %.				
2 nd day	Immersion steeping for 4 h at 14.5 °C; Dry steeping for 20 h at 14.5 °C, relative air humidity 85 %.				
3 rd day	Immersion steeping for 2 h at 14.5 °C, relative air humidity 85 %.				
3 rd - 6 th day	Germination was carried out according to the scheme: 96 h at 14.5 °C Relative air humidity in each procedure was 85 %				
7 th day	Kilning was performed for 19 h, according to standard procedures for pale malt, after last germination hour; malt degermination; packing in paper bags and storage				

Table 1. General micromalting scheme of wheat samples

Wheat beer production

Wheat beer was produced according to MEBAK (29). Mashing (4.1.4.2.) and fermentation (2.11.2) were carried out according to the attenuation limit determination method [29].

Wort production

Mashing step

Dried wheat malt was milled in a laboratory mill (Newman Industries, Sale Creek, TN). Fifty grams of wheat malt was mashed with 200 mL of water ($45 \pm 1 \,^{\circ}$ C) and held in a water bath for 30 minutes with constant stirring ($80 - 100 \,\text{rpm}$). Over the next 25 minutes, the mash temperature was increased to 70 °C at approximately 1 °C per minute. When the target temperature was reached, 100 mL of 70 °C water was added and kept for one hour at 70 °C. The mash was then cooled down to room temperature and filtered through folded filter paper. Samples of spent grains and unfermented wort were frozen until further mycotoxicological analysis.

Wort fermentation

Wort fermentation was performed according to the attenuation limit determination method for wort and beer (29). Two hundred milliliters of wort was transferred into an Erlenmeyer flask and inoculated with 15 g of yeast. A fermentation airlock was placed onto the flask. Fermentation at room temperature followed for 24 hours with constant stirring. After the fermentation was complete, the beer was decanted from the spent yeast. Beer and spent yeast samples also underwent toxicological analysis.

Mycotoxicological analysis

Chemicals and apparatus

A Ridascreen® FAST DON kit for ELISA was provided by R-Biopharm (Darmstadt, Germany). Each kit contains a microtiter plate with 96 wells coated with capture antibodies, standard solutions of DON (0, 0.222, 0.666, 2 and 6 ng/mL), peroxidase conjugated DON, anti-DON antibody, substrate/chromogen solution, stop solution and washing buffer (salt). DON standard (D 0156; purity \geq 98.00 %) from Sigma Aldrich Chemie GmbH (Steinheim, Germany) was used for sample fortification. ELISA was performed by the ChemWell auto-analyzer (Awareness Technology, Inc., USA).

Sample preparation and extraction procedure

Samples of wheat, green malt, dry malt, spent grains and germs/rootlets were ground on an analytical mill (Cylotec 1093, Tecator). After grinding, 5 g of sample was extracted with 100 mL of distilled water. The extraction was performed with vigorous shaking in a shaker for 3 minutes and followed by filtering of the extract through filter paper (Whatman, Black Ribbon). Wort, spent grains and spent yeast were first prepared by removing the excessive CO_2 by shaking followed by filtration before they were used for the assay. The filtrate obtained was further diluted with distilled water and applied onto the ELISA kit wells. When calculating the concentration of DON in the samples, the results obtained from the calibration curve were multiplied by the corresponding dilution factor.

Analysis of DON

Competitive ELISA was performed according to the manufacturer's instructions. All standards and samples were analyzed in duplicate. Enzyme conjugate (50 μ L) and anti-DON antibody solution (50 μ L) were added to 50 μ L of standards and prepared samples. The microwell holder was shaken gently and incubated for 5 min at room temperature. After incubation, the wells were washed 3 times with 250 μ L of distilled water. Then, 100 μ L of substrate/chromogen solution was added to each well and incubated for 3 min at room temperature in the dark. The reaction was stopped by adding 100 μ L of stop solution and absorbance was measured at 450 nm. Statistical data analysis was performed by using the Statistica Ver. 6.1. Software (StatSoftInc. 1984-2003, USA). The statistical significance was set at 95 % (P =0.05).

Method validation

The estimated limits of detection (LOD) and limits of quantitation (LOQ) were calculated from the mean value of ten determinations of a blank wheat, malt and beer material plus twoand tenfold standard deviation. The determined values for LOD were less than 200 μ g/kg and for LOQ they were 200 to 220 μ g/kg, depending on the material. The method's recovery was determined at three different levels by spiking blank wheat, malt and beer samples with DON standard to yield concentrations equivalent to 500, 1000 and 5000 μ g/kg (six replicates *per* concentration level *per* material) and calculated from five-point calibration curves.

Material	Spiked concentration (µg/kg)	Determined concentration (µg/kg)	Mean recovery (%)	Coefficient of variation (%)
	500	508	101.6	6.5
Wheat	1000	983	98.3	5.3
	5000	4461	89.2	4.2
	500	546	109.2	7.3
Malt	1000	1162	116.2	6.4
	5000	5562	111.2	8.8
	500	578	115.6	6.3
Beer	1000	1123	112.3	5.7
	5000	5902	118.0	7.9

 Table 2. Determination of recovery (n=6) in uncontaminated wheat, malt and beer samples spiked with DON

Data analysis

Differences between the average values were analyzed using the analysis of variance (ANOVA) and the Fisher's least significant difference test (LSD), with statistical significance being set at P<0.05. Statistical analysis was carried out using Statistica Ver. 8.0 StatSoft Inc. Tulsa, OK, USA.

Results and discussion

In this study, the isolate of *F. culmorum* was used rather than *F. graminearum*, a more prevalent specie in Croatia, because it is more aggressive. In general, more aggressive isolates produce higher DON concentrations, indicating that DON can play a role as a virulence factor (30). Relative grain yield loss (RGYL), the difference between control and inoculated treatments, was 16 % (data not shown). Field severity in all three replications was 40 %. It was reported that the FHB-resistant wheat genotypes accumulate far less DON than susceptible ones (31; 32).

The results from method validation are presented in **Table 2**. Validation resulted in mean values of recoveries ranging from 89.2 % in wheat to 118.0 % in beer with coefficients of variation from 4.2 to 8.8 %.

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Sample	Determined contamination level (%)				
	Wheat	Green malt	Dry malt		
1	0	6	0.5		
2	7.5	100	2.5		
3	19.5	100	1		
4	25.5	100	1.5		
5	35	100	7.5		
6	52	100	12		

Table 3. Microbiological analysis of samples

Table 4. Micotoxicological analysis of samples

Sample	DON concentrations (µg/kg)						
	1	2	3	4	5	6	
Wheat	$0.00b\pm0.00$	$1108.33h\pm6.51$	$1958.66h \pm 10.50$	$3247.00h\pm2.00$	$4604.33f \pm 6.50$	$4810.66g \pm 0.58$	
Green malt	$0.00b\pm0.00$	$1746.33g \pm 1.53$	$2694.66g \pm 4.51$	$3721.00g \pm 3.00$	$3873.66g \pm 1.52$	$5122.00f \pm 1.00$	
Dry malt	$0.00b\pm0.00$	$6962.33a \pm 7.51$	7021.33a ± 2.51	7712.66b ± 1.15	$8091.66c \pm 2.51$	$8757c\pm2.00$	
Germ/rootlets	$0.00b\pm0.00$	$5022.00b \pm 8.00$	$6412.66b \pm 2.51$	8312.00a ± 3.00	10635.33a ± 1.52	$12629.66a \pm 3.51$	
Wort	$0.00b\pm0.00$	3982.00d ± 1.00	$5126.66c \pm 0.57$	$6325.00c \pm 1.00$	$8314.00b \pm 4.00$	9511.66b ± 0.57	
Spent grains	$0.00b \pm 0.00$	$3512.00e \pm 2.00$	$3432.66f \pm 4.04$	$3745.33f \pm 1.52$	$3721.33h \pm 4.52$	$4532.00h \pm 1.00$	
Beer	$0.00b\pm0.00$	$4565.00c \pm 4.00$	$4535.00d \pm 4.50$	$4814.00d \pm 0.00$	$5021.00e \pm 0.58$	$5215.00e \pm 0.58$	
Spent yeast	$762.33a \pm 6.50$	3415.33f ± 0.57	$4231.33e \pm 5.51$	$4615.33e \pm 1.52$	5537.66d ± 1.52	$6480.00d \pm 4.00$	

Values are means \pm SD obtained with three measurements. Values displayed in the same row and tagged with different letters (a-h) are significantly different (P<0.05)

Six wheat samples with different levels of *F. culmorum* contamination were prepared for this research, as shown in **Table 3**. **Table 4** shows levels of the mycotoxin DON in wheat, green malt, dry malt, wort, spent grains, beer and spent yeast.

All results are consistent with previous research and show that higher initial contamination levels of wheat due to *F. culmorum* fungi results in higher levels of DON in all other products (33; 34; 35). This investigation shows that even samples with no visible signs of *F. culmorum* contamination (relevant red grains) - sample "1" contain DON. Toxicological analysis of green malt showed an increase in DON with regard to wheat, which indicates that DON is being synthesized *de novo* during the grain's germination period. Earlier research [18] showed that during steeping, DON levels in grain decreased due to its water-solubility that allows DON to transfer into steeping water. In this research it was also confirmed that after five days of germination, levels of DON are significantly higher in green malt than in steeped grain (18 - 114 %).

Nevertheless, it appears that the highest amount of DON is synthesized during the drying phase. This increase in DON levels in malt samples is consistent with initial *F. culmorum* contamination levels of wheat, which means that wheat with a high *F. culmorum* contamination rate will result in malt with high DON levels. *Fusarium* fungi synthesize mycotoxins when they are found in unfavorable conditions, which is the case with the first

drying phase of green malt (temperature 50 °C, grain moisture approximately 45 %) (36; 37; 38). Unlike fungi mycelium that dies out when exposed to high temperatures in the later phases of drying (17), DON synthesized in the earlier phases of malting (steeping and germination) will not decompose during drying because it is thermostable (39).

Furthermore, the results indicate that mycotoxins are not uniformly distributed in dry malt because it is apparent that DON levels in the germ/rootlets are significantly higher than in malted grain. The germ is a place of the most intense fungal growth, which explains why DON levels in germ are much higher than in malted grain (40). The higher the initial *Fusarium* contamination, the poorer the germ development will be (41). Additionally, degermination lowers mycotoxicological contamination. This is consistent with the study that was conducted on the distribution of another *Fusarium* mycotoxin, zearalenone (ZEA) in malted barley fractions (germ, bran and flour) dependent on *F. graminearum* growing conditions (40). This research showed that ZEA levels in malted bran and germ were significantly higher than in malt flour (endosperm) in almost all growing conditions.

Results in Table 4 show that DON is very stable throughout the beer production process and is easily extracted from the raw material into the final product. DON also resides in by-products: germ/rootlets, spent grains and spent yeast [18]. During mashing, DON (because it is watersoluble) is extracted into wort. DON concentrations in wort samples increase with the high contamination rate of malt, indicating that the highest transfer of DON into wort was found in a sample that had the highest contamination rate. The research conducted by SCHWARZ & al. (18) found that 80 - 93 % of DON transfers from malt to wort. Results obtained in this investigation indicate that DON concentrations in wort are lower than DON concentrations in malt, except in the last two samples with the highest F. culmorum contamination level. This decrease in concentration can be explained by the fact that DON resides on spent grains after the filtration of wort. In the research that NIESSEN (41) conducted, a significant increase in DON levels during mashing was observed because of DON being released from protein conjugates. The same research identified mashing as the key phase affecting changes in DON concentrations during beer production. In certain conditions and technological parameters such as water/raw material ratio, temperatures and enzymatic activity, a higher DON concentration was noticed in wort (600 %) than in malt. Furthermore, the obtained results indicate that DON concentrations in beer samples also increase when malt contamination rates increase, but the percentage of DON transfer from wort into beer decreases with the increase of malt contamination. BOEIRA & al. (42) explained that adsorption of DON onto yeast cells or the metabolism of DON by yeast extracellular mechanisms may account for the reduction of DON in beer compared with the DON concentration in wort. Higher DON concentrations in beer can be explained by an increase in water, which allows DON to dissolve, as there is less dry matter after fermentation than after malting. Many authors positively correlate mycotoxin levels with *Fusarium* contamination levels (34; 43; 44; 35; 45) and the results of this study further validate this correlation.

It is assumed that trichothecenes are partially removed with spent grains and this is especially true in the case of less soluble mycotoxins, such as zearalenone (46). DON concentrations in spent grains are somewhat similar regardless of the contamination rate of malt, but in spent yeast samples an increase in DON concentration follows an increase in the contamination rate. Because spent grains are used as animal feed, recommended concentrations of DON in animal feed range from 5 to 10 μ g/g(47). The results indicate that wheat with an initial *F. culmorum* contamination level higher than 10 % (sample "3") exceeds the limit of 1250 μ g/kg which means that this wheat is not suitable for beer production, because of the additional increase in DON that occurs during malting. However, although the

DON level trends in different fractions during this research were in accordance with other studies, an overestimation occurred when comparing the absolute levels with those reported in other studies. Because some antibodies can cross-react to DON metabolites (DON-3-glucoside and ADONs) present in the analyzed matrix, overestimation of DON levels can occur when using ELISA (48). A slight overestimation of the DON concentrations in malt and beer using the ELISA method was also observed in the validation process of this study (recovery > 100 %; Table 2). ZACHARIASOVA & al. (49) suggested that overestimating the DON levels in beer samples is due to DON-3-glucoside and ADONs occurring in fermented foods at higher levels than in unprocessed cereals. Future studies must include 'masked' mycotoxins, considering the overestimation factor of the data obtained in this research.

Conclusions

Despite many efforts to control *Fusarium* infections of barley and wheat used for malting and brewing, as well as to eliminate the presence of its mycotoxins in beer and beer byproducts the fast and affordable methods for quick estimation of the *Fusarium* contamination level and mycotoxins concentration is still missing. Studies that correlate the *Fusarium* contamination level with DON content in the raw material, malt and beer stress out the necessity of developing such methods in the future, to ensure the safety of food chain.

Future studies must include 'masked' mycotoxins, considering the overestimation factor of the data obtained in this research.

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References

- 1. KOCIĆ-TANACKOV SD, ŠKRINJAR MM, GRUJIĆ OS, PEJIN JD, Fungal growth during malting of barley. 51: 60 (2005).
- 2. NOOTS I, DELCOUR JA, MICHIELS CW, From field barley to malt: detection and specification of microbial activity for quality aspects. *Crc Critical Reviews in Microbiology*, 25: 121-153 (1998).
- **3.** SIRANIDOU E, KANG Z, BUCHENAUER H, Studies on symptom development, phenolic compounds and morphological defence response in wheat cultivars differing in resistance to *Fusarium* head blight, *Journal of Phytopathology*, 150: 200-208 (2001).
- **4.** VANNE L, HAIKARA A, Mycotoxins in the total chain from barley to beer. Proceedings of the 28th European Brewery Convention Congress, 838-848 (2001).
- **5.** PERKOWSKI J, KIECANA I, KACZMAREK Z, Natural occurrence and distribution of *Fusarium* toxins in contaminated barley cultivars, *European Journal of Plant Pathology*, 109: 331-339 (2003).
- 6. KRSTANOVIĆ V, KLAPEC T, VELIĆ N, MILAKOVIĆ Z, Contamination of malt barley and wheat by *Fusarium graminearum* and *Fusarium culmorum* from the crop years 2001-2003 in Eastern Croatia, *Microbiological Research*, 160: 353-359 (2005).
- 7. BAI G, SHANER G, Scab in wheat: Prospects for control. Plant Disease, 78: 760-766 (1994).
- 8. MCMULLEN M, JONES R, GALLENBERG D, Scab of wheat and barley: Are-emerging disease of devastating impact, *Plant Disease*, 81: 1340-1348 (1997).
- **9.** SPANIC V, LEMMENS M, DREZNER G, Morphological and molecular identification of *Fusarium* species associated with head blight on wheat in East Croatia, *European Journal of Plant Pathology*, 128: 511-516 (2010).

- **10.** PLEADIN J, VAHČIĆ N, PERŠI N, ŠEVELJ D, MARKOV K, FRECE J, *Fusarium* mycotoxins' occurrence in cereals harvested from Croatian fields, *Food Contaminants*, 32: 49-54 (2013).
- 11. PLACINTA CM, D'MELLO JPF, MACDONALD AMC, A review of worldwide contamination of cereal grains and animal feed with *Fusarium* mycotoxins, *Animal Feed Scence and Technology*, 78: 21-37 (1999).
- 12. VÁŇOVÁ M, HAJŠLOVÁ J, HAVLOVÁ P, MATUŠINSKY P, LANCOVÁ K, SPITZEROVÁ D, Effect of spring barley protection on the production of *Fusarium* spp. mycotoxins in grain and malt using fungicides in field trials, *Plant Soil and Environment*, 50: 447-455 (2004).
- **13.** SALAS B, STEFFENSON BJ, CASPER HH, TACKE B, PROM LK, FETCH TG Jr, SCHWARZ PB, *Fusarium* species pathogenic to barley and their associated mycotoxins, *Plant Disease*, 83: 667-674 (1999).
- 14. LEGZDINA I, BUERSTMAYR H, Comparison of infection with *Fusarium* head blight and accumulation of mycotoxins in grain of hulles and covered barley, *Journal of Cereal Science*, 40: 61-67 (2004).
- **15.** ROTTER BA, PRELUSKY DB, Toxicology of deoxynivalenol (vomitoxin), *Journal of Toxicology and Environmental Health*, 48: 1-34 (1996).
- 16. VAUGHAN A, O'SULLIVAN T, van SINDEREN D, Enhancing the microbiological stability of malt and beer, *Journal of the Institute of Brewing*, 111: 355-371 (2005).
- LANCOVA K, HAJSLOVA J, POUSTKA J, KRPLOVA A, ZAACHARAISOVA M, DOSTALEK P, SACHAMBULA L, Transfer of *Fusarium* mycotoxins and "masked" deoxynivalenol (deoxynivalenol-3glucoside) from field barley through malt to beer, *Food Additives and Contaminants*, 25: 732-744 (2008).
- **18.** SCHWARZ PB, CASPER HH, BEATTIE S, Fate and development of naturally occurring *Fusarium* mycotoxins during malting and brewing, *Journal of American Society of Brewing Chemists*, 53: 121-127 (1995).
- **19.** VARGA E, MALACHOVA A, SCHWARTZ H, KRSKA R, BERTHILLER F, Survey of deoxynivalenol and its conjugates deoxynivalenol-3-glucoside and 3-acetyl-deoxynivalenol in 374 beer samples, *Food Additives and Contaminants A*, 1: 137-146 (2012).
- 20. European Commision (EC) No 1881 (2006) Official Journal of the European Union L 364/5.
- **21.** NOOTS I, DERYCKE V, JENSEN HE, MICHIELS C, DELCOUR JA, COPPENS T, Studies on Barley Starchy Endosperm Cell Wall Degradation by Rhizopus VII, *Journal of Cereal Science*, 37: 81-90 (2003).
- 22. WOLF-HALL CE, Mold and mycotoxin problems encountered during malting and brewing, *International Journal of Food Microbiology*, 119: 89-94 (2007).
- **23.** CAVAGLIERI LR, KELLER KM, PEREYRA CM, GONZÁLEZ PEREYRA ML, ALONSO VA, ROJO FG, Fungi and natural incidence of selected mycotoxins in barley rootlets, *Journal of Stored Products Research*, 45: 147-150 (2009).
- 24. AWAD WA, GHAREEB K, BÖHM J, ZENTEK J, Decontamination and detoxification strategies for the *Fusarium* mycotoxin deoxynivalenol in animal feed and the effectiveness of microbial degradation, *Food Additives and Contaminants A*, 27: 510-520 (2010).
- **25.** SNIJDERS CHA, VAN EEUWIJK FA, Genotype X strain interactions for resistance to *Fusarium* head blight caused by *Fusarium culmorum* in winter wheat, *Theoretical and Applied Genetics*, 81: 239-244 (1991).
- 26. LEMMENS M, BUERSTMAYR H, RUCKENABAUER P, Variation in *Fusarium* head blight susceptibility of international and Austrian wheat breeding material, *Die Bodenkultur*, 44: 65-78 (1993).
- 27. ZADOKS JC, CHANG TT, KONZAC FC, A decimal code for the growth stages of cereals, *Weed Research*, 14: 415-421 (1974).
- **28.** NELSON PE, TOUSSOUN TA, MARASAS WFO, *Fusarium* species. An Illustrated Manual for Identification. The Pennsylvania State University Press, University Park Londra, PA, 193 pp (1983).
- **29.** LESLIE JF, SUMMERELL AB, The *Fusarium* Laboratory Manual. Blackwell Publishing Professional, Ames, IA, 388 pp (2006).
- **30.** Mitteleuropäische Brautechnische Analysenkommision: Brautechnische Analysenmethoden, Bd. I, 3rd Ed. MEBAK (1997).
- **31.** ILHAM M, REFAI E, The relation between virulence of some Egyptian isolates of *Fusarium graminearum* and deoxynevalenol production in some maize cultivars, *Journal of Biological Sciences*, 3: 103-111 (2003).
- **32.** GOSWAMI RS, KISTLER HC, Pathogenicity and in planta mycotoxin accumulation among members of the *Fusarium graminearum* species complex on wheat and rice, *Phytopathology*, 95: 1397-1404 (2005).
- **33.** WILDE F, MIEDANER T, Selection for *Fusarium* head blight resistance in early generations reduces the deoxynivalenol (DON) content in grain of winter and spring wheat, *Plant Breeding*, 125: 96-98 (2006).
- 34. HART LP, PESTKA JJ, LIU MT, Effect of kernel development and wet periods on production of deoxynivalenol in wheat infected with *Gibberella zeae, Journal of Phytopathology*, 74: 1415-1418 (1984).

- **35.** SCHWARZ PB, SCHWARZ JG, ZHOU A, PROM LK, STEFFENSON BJ, Effect of *Fusarium graminearum* and *Fusarium poae* infection on barley and malt quality, *Monatsschrift Fur Brauwissenschaft*, 54: 55-53 (2001).
- **36.** SMITH J, MOSS M, Mycotoxins formation, analysis and significance. NewYork: John Wiley & Sons (1985).
- **37.** MEDENTSEV AG, ARINBASAROVA AY, AKIMENKO VK, Adaptation of the phytopathogenic fungus *Fusarium decemcellulare* to oxidative stress, *Microbiology*, 70: 26-30 (2001).
- **38.** BOEIRA LS, BRYCE JH, STEWART GG, FLANNIGAN B, Influence of cultural conditions on sensitivity of brewing yeasts growth to *Fusarium* mycotoxins zerealenone, deoxynivalenol and fumonisin B1, *Dechema Monographs*, 50: 69-81 (2002).
- **39.** WOLF CE, BULLERMAN LB, Heat and pH alter the concentration of deoxynivalenol in an aqueous environment, *Journal of Food Protection*, 61: 661-667 (1998).
- **40.** HABSCHIED K, ŠARKANJ B, KLAPEC T, KRSTANOVIĆ V, Distribution of zearalenone in malted barley fractions dependent on *Fusarium graminearum* growing conditions, *Food Chemistry*, 129: 329-332 (2011a).
- **41.** NIESSEN LM, Entwicklung und Andwendung immunchemischer Verfahren zum Nachwies wichtiger *Fusarium*-toxine bei der Bierbereituing sowie mycologiche Untersuchungen im Zuasammenhang mit dem Widwerded von bieren. Doctoral thesis, Tehnical University, Munick (1993).
- **42.** BOEIRA LS, BRYCE JH, STEWART GG, FLANNIGAN B, Inhibitory effect of *Fusarium* mycotoxins on growth of brewing yeasts. 2. Deoxynivalenol and nivalenol, *Journal of the Institute of Brewing*, 105: 376-381 (1999).
- **43.** LORI GA, SISTERNA MN, HAIDUKOWSKI M, RIZZO I, *Fusarium graminearum* and deoxynivalenol contamination in the durum wheat area of Argentina, *Microbiological Research*, 158: 29-35 (2003).
- 44. LAMPER C, TEREN J, BARTOK T, KOMOROCZY R, MESTERHAZY A, SAGI F, Predicting DON contamination in *Fusarium*-infected wheat grains via determination of ergosterol content, *Cereal Research Communication*, 28: 337-344 (2000).
- **45.** VELIĆ N, PAVLOVIĆ H, ĆOSIĆ J, KANIŽAI G, KRSTANOVIĆ V, A survey of *Fusarium* graminearum and deoxynivalenol contamination of malt barley from the crop year 2004 in Eastern Croatia, *Cereal Research Communication*, 35: 1293-1296 (2007).
- **46.** HABSCHIED K, VELIĆ N, TIŠMA M, KRSTANOVIĆ V, The effect of barley and wheat microflora on malt and beer quality, *Croatian Journal of Food Technology, Biotechnology and Nutrition*, 6: 100-111 (2011b).
- 47. TRUCKSESS MW, POHLAND EE, Mycotoxin protocols. New Jersey: Humana Press Inc (2001).
- **48.** KOSTELANSKA M, HAJSLOVA J, ZACHARIASOVA M, MALACHOVA A, KALACHOVA K, POUSTKA J, FIALA J, SCOTT PM, BERTHILLER F, KRSKA R, Occurrence of deoxynivalenol and its major conjugate, deoxynivalenol-3-glucoside, in beer and some brewing intermediates, *Journal of Agricultural and Food Chemistry*, 57: 3187-3194 (2009).
- **49.** ZACHARIASOVA M, HAJSLOVA J, KOSTELANSKA M, POUSTKA J, KRPLOVA A, CUHRA P, HOCHEL I, Deoxynivalenol and its conjugates in beer: a critical assessment of data obtained by enzyme-linked immunosorbent assay and liquid chromatography couple to tandem mass spectrometry, *Analitica Chimica Acta*, 625: 77-86 (2008).