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Occurrence of potentially toxigenic *Fusarium verticillioides* and low fumonisin B_1 content on barley grain in Bosnia and Herzegovina

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Summary

A study was conducted to determine the mycobiota, the presence of potential fumonisin producers, and eventual fumonisin B_1 contamination on four barley grain samples from Bosnia and Herzegovina. *Alternaria* spp. were the dominant fungi, detected on 82 %, 77 %, 78 % and 82 % of kernels in different samples. *Fusarium* spp. was found in all samples, on 26 %, 40 %, 49 % and 60 % of kernels. Among *Fusarium* spp., *F. verticillioides* was the most frequent in three samples (40 %, 49 % and 60 %), while *F. graminearum* was the most frequent in one sample (12 %). *F. avenaceum*, *F. sporotrichioides*, *F. solani*, *F. semitectum*, *F. tricinctum*, *F. equiseti* and *F. oxysporum* were found in lower percentages. Other fungal species found in all samples were *Cladosporium* spp., *Epicoccum* spp., *Gonatobotrys* spp. and *Drechslera* spp. Contamination of grain with *Aspergillus* spp. and *Penicillium* spp. was very low. PCR amplification with FUM5/6 primer pairs was performed on 29 *F. verticillioides* isolates from the grain, and all isolates yielded 419 bp amplification products. Fumonisin B_1 content in grain determined by ELISA was very low (5.35, 1.68, 1.48, and 1.01 ng/g), with an average of 2.40 ng/g for all four samples.

Introduction

Cereal grain is usually infected or colonised by different pathogenic or saprophytic fungi during the vegetation period. Numerous fungal species are reported to occur on barley grain (Rohácik and Hudec, 2007; Medina et al., 2006; Andersen et al., 1996), some of which are economically important as seed-borne pathogens (Maude, 1996), or producers of mycotoxins, fungal metabolites toxic to humans or animals (Bottalico and Perrone, 2002). Different *Fusarium* species, causal agents of barley head blight (McMullen et al., 1997) and producers of numerous mycotoxins (De Nijs et al., 1996), are among those fungi which regularly colonize barley grain (Leslie and Summerell, 2006; Bottalico and Perrone, 2002). Barley grain from different European countries was found to be contaminated with one or more mycotoxins produced by *Fusarium* species, mostly with zearalenone (ZEN) or trichothecenes like deoxinivalenol (DON) (Malachova et al., 2010; Tabuc et al., 2009; Bottalico and Perrone, 2002). These mycotoxins are produced by *Fusarium graminearum*, *F. culmorum* or *F. crookwellense* (Leslie and Summerell, 2006), pathogenic species which cause head blight of barley and other small-grain cereals. However, numerous other *Fusarium* species can colonize barley grain as secondary invaders or saprophytes. Several recent studies showed that *F. verticillioides* and *F. proliferatum* are common on barley grain (Maenetje and Dutton, 2007; Medina et al., 2006). As those species are the main producers of fumonisins, cancerogenic mycotoxins (Leslie and Summerell, 2006), it would be important to find out whether these toxins are produced on barley grain. Fumonisins are mycotoxins mainly found in maize (Munkvold and Desjardins, 1997), but there are reports on their occurrence in other plants (Seefelder et al., 2002; Logrieco et al., 1998). The presence of fumonisin B_1 was detected in wheat (Ivic et al., 2008; Desjardins et al., 2007), and small quantities of these mycotoxins were found in beer (Torres et al., 1998). These studies indicate that fumonisins could be found on barley grain as well.

Studies on mycotoxin production on molecular and genetic level have lead to the development of PCR-based detection of fungal genes needed for different mycotoxin production (Niessen, 2007; Nicholson et al., 2004; Niessen et al., 2004). PCR primers based on sequence data of polyketide synthase gene (FUM 1) were recently developed for the detection of fumonisin-producing *F. verticillioides* (Baird et al., 2008). Although not without certain constraints (Paterson, 2006), PCR amplification of FUM genes could become rapid and reliable method for detecting potential fumonisin-producing fungi in plants or plant parts. It could also be used for screening the potential ability of *F. verticillioides* isolates to produce fumonisins.

In a routine microbiological analysis of stored barley grain from Bosnia and Herzegovina during the last few years, a relatively high level of contamination with *Fusarium* spp. of the *Liseola* section was observed, resembling mostly *F. verticillioides* or *F. proliferatum*. The objectives of this study were to determine the presence of potential fumonisin B_1 producers and eventual fumonisin B_1 contamination on barley grain in Bosnia and Herzegovina. The other objectives were to determine eventual differences in mycobiota on several grain samples from the same region, especially regarding the presence of *Fusarium* species.

Materials and methods

Grain origin, sampling, and mycological analysis

Spring barley (cv. Novosadski 294) was grown in 2008 on different locations around the area of Posusje, Bosnia and Herzegovina. Data on temperatures and rainfall in the area of Posusje in 2008 (April, May and June) was obtained from Federal Hydrometeorological Institute in Sarajevo. Grain was harvested in June, and approximately one kg of randomly selected harvested grain was collected for mycobiota and mycotoxin analysis. For mycological analysis, 100 kernels in four replicates was placed on moist blotter and incubated under 12/12 h photoperiod at 22 °C for 14 days. After incubation, each kernel was checked for the presence of fungi under the stereomicroscope and microscope. Fungi other than *Fusarium* spp. on kernels were identified only to the genus level based on the conidial morphology according to descriptions of Barnett and Hunter (1998) and Samson et al. (1984). If a colony of *Fusarium* was detected, kernel was placed on potato-dextrose agar (PDA) and incubated at the same conditions as described above for additional seven days. *Fusarium* species were determined according to Leslie and Summerell (2006) from colonies developed on PDA, while non-sporulating colonies or colonies resembling *F. graminearum* or *F. avenaceum* were subcultured using a single-spore technique described by Leslie and Summerell (2006), transferred to carnation leaf agar (CLA), and identified. Mycobiota on kernels was expressed as a percentage of kernels on which certain fungal genera or species were found.

F. verticillioides isolation, identification, and PCR amplification of FUM fragments

From PDA cultures, 29 isolates identified as *F. verticillioides* were collected using a single-spore technique (LESLIE and SUMMERELL, 2006). Five isolates were collected from the Posusje I sample, while 8 isolates were collected fom each of the other three grain samples. A single-spore isolate of *F. semitectum* collected from the Posusje III sample was used in PCR reaction as a negative control. For DNA extraction, isolates were grown on cellophane-layer PDA, from where mycelia was harvested and ground to a powder with a liquid nitrogen. Fungal DNA was extracted using DNeasy® Plant Mini Kit (Quiagen Inc., USA) according to manufacturer's instructions and checked on agarose gel. Two to five µl of dilluted DNA extract was used in 50 µl PCR reactions containing 5 µl of 10x PCR buffer, 4 µl of 2.5 mM dNTPs, 2.5 µl of $MgCl_2$, 1 U of Taq polymerase and 25 pmoles of FUM5F and FUM6R primers (BAIRD et al., 2008). PCR conditions were the following: 95 °C for 3 min, 33 cycles of 95 °C for 1 min, 60 °C for 1 min, and 72 °C for 3 min, with a final extension on 72 °C for 5 min. PCR products were visualised and photographed after electrophoresis through 1.5 % TBE agarose gels, stained with ethidium-bromide.

All isolates used in this research were stored on water agar (WA), and they are available at the Institute for Plant Protection collection in Zagreb, Croatia.

Fumonisin B_1 analysis

ELISA test (Fumonisin B1 EIA kit, Euro-Diagnostica B.V., Arnhem, The Netherlands) was used for fumonisin B_1 quantification on grain of each sample. Approximately 50 g of grain was pulverised, extracted with 80 % methanol and filtrated through Whatman No.1 filter paper. An aliquot of 50 µl of extract was used for analysis in two replicates. ELISA was performed according to the manufacturer's instructions.

Results

Colonies of different fungi were detected on almost all analysed barley kernels in all periods of analysis (Tab. 1). Species from two or more genera were often found to be present on the same kernel. Mycobiota was very similar in all four samples. *Alternaria* spp. were the dominant fungi on grain, found on average 80 % of kernels. *Fusarium* spp. were the second in prevalence on grain, detected on average 44 % of kernels. *F. verticillioides* was found to be the dominant *Fusarium* species in three samples, while *F. graminearum* was found in the highest percentage in one sample. Some *Fusarium* species sporulated well on kernels after the incubation (e.g. *F. avenaceum*, *F. sporotrichioides*, *F. verticillioides* or *F. semitectum*) while most of the colonies later determined to be *F. graminearum* developed as sterile mycelium on kernels and had to be transferred and identified on PDA and CLA.

Average temperatures in 2008 in period from "boot" stage of experimental barley to the harvest (April, May and June) were similar to the 10-year average (Tab. 2). However, average rainfall in April and June was higher than average, while in May it was almost 50 mm lower than average.

Tab. 2: Average temperatures (°C) and rainfall (mm) in Posusje for April, May and June 2008, compared to 10-year average.

Month	2008		10-year average	
	Temperature (°C)	Rainfall (mm)	Temperature (°C)	Rainfall (mm)
April	14.0	162.0	16.5	119.2
May	20.0	30.3	21.9	87.8
June	23.5	121.9	24.6	82.7

Tab. 1: Contamination of barley grain with fungi (%) and fumonisin B_1 (ng/g).

Fungal genera/species	Grain samples				Average
	Posusje I	Posusje II	Posusje III	Posusje IV	
	% of kernels contaminated				
Alternaria spp.	82	77	78	82	79.8
Cladosporium spp.	24	18	11	3	14
Epicoccum spp.	20	10	7	11	12
Drechslera spp.	17	9	10	14	12.5
Gonatobotrys spp.	14	27	17	21	19.8
Khuskia oryzae	0.3	1	3	1	1.4
Aspergillus spp.	0.5	0.8	2	0.3	0.9
Penicillium spp.	0.5	2	0.8	3	1.6
Gliocladium spp.	0.3	-	-	-	0.1
Fusarium graminearum	12	10	8	10	10
Fusarium avenaceum	2	1	5	0.8	2.2
Fusarium verticillioides	6	26	32	42	26.5
Fusarium solani	2	-	0.5	-	0.6
Fusarium sporotrichioides	2	1	2	2	1.8
Fusarium semitectum	0.8	0.5	1	5	1.8
Fusarium tricinctum	0.5	1	-	0.3	0.5
Fusarium equiseti	0.3	-	-	-	0.1
Fusarium oxysporum	0.5	0.5	0.3	0.3	0.4
Fumonisin B_1 content (ng/g)	5.350	1.680	1.482	1.099	2.40

PCR products of 419 bp amplified with FUM5F/FUM6R primer pairs were recorded for all 29 *F. verticillioides* isolates. (Fig. 1). No products were visible for *F. semitectum* negative control isolate and for water blanks.

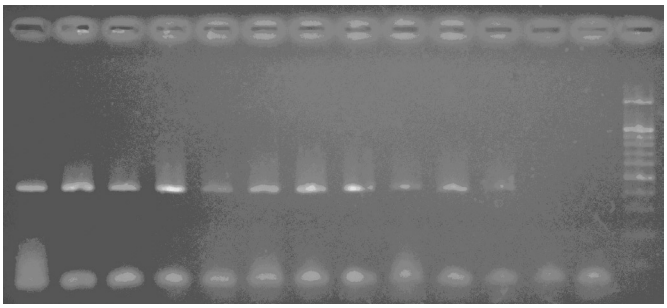

Fig. 1: PCR products of *F. verticillioides* isolates amplified with FUM5F/FUM6R primer pairs. Lanes 1-11: Fv26; Fv27; Fv28; Fv29; Fv30; Fv32; Fv33; Fv34; Fv37; Fv39; Fv40 (all *F. verticillioides*); lane 12: Fs36 (*F. semitectum*); lane 13: water blank; lane 14: 1 kb DNA ladder.

Discussion

Alternaria, *Gonatobotrys* and *Fusarium* species were found in the highest percentage on barley grain during the storage. Similar percentages of *Alternaria* spp. contamination was reported from other studies (ROHÁCIK and HUDEC, 2007; MEDINA et al., 2006; ANDERSEN et al., 1996). The presence of *Epicoccum*, *Cladosporium* and *Drechslera* species in all samples is not unexpected, as those species often colonize seed and grain of various plants (MAUDE, 1996; SAMSON et al., 1984). Population structure of *Fusarium* spp. on grain was also similar in all four samples. The only noticeable difference was between relatively low contamination level of *F. verticillioides* on Posusje I sample and the other three samples, where *F. verticillioides* was detected in high percentages. Considering the presence of *F. graminearum*, *F. avenaceum* and other species found in this study, all of them are reported as the usual colonizers of barley grain (LESLIE and SUMMERELL, 2006; KRSTANOVIC et al., 2005; BOTTALICO and PERRONE, 2002). It could be assumed that the high rainfall in June, during the ripening stages of barley, was conductive to the colonization of saprophytic fungi, like *Alternaria*, *Epicoccum* or *F. verticillioides*, on developing grain. On the other hand, low rainfall conditions in May, during the flowering stage, might has not be favourable to the development of Fusarium head blight and more extensive colonization of grain with pathogenic *F. graminearum*.

Relatively high contamination level of *F. verticillioides* detected (average 27 %) can indicate the possibility of fumonisin contamination. *F. verticillioides*, *F. proliferatum* and fumonisin B_1 were detected on barley grain in South Africa (MAENETJE and DUTTON, 2007). *F. verticillioides* was the most prevalent fungal species found on barley rootlets used as feedstuff raw material in Brazil, and all samples were contaminated with fumonisin B_1 (CAVAGLIERI et al., 2009). Based on their results, CAVAGLIERI et al. (2009) pointed out that the presence of *F. verticillioides* on barley rootlets could be correlated with fumonisin B_1 contamination. In the study of MEDINA et al. (2006), *F. verticillioides* and *F. proliferatum* were also among the most frequent *Fusarium* spp. found on barley grain. MEDINA et al. (2006) studied the production of fumonisin B_1 by 31 *F. verticillioides* and 18 *F. proliferatum* isolates from barley grain, and most of them were capable of fumonisin production on a rice grain medium.

Amplification of polyketide synthase gene with FUM5/6 primer pairs showed that all 29 *F. verticillioides* isolates tested are potential fumonisin producers. Considering that these isolates were randomly collected from the kernels, it can be assumed that the high percentage of *F. verticillioides* on analyzed barley grain is capable of producing fumonisins. However, fumonisin B_1 content in all four samples was very low. It is interesting to notice that the relatively highest amount of fumonisin B_1 was found in the sample Posusje I, on which low *F. verticillioides* contamination was detected, while the relatively lowest content of fumonisin B_1 was found on sample Posusje IV, heavily contaminated with *F. verticillioides*. To a certain extent, such results can show that in some cases mycotoxin content can not be predicted on the basis of the extent of fungal contamination. Beside various environmental factors (MARÍN et al., 1999a; MARÍN et al., 1999b), it is also known that the quantity of fumonisin B_1 produced in different conditions is dependent on strain of *F. verticillioides* (MEDINA et al., 2006; REYONSO et al., 2004).

Average fumonisin B_1 content of 2.40 ng/g detected in this study is far below those reported to occur on maize (LOGRIECO et al., 2002). One of the reasons could be in the difference between saprophytic nature of *F. verticillioides* on barley and parasitic nature of the same fungal species on maize. *F. verticillioides* is considered to be a saprophyte on barley grain (LESLIE and SUMMERELL, 2006). On the other hand, this fungus is the causal agent of Fusarium ear rot of maize, and high fumonisin B_1 content in maize grain is often correlated with ear rot (MUNKVOLD and DESJARDINS, 1997). When the fungus is a parasite, it has an ability to grow through plant tissue without competing with other micro-organisms, and consequently to develop much more mycelial mass. The other reason for low fumonisin B_1 contamination of analyzed grain may be in environmental factors not conductive for fumonisin production. Fumonisin production is dependent on many factors, out of which temperature and water availability are among the most important ones (MARÍN et al., 1999a). Comparing the kernel development period in barley and maize in Europe, it is clear that the maize grain is developing and maturing in warmer periods of the season. It is not known when *F. verticillioides* colonize barley kernels in the field. If such colonization occurs late in the season, when kernels are approaching maturity, the fungus may simply not have enough time to produce higher quantities of mycotoxins. The moisture content of maturing barley kernels could descend to the level which does not support further *F. verticillioides* growth or fumonisin production. In cases of Fusarium ear rot of maize, the moisture content of grain and humidity under the husks usually allows *F. verticillioides* or *F. proliferatum* intensive growth. Finally, it could be assumed that barley grain is simply not a good substrate for fumonisin production. MARÍN et al. (1999b) compared fumonisin B_1 production by *F. verticillioides* and *F. proliferatum* isolates on maize, barley and wheat grain in controlled conditions. Fumonisin B_1 production by both *Fusarium* species was much higher on maize grain than on wheat or barley grain.

Considering all of the theories which could explain the results of this study, it can be assumed that barley grain as an unsuitable substrate, late colonisation, and saphrophytic growth of *F. verticillioides* could all, in certain amount, contribute to the low level of fumonisin production on grain. The results of the present study showed that relatively high contamination levels of fumonisin-producing *Fusarium* species on grain sometimes may not lead to high levels of fumonisin contamination.

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