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Occurrence of Fusarium Head Blight and Mycotoxins as well as Morphological Identification of *Fusarium* Species in Winter Wheat in Kosovo

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Wheat (*Triticum aestivum* L.) is an important cereal crop in Kosovo and a major component of population food. Fusarium head blight (FHB) of wheat is an economically very significant disease. FHB leads to various losses in quality like reduced germination of seeds, reduced baking quality and reduced nutritional quality through mycotoxin contamination. In 2010 and 2011 the incidence and identity of the *Fusarium* spp. infecting wheat in Kosovo as well as mycotoxin contamination was investigated. The results of two years research work show that the incidence of FHB on winter wheat in 2010 was low (<6%). In the year 2011 the disease incidence was clearly higher (up to 31%). Based on morphological characters, *F. graminearum* was the most frequently *Fusarium* sp. identified on wheat kernels in the year 2010 (100%) and 2011 (98%). Less frequently isolated species included *F. cerealis* (<1%) and *F. avenaceum* (<1%). Wheat flour samples were analyzed by liquid chromatography coupled to mass spectrometry and found to be contaminated with a variety of mycotoxins, most importantly deoxynivalenol and zearalenone. This is the first report on the incidence as well as on the identification of *Fusarium* species isolated from naturally infected winter wheat in Kosovo.

Keywords: Fusarium head blight, *Fusarium* species, *Triticum aestivum*, Kosovo

Introduction

In Kosovo approximately 80 000–100 000 ha of wheat are cultivated annually (Statistical Office of Kosovo 2009). The seeds for cultivation are largely imported from neighbouring countries. To date no cultivars for multiplication are available. Also, the average

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wheat yield of 2,500–3,000 kg ha^{-1} (Statistical Office of Kosovo 2009) does not fulfil the domestic demand of Kosovo. As a consequence the selection of suitable wheat cultivars for the producing region Kosovo is crucial in order to increase crop yield and to improve quality. In addition to the parameters "quality and yield", fungal diseases represent a central problem.

Fusarium head blight (FHB) in wheat is a widespread disease around the world including all European cereal-growing areas (Bottalico and Perrone 2002). The extent of the damage increases with the intensification of grain production. The disease leads to various losses in quality, including reduced germination in seeds, reduced baking quality due to the breakdown of wheat storage proteins and reduced nutritional quality through contamination with mycotoxins (Champeil et al. 2004; Mesterházy 2005). Based on economic and environmental aspects, host plant resistance is considered to be the most appropriate method to control FHB (Ruckenbauer et al. 2001).

In extreme cases the wheat heads infected with *Fusarium* are completely bleached (white heads) and show a limited kernel set. The kernels are often shrivelled. Usually individual spikelets or parts of the wheat head are infected. The infected parts die off and appear somewhat lighter (straw like colour). Often, a salmon-coloured coating (spore agglomeration) also occurs on the chaff. A white, rose or red colouring of the grains can also be a consequence of this disease. If the infection of the wheat takes place in a very early phase during flowering, already the young, green wheat heads can be strongly attacked by fungi. The fungus is seed-borne or initiates from the soil, however, the most important source of infection is represented by crop debris from the previous crop on the soil surface on which the fungi prevail over winter (Sutton 1982). Maize as a preceding crop promotes the incidence of *Fusarium* spp. including *F. graminearum* in winter wheat (Dill-Macky and Jones 2000; Schaafsma et al. 2005; Blandino et al. 2010; Vogelgsang et al. 2011). An explanation for this fact is the infection progress of the pathogen. Already at the time of maize maturation onset, *Fusarium* can penetrate the lower stem portion of the maize plant. After the harvest, most of the maize stubble is colonized with *Fusarium*. The maize straw remaining on the soil, with its high nitrogen content, appears to be a suitable culture medium for the cultivation of the main progeny form of the fungi in the next vegetation period. The ascospores produced by *Gibberella zeae* in the perithecia can be actively ejected and are the source of the new infection. This takes place in a monocyclic occurrence from the spore repositories on the soil surface directly onto the wheat heads. The level of infection highly depends on the year and location and is also largely influenced by weather conditions (Lemmens et al. 2004; Lori et al. 2009; Lakhesar et al. 2010).

Fusarium head blight in wheat is caused by several species of *Fusarium*. In general, the causal agents of FHB in Europe are primarily *F. graminearum* (teleomorph *Gibberella zeae*), *F. culmorum* (teleomorph unknown) and *F. avenaceum* (teleomorph *G. avenacea*). Less frequently represented species are *F. poae*, *F. cerealis*, *F. equiseti*, *F. sporotrichioides*, *F. tricinctum* and, to an even lesser extent also *F. acuminatum*, *F. subglutinans*, *F. solani*, *F. oxysporum*, *F. verticillioides*, *F. semitectum* and *F. proliferatum* (Bottalico and Perrone 2002; Lemmens et al. 2004; Stepień et al. 2008).

The aim of this study was 1) to demonstrate the possible role of Fusarium head blight of wheat in Kosovo, 2) to identify the *Fusarium* species isolated from naturally infected wheat kernels, and 3) to demonstrate that the mycotoxins predicted to be produced by these *Fusarium* spp. were indeed present in kernels samples. Within the framework of this project, field and laboratory tests were carried out on wheat in 2010 and 2011 at five locations from the two most important wheat growing regions in Kosovo (the Kosovo region and the Dukagjini region).

Materials and Methods

Locations and cultivars

During the vegetative period in 2010 and in 2011 five locations within two regions of Kosovo were selected (Fig. 1). The first region was the Kosovo region with the locations Baks and Skifter: altitude around 550 meters, mean annual temperature 16.5 °C, soil type: vertisol and chernozem. The second region was the Dukagjini region (Gurakoc, Novosellë, Prizren): altitude around 370 meters, mean annual temperature 17.5 °C, soil type:

Figure 1. Map of Kosovo showing the locations where visual field analyses and *Fusarium*-infected kernels were collected in 2010 and 2011 (map modified after Nord Nord West Version of 23 November 2009). http://commons.wikimedia.org/wiki/File:Kosovo_location_map.svg?uselang=en (Accessed 15 December 2012) published under the licence of Creative CommonsAttribution-ShareAlike 3.0 Unported (CC BY-SA 3.0), http://creativecommons.org/licenses/by-sa/3.0/deed.en

alluvial, brown, alluvium/pseudogley and pelosol, continental climate. The preceding crop was maize in all cases. The fields were selected from local farmers and were located in the main wheat growing regions in Kosovo. The sampled wheat cultivars depending on the location were Evropa, Renesanca and Ritma. For these genotypes there is no information available on the resistance to Fusarium head blight. Evropa was sampled at the locations Prizren and Skifter in 2010 and 2011, at Baks and at Gurakoc in 2011. Renesanca was investigated at Novosellë in 2010 and 2011 and at Gurakoc in 2010. Ritma was sampled only at the location Baks in 2010. The areas of the plots were 0.75 ha in Baks, 0.4 ha in Skifter, 0.4 ha in Novosellë, 0.3 ha in Gurakoc and 0.7 ha at the location Prizren. The number of replications was four. As usual in Kosovo, fertilization was done with N:P:K 120:80:80 kg ha^{-1}. To control weeds the following herbicides were used depending on the location: Senkor, Agrosan, Mustang and Maton 2.4-D. The rates were applied according to the protocol of the producers. No fungicides were applied.

Field analyses

For disease assessment wheat heads were rated at the hard dough stage. One hundred wheat heads from each replication were harvested by hand and subsequently analyzed in the laboratory for disease incidence (DI; percentage of diseased ears) and disease severity (DS) on a 0 to 5 scale in which 0 equals no symptoms, 1 = 1–5%, 2 = 6–10%, 3 = 11–25%, 4 = 26–50% and 5 equals infection of over 50% of spikelets of the wheat ear (EPPO 1997).

Laboratory analysis

To isolate *Fusarium* species, kernels with visible symptoms were collected directly during the visual analysis from the wheat ears. They were surface-sterilised in 80% ethanol for two minutes, air dried and transferred onto **S**ynthetic **N**utrient-poor **A**gar (SNA) (1 g KH_2PO_4; 1 g KNO_3; 0.5 g $MgSO_4 \cdot 7\ H_2O$; 0.5 g KCl; 0.2 g glucose; 0.2 g saccharose; 1 L H_2O dest.; 22 g agar; 0.6 mL 1N NaOH) supplemented with antibiotics (Nirenberg 1981; modified). After 7–14 days incubation in an incubator (temperature 25 °C and humidity 80%, illumination with dark UV light), a single spore technique was used to obtain pure cultures for identification. Potato dextrose agar (PDA) (Difco, Detroit, MI) medium was used to assess colony characteristics such as pigmentation and growth rate. SNA medium, supplemented with a piece of filter paper to promote sporulation, was used to examine conidial morphology and to detect the presence of chlamydospores. Black UV light (36 W) was used to improve sporulation of isolates. Individual *Fusarium* species were identified (Nirenberg 1981; Nelson et al. 1983; Singh et al. 1991). From the 250 kernels we collected in total during both years, 228 kernels were infected with *Fusarium* species. From the other kernels no *Fusarium* was growing out or we were not able to obtain pure *Fusarium* colonies due to contamination with other fungi.

Mycotoxins

Detection and quantification of the mycotoxins in wheat was carried out by high performance liquid chromatography-tandem mass spectrometry. A multi-mycotoxin method for wheat (Sulyok et al. 2006) was enhanced to cover a total of 186 mycotoxins and other fungal and bacterial metabolites (Vishwanath et al. 2009). The kernel samples were taken after threshing with a plot combiner and were milled. Only samples from 2011 were analyzed (Table 2) and the 4 replications were pooled. From 1 kg milled wheat a representative sub-sample of 10.00 ± 0.01 g ground wheat was extracted for 90 min on a rotary shaker using 40 mL acetonitrile/water/acetic acid (79/20/1, v/v/v). The centrifuged extract was diluted 1+1 with acetonitrile/water/acetic acid (20/79/1, v/v/v). Five µL of the diluted extract were injected using an Agilent 1100 HPLC system (Waldbronn, Germany). Separation was achieved on a 150 × 4.6 mm i.d., 5 µm particle size, Phenomenex Gemini RP-C18 column (Torrance, USA) using a linear aqueous ammonium acetate/acetic acid/methanol gradient from 10 to 97% organic solvent at a flow rate of 1 mL/min at 25 °C. Detection of mycotoxins was achieved using an ABSciex4000 QTrap mass spectrometer (Foster City, USA) equipped with a TurboV electrospray source. Two injections were used for each sample, one for each ion source polarity. The scheduled single reaction monitoring mode was used to acquire data, which were further evaluated using Analyst version 1.5.2. Mixed standard solutions containing all 186 analyses at different concentration were used for external calibration. All data were corrected for the apparent recovery according to Sulyok et al. (2006).

Statistical analyses

Data taken were analyzed for variance (ANOVA) and the means as well as the standard deviations ($n = 4$) were calculated. The analysis of variance was performed only for the disease incidence and severity. SPSS Version 15.0 for MS Windows was used for the analyses.

Results

Generally in 2010 the disease incidence (DI) was low and in all cultivars below 6% (Table 1). In 2011 the DI was higher and varied from 4 to 31%. Also, disease severity (DS) showed a similar picture: DS was higher in the second season (from 11 to 24%). The highest DI and disease severity (DS) in both years showed the cultivar Evropa at the location Prizren.

Identified *Fusarium* spp. from each cultivar in 2010 and 2011 are summarized in Table 1. Based on morphological characters *F. graminearum* was the only species identified in 2010. Two *Fusarium* strains isolated from the cultivar Renescanca at the location Novosellë could not be clearly identified at the species level. In 2011, the most frequently *Fusarium* spp. identified was again *F. graminearum* (98%). Less frequently isolated species included *F. cerealis* and *F. avenaceum* (<1%).

Table 1. Percentage of disease incidence and disease severity of *Fusarium* head blight on winter wheat, total number of infected kernels and *Fusarium* species (%) identified on wheat kernels

Location, Wheat cultivar (Year of cultivation)	2010							2011					
	DI	DS	TNIK	*F. graminearum*	*F. avenaceum*	*F. cerealis*	*Fusarium* spp.	DI	DS	TNIK	*F. graminearum*	*F. avenaceum*	*F. cerealis*	*Fusarium* spp.
Baks, Ritma (2010) Evropa (2011)	4.3±2.2	8.5±3.9	14	100				n.d.	n.d.					
Gurakoc, Renesanca (2010) Evropa (2011)	n.d.	n.d.						4.0±2.4	11.1±5.6	15	93			7
Novosellë, Renesanca (2010 and 2011)	0.5±0.6	2.5±2.9	2				100	11±4.1	11±1.8	41	100			
Prizren, Evropa (2010 and 2011)	5.5±3.1	17±3.3	15	100				31±3.5	23±13.1	116	99		1	
Skifter, Evropa (2010 and 2011)	3.3±2.1	8±4.8	11	100				3.5±2.6	16±4.8	14	86	7		7

DI and DS values are means ± standard deviation ($n = 4$).
DI: disease incidence; DS: disease severity; TNIK: total number of investigated infected kernels; n.d.: no diseased ears were found.

Table 2. Mycotoxin contamination (µg/kg) in wheat from Kosovo in 2011. The four replications were pooled for the analyses

Location, Wheat cultivar	MON	NIV	DON	D3G	3ADON	15ADON	ZEN	FB1	ENNA	ENNA1	ENNB	ENNB1	ENNB2	CUL
Baks, Evropa	<LOQ	<LOQ	<LOQ	<LOQ	4.3	14	280	57	<LOQ	<LOQ	<LOQ	0.1	<LOQ	<LOQ
Gurakoc, Evropa	<LOQ	28	420	170	74	13	340	11	0.2	1.2	3.2	3.6	<LOQ	91
Novosellë, Renesanca	80	24	2860	1030	30	150	340	22	0.8	12	68	57	4.4	640
Prizren, Evropa	<LOQ	31	6310	2300	73	360	350	19	0.1	0.9	3.4	3.4	0.2	1210
Skifter, Evropa	<LOQ	7.3	430	780	<LOQ	29	340	41	<LOQ	<LOQ	0.5	0.4	<LOQ	150

MON, moniliformin; NIV, nivalenol; DON, deoxynivalenol; D3G, deoxynivalenol-3-glucoside; 3ADON, 3-acetyl-DON; 15ADON, 15-acetyl-DON; ZEN, zearalenone; FB1, fumonisin B_1; ENNA, enniatin A; ENNA1, enniatin A1; ENNB, enniatin B; ENNB1, enniatin B1; ENNB2, enniatin B2; CUL, culmorin; <LOQ, detected but below limit of quantification.

Regarding mycotoxins, the following substances were detected in the wheat samples from 2011 (Table 2): moniliformin (MON), nivalenol (NIV), deoxynivalenol (DON), deoxynivalenol-3-glucoside (D3G), 3-acetyl-DON (3ADON), 15-acetyl-DON (15ADON), zearalenone (ZEN), fumonisin B_1 (FB1), enniatin A (ENNA), enniatin A1 (ENNA1), enniatin B (ENNB), enniatin B1 (ENNB1), enniatin B2 (ENNB2) and culmorin (CUL), all of which are produced by various *Fusarium* spp. As expected from the high DI and DS, the sample obtained from Prizren was also the one with the highest concentration of mycotoxins, exceeding 6 mg/kg DON, 2 mg/kg D3G and 1 mg/kg CUL. Interestingly, the level of ZEN found in the samples was very constant (at around 300 µg/kg), regardless of the location. Enniatins were found in all samples at very low concentrations, with the exception of the wheat from Novosellë, which was contaminated with ENNB and ENNB1 at levels exceeding 50 µg/kg. In the sample from Baks, which showed no FHB symptoms *in planta*, with the exception of ZEN, no other mycotoxins could be found in relevant concentrations.

Discussion

The purpose of this study was to analyze the incidence and identity of the *Fusarium* species isolated from naturally infected winter wheat kernels in Kosovo as well as mycotoxin contamination. In particular, through relevant field analyses and laboratory tests, this two-year research work generated important information about the *Fusarium* spp. encountered in Kosovo on naturally infected wheat and their contamination with mycotoxins.

The results showed that Fusarium head blight on wheat is an important problem in Kosovo. The disease incidence of FHB on wheat on the sampled fields varied in both years: in 2010 it was low, but in 2011 the disease incidence and severity was substantially higher. In one location 31% of the investigated ears showed symptoms. On the other hand, the disease was not detected on one location in each season. It is very well known that the level of incidence of FHB is highly depending on the year, location, cultivar and weather conditions (Dill-Macky and Jones 2000; Champeil et al. 2004; Lori et al. 2009). The preceding crop in all locations was maize, which promotes the incidence of *Fusarium* spp. in winter wheat (Vogelgsang et al. 2011). The highest percentage of DI and DS of FHB was recorded at the location Prizren, exactly the same place where we have also seen the highest DI of *Fusarium* ear rot in the preceding maize crop (Shala-Mayrhofer et al. 2013). It has to be mentioned, that at this location irrigation was used during maize cultivation. We hypothesize that irrigation resulting in a high ear rot disease level in maize is responsible for a high inoculum potential in the following wheat crop.

Based on morphological characters, *F. graminearum* was the most frequent species identified. Less frequently isolated species included *F. cerealis* and *F. avenaceum*. The latter were found only in 2011. These toxigenic *Fusarium* species isolated from wheat are in good accordance with previous reports in other European countries (Mesterházy 2005; Bottalico and Perrone 2002) as well as in Serbia (Lević et al. 2009) and in Croatia (Ivič et al. 2009; Spanič et al. 2010).

Mycotoxins are secondary metabolic products of various fungi, which play an important role in the spoiling of animal feed and human food products, and represent one of the most serious consequences of *Fusarium* infection. While the overall sample set was very small, still high amounts of toxins were found in the sample from Prizren and Novosellë. The values found for DON in those two samples are exceeding the regulated maximum level (1250 µg/kg) for human consumption in the EU (Commission Regulation (EC) No. 1881/2006). *F. graminearum* and *F. cerealis* can also produce NIV and ZEN. All investigated wheat samples would exceed the European Union regulated limit for ZEN (100 µg/kg) for unprocessed cereals intended for food. We have no explanation for this high level of ZEN and it should be stated that a larger set of samples should be investigated to confirm this. All other detected toxins are either not regulated in the EU or well below the maximum level in case of FB1. Interestingly, culmorin (Ghebremeskel and Langseth 2001) and hydroxyculmorins (not shown) – secondary metabolites from *F. graminearum* or *F. culmorum* – were found in the majority of the samples. The toxicity of culmorin is rather low, therefore the found values are of little concern. *F. graminearum* probably causes the contamination with the monoacetyl-deoxynivalenols. *F. avenaceum* could be responsible for the MON and ENN contamination of the kernels. Fumonisins are toxins known to be produced by *F. proliferatum* and *F. verticillioides* which are important *Fusarium* ear rot pathogens in maize. They are considered to be less pathogenic or opportunistic *Fusarium* species on small grain cereals (Bottalico and Perrone 2002). Last but not least the fraction "masked" trichothecenes (deoxynivalenol-3-glucoside) should be mentioned. D3G is a plant metabolite of DON. The D3G concentrations in 3 out of 4 samples were in the range of 36–40% of the non-conjugated mycotoxin, but exceeds the contamination with DON in the sample from Skifter.

In conclusion we found that Fusarium head blight on wheat in Kosovo is present and the incidence can be high. The most frequently *Fusarium* sp. identified on wheat kernels was *F. graminearum.* Wheat samples were contaminated with up to 14 different Fusarium mycotoxins including D3G. Based on these results, further research should be done in Kosovo and the mycotoxins deoxynivalenol and zearalenone should be monitored to control food and feed quality. Furthermore, suitable wheat varieties should be selected for the producing region in Kosovo in order to increase FHB resistance and crop yield.

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