CAPACITY OF FUSARIUM SPECIES ISOLATED FROM BREWER’S BARLEY TO SYNTHESISE ZEARALENONE

ABSTRACT: Fungi of the genus Fusarium, known as toxigenic species, are very often parasites and contaminants of brewer’s barley. In this paper, the composition of the genus Fusarium species in brewer’s barley samples and their potential in the zearalenone synthesis were investigated.

The tests were done on different brewer’s barley varieties, crop 2003, samples (SSK1, SSK2, SSK3, SSK4, SSK5, SSK6, SSK7, SSK8, SSK9, SSK10 and SSK12) from Kragujevac locality. The isolation and identification of the Fusarium species were done according to the methods described by Nelson et al. (1983). The identified Fusarium species (6) were tested for their capacity to synthesise zearalenone. The isolates were cultured on sterilised barley grains at the temperature of 25°C for 14 days, and then the zearalenone concentration was determined by the fluorometric method on the fluorometer “VIS CAM” series 4.

The following seven Fusarium species were isolated from barley samples: F. acuminatum, F. avenaceum, F. culmorum, F. equiseti, F. poae, F. sporotrichioides and F. triticum. F. poae was the most distributed species (10.26%). The zearalenone concentration within the range of 12.0 to 430.0 μg·kg⁻¹ was determined in cultures of barley grain inoculated with F. avenaceum (SSK6 and SSK12), F. culmorum (SSK8), F. triticum (SSK1), F. sporotrichioides (SSK7 and SSK12) and F. poae (SSK5, SSK9 and SSK10). Isolates of F. equiseti (SSK2) and F. poae (SSK6) did not express capacity to synthesise this toxic metabolite.

KEY WORDS: barley, Fusarium species, zearalenone

INTRODUCTION

Barley grains similar to other cereal grains are a good substrate for the development of numerous microorganisms, especially fungi (Škriñar, 2001; Kocić-Tanackov and Škriñar, 2004). The process of contamination and fungi development actually starts in the field (Schollenberger et al., 2005), during harvest, transport and storage or is activated in the certain stages of the grain technological processing. Fungi of the genus Fusarium, a
common contaminant of cereal grains (Đuraković and Đuraković, 2003), can cause the loss of colour, change of flavour or essence, loss in the nutritive value, quality of milling, cooking and roasting, contamination with mycotoxins, etc. (Šarić i sar., 1980; Bočarov-Stančić, 2001). Infection with Fusarium species is very critical for barley that is malted and which malt is used in brewing beer.

Species of the genus Fusarium can deteriorate brewer’s barley quality in several ways:

— they decrease the average size, weight and nutritive value of the grain (Salas et al., 1999; Vanne and Haikara, 2001);
— they inhibit barley grain germination during the process of malting, decrease the α-amylase activity and cause beer gushing (Noots et al., 1998; Kleemola et al., 2001; Schwarz et al., 2001);
— they synthesise alkaline proteases that hydrolyse brewer’s barley proteins (Pekkarinen et al., 2003);
— they produce mycotoxins (zearalenone, DON, DAS, fumonisine, moniliformin, etc.) (Schollenberger et al., 2005) that are thermostable and are not destroyed during the malting process, wort production, beer pasteurisation and therefore can be transmitted to beer (Scott, 1996).

Due to all above mentioned and especially due to their capacity to produce mycotoxins that are harmful to human health, the objective of the present study was to analyse both, the composition of the genus Fusarium species in brewer’s barley samples that is used as a raw material in the beer production and the potential of isolated species to synthesise zearalenone.

MATERIAL AND METHODS

Samples of brewing barley. Two-rowed winter barley samples, crop 2003, were collected in storage rooms at Kragujevac locality. A total of 3 kg each was sampled from the following 11 different varieties of brewer’s barley: SSK1, SSK2, SSK3, SSK4, SSK5, SSK6, SSK7, SSK8, SSK9, SSK10 and SSK12.

Isolation and identification of fungi. A total of 100 barley kernels was taken from each sample and treated by shaking with 100 ml of 4% NaOCl solution for 2 minutes. Then, the sample was rinsed twice with 100 ml of sterile distilled water. Filter papers soaked with 10 ml of sterile distilled water were placed in four 130 mm Petri dishes. Twenty five barley kernels were placed in each of four Petri dishes. The incubation of cultivated samples was proceeded at the temperature of 25°C during the 14-day period. An additional wetting of the filter paper with 7 ml of sterile distilled water was done on the 7th day. In order to obtain a pure culture and identification fungi, on the basis of the macro-morphological properties of colonies, fungi were re-cultivated to the Sabouraud maltose agar (SMA) or to the Capek medium. According to macro-morphological properties, all colonies, which were assumed to be representatives of the genus Aspergillus or Penicillium, were transferred to the Capek medium,
while all other colonies remained on the Sabouraud maltose agar (SMA). Cultivated Petri dishes were incubated at 25°C for 7 days. Subsequent to the incubation, isolates, determined to belong to the genus *Fusarium*, were used for producing monosporous cultures on the potato dextrose agar (PDA) and 2% carnation leaf agar (CLA) according the procedure described by Nelson et al. (1983). In order to stimulate the formation of conidiogenic structures, cultivated media were incubated in the 12 h UV light/dark cyclic regime. Monosporous cultures were incubated under stated conditions at the temperature of 25°C for 10 to 14 days.

The determination of isolated pure fungal cultures was done in accordance with taxonomic properties described by Ellis (1971, 1976), Nelson et al. (1983) and Samson and van Reenen-Hoekstra (1988).

*In vitro* zearalenone production. Six species of the genus *Fusarium* were investigated for their potential in zearalenone synthesis. Zearalenone-free barley sample were used for this investigation. Eleven sub-samples of 20 g, taken from the primary sample, were coarsely milled and autoclaved at 121°C for 30 minutes. Afterwards they were inoculated with a suspension of *Fusarium* spp. Number *Fusarium* spp. spores was determined according to the dilution method. The inoculum was incubated at 25°C for 14 days, with the periodic addition of sterile distilled water and daily shaking of the sample.

**Zearalenone analysis.** The zearalenone isolation was done in barley samples naturally contaminated with species of the genus *Fusarium* and in barley samples that had been inoculated with cultures of *Fusarium* spp. The zearalenone extraction from the barley samples was done by mixing 20 g of milled sample with 2 g NaCl and 50 mL of the mixture of 90:10 acetonitrile-water in the high-speed glass blender for 2 minutes. The obtained extract was filtrated through the filter paper, and then 10 mL of the filtrate was mixed with 40 mL of washing buffer (PBS/0.1% Tween-20, VICAM). The homogenised mixture was filtrated through the 1.0 μg micro fibre filter. The obtained filtrate (10 mL) was passed through a Zearalenone Test immunoaffinity column (1—2 drops/second). 10 mL of washing buffer and 10 mL of distilled water (1—2 drops/second) were passed through the column. Zearalenone was rinsed out from the column with 1.0 mL of methanol (HPLC purity) into the glass test tube by speed of approximately 1 drop/second. Then, 1 mL of the Zearala Test developer (VICAM) was added to the tube and the content was well homogenised. The test tube was placed into the graduated fluorometer (VICAM series 4), in which the zearalenone concentration was read in PPB (VICAM, 1997).

**RESULTS AND DISCUSSION**

**Isolated fungal species.** Fourteen different fungal species were isolated from two-rowed winter barley samples. On the basis of their taxonomic properties these species were classified into the following genera: *Alternaria, Cladosporium, Fusarium, Mycelia sterilia, Rhizopus, Scopulariopsis* and *Ulocladium* (Figure 1).
Out of totally isolated mycopopulations, the highest distribution (70%) in all samples was determined for the species of the genus *Alternaria* (Fig. 1): *A. alternata* (54%), *A. brassicicola* (14%) and *A. tenuissima* (2%). *A. brassicicola* was distributed with 19% within the genus *Alternaria*.

The genus *Fusarium* in the studied barley samples ranked second by its distribution (19%), but it was represented by a greater number of different species (7) than the genus *Alternaria*. The species *F. acuminatum*, *F. avenaceum*, *F. culmorum*, *F. equiseti*, *F. poae*, *F. sporotrichioides* and *F. tricinctum* were isolated from the genus *Fusarium*. *F. poae* dominated in all barley samples (Figure 2).
One species each was isolated from genera *Cladosporium*, *Rhizopus*, *Sco- pulariopsis* and *Ulocladium* as follows: *C. herbarum*, *R. stolonifer*, *S. fusca* and *U. charatum*.

The obtained data on the distribution of genera *Alternaria* and *Fusarium* are in accordance with the literature data obtained by international and national researchers. Mycological studies on 260 samples of wheat, barley and oat showed similar results in relation to the frequencies of these two genera in Norway (Kosiak et al., 2004). A high distribution of *F. poae* (approximately 20%) was determined in barley grain in the U.S.A. (Salas et al., 1999) and Czech Republic (Hyssek et al., 2000), as well as, in oat grain in Canada (Tekausz, 2002). The dominance of *Alternaria* spp. (up to 72%) and *Fusarium* spp., especially *F. poae* (53%), in barley, i.e. wheat grain, was determined by Bočarov-Štančić et al. (2001), i.e. Balaž et al. (2003) and Doupuda and Levič (2004), respectively.

**Zearalenone content.** The determined zearalenone content in barley grain samples naturally contaminated with species of the genus *Fusarium* ranged from 5.2 to 52.0 μg · kg⁻¹ (Table 1). These levels were higher than the maximum tolerable levels for zearalenone in cereals according to the Regulations on critical amounts of pesticides, metal, metalloids and other toxic substances, homotherapeutics, antibiotics and other substances included into food commodities (Official Gazette of FRY, Issue 5, 1992, Article 15).

<table>
<thead>
<tr>
<th>Sample number</th>
<th>Barley sample</th>
<th>Zearalenone content (μg · kg⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>SSK1</td>
<td>22.0</td>
</tr>
<tr>
<td>2.</td>
<td>SSK2</td>
<td>5.2</td>
</tr>
<tr>
<td>3.</td>
<td>SSK3</td>
<td>8.6</td>
</tr>
<tr>
<td>4.</td>
<td>SSK4</td>
<td>5.8</td>
</tr>
<tr>
<td>5.</td>
<td>SSK5</td>
<td>7.1</td>
</tr>
<tr>
<td>6.</td>
<td>SSK6</td>
<td>8.2</td>
</tr>
<tr>
<td>7.</td>
<td>SSK7</td>
<td>52.0</td>
</tr>
<tr>
<td>8.</td>
<td>SSK8</td>
<td>24.0</td>
</tr>
<tr>
<td>9.</td>
<td>SSK9</td>
<td>37.0</td>
</tr>
<tr>
<td>10.</td>
<td>SSK10</td>
<td>19.0</td>
</tr>
<tr>
<td>11.</td>
<td>SSK12</td>
<td>16.0</td>
</tr>
</tbody>
</table>

The zearalenone content in barley samples inoculated with cultures of *Fusarium* spp. ranged from 0.0 to 430 μg · kg⁻¹ (Table 2). After the 14-day incubation on sterilised barley grains, *F. avenaceum* isolates, originating from barley samples SSK6 and SSK12, synthesised 430—330 μg · kg⁻¹ of zearalenone.
Table 2. Zearalenone content (μg · kg⁻¹) in barley samples incubated with isolated cultures of *Fusarium* spp.

<table>
<thead>
<tr>
<th>Sample number</th>
<th><em>Fusarium</em> species</th>
<th>Origin of isolate of <em>Fusarium</em> spp. (barley variety)</th>
<th>Concentration of spores in suspension for barley grain inoculation (spore ml⁻¹)</th>
<th>Zearalenone (μg · kg⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td><em>F. avenaceum</em></td>
<td>SSK6</td>
<td>8 x 10⁵</td>
<td>430.00</td>
</tr>
<tr>
<td>2.</td>
<td><em>F. avenaceum</em></td>
<td>SSK12</td>
<td>1 x 10⁵</td>
<td>330.00</td>
</tr>
<tr>
<td>3.</td>
<td><em>F. culmorum</em></td>
<td>SSK8</td>
<td>1 x 10⁵</td>
<td>220.00</td>
</tr>
<tr>
<td>4.</td>
<td><em>F. equiseti</em></td>
<td>SSK2</td>
<td>1 x 10⁵</td>
<td>0.00</td>
</tr>
<tr>
<td>5.</td>
<td><em>F. tricinctum</em></td>
<td>SSK1</td>
<td>4 x 10⁵</td>
<td>220.00</td>
</tr>
<tr>
<td>6.</td>
<td><em>F. sporotrichioides</em></td>
<td>SSK7</td>
<td>1 x 10⁶</td>
<td>290.00</td>
</tr>
<tr>
<td>7.</td>
<td><em>F. sporotrichioides</em></td>
<td>SSK12</td>
<td>8 x 10⁶</td>
<td>340.00</td>
</tr>
<tr>
<td>8.</td>
<td><em>F. poae</em></td>
<td>SSK5</td>
<td>2 - 10⁵</td>
<td>120.00</td>
</tr>
<tr>
<td>9.</td>
<td><em>F. poae</em></td>
<td>SSK9</td>
<td>2 - 10⁵</td>
<td>120.00</td>
</tr>
<tr>
<td>10.</td>
<td><em>F. poae</em></td>
<td>SSK10</td>
<td>16 - 10⁶</td>
<td>140.00</td>
</tr>
<tr>
<td>11.</td>
<td><em>F. poae</em></td>
<td>SSK6</td>
<td>1 - 10⁵</td>
<td>0.00</td>
</tr>
</tbody>
</table>

An isolate of *F. sporotrichioides*, originating from the barley sample SSK7, synthesised 290.0 μg · kg⁻¹ of zearalenone while another, originating from the sample SSK12, synthesised 340.00 μg · kg⁻¹ of zearalenone. *F. poae* isolates, originating from three different barley samples (SSK5, SSK9 and SSK10), synthesised 120.00—140.00 μg · kg⁻¹ of zearalenone. *F. culmorum* and *F. tricinctum* synthesised equal content of zearalenone (220.00 μg · kg⁻¹), while a *F. equiseti* isolate and a *F. poae* isolate did not synthesise zearalenone.

In cases when a greater initial number of spores for the same species of the genus *Fusarium* was used for the inoculation of sterilised barley grain, the fungi produced a higher zearalenone content (Table 2): two isolates of *F. avenaceum* (samples No. 1 and 2) and *F. sporotrichioides* (samples No. 6 and 7), and especially three isolates of *F. poae* (samples No. 8, 9, and 10).

In comparison with other species, the lowest zearalenone content in three isolates of *F. poae* (samples No. 8, 9 and 10) or even non detectable contents of this mycotoxin in cultures of *F. equiseti* (sample No. 4) and *F. poae* (sample No. 11) most probably point out to the fact that these species or particular isolates had no genetic background for the zearalenone synthesis.

**CONCLUSIONS**

According to the obtained results on the composition of mycopopulations and zearalenone content in naturally and artificially inoculated brewer’s barley grains with species of the genus *Fusarium* the following conclusions can be drawn:

— fungi of the genera *Alternaria* (70%) and *Fusarium* (19%) dominate in samples of brewer’s barley, while percentage of remaining fungal genera

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ranged from 6—2% \((\text{Mycelia sterilia, Ulocladium, Scopularipsis})\) to 0.5% \((\text{Rhizopus, Cladosporium})\);

— \(A.\ alternata\) (54.6\%) and \(F.\ poae\) (10.26\%), were the most distributed within the genus \(\text{Alternaria}\), i.e. \(\text{Fusarium}\), respectively;

— several species of the genus \(\text{Fusarium}\) \((\text{F. acuminatum, F. avenaceum, F. culmorum, F. equiseti, F. poae, F. sporotrichioides and F. tricinctum})\) were isolated from brewer's barley grain;

— natural occurrence of zearalenone varied from 5.2 to 52.0 \(\mu\text{g} \cdot \text{kg}^{-1}\), i.e. it was higher than the maximum tolerable levels prescribed by legal regulations for content in cereals;

— the zearalenone content varied from 120.0 to 430.0 \(\mu\text{g} \cdot \text{kg}^{-1}\) in cultures of \(F.\ avenaceum\) (2), \(F.\ culmorum\) (1), \(F.\ tricinctum\) (1), \(F.\ sporotrichioides\) (2) and \(F.\ poae\) (3);

— the highest, i.e. lowest potential in zearalenone synthesis was expressed by the isolates of \(F.\ avenaceum\) and \(F.\ sporotrichioides\), i.e. \(F.\ equiseti\) and \(F.\ poae\), respectively;

— only one isolate of \(F.\ equiseti\) (sample No. 4) and of \(F.\ poae\) (sample No. 11) did not synthesised zearalenone;

— the same species of the genus \(\text{Fusarium}\) synthesise a higher zearalenone content if a higher concentration of spore suspension is used for the inoculation of sterilised barley grain.

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СПОСОБНОСТ СИНТЕЗЕ ЗЕАРАЛЕНОНА Fusarium ВРСТА ИЗОЛОВАНИХ ИЗ ПИВСКОГ ЈЕЧМА

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Резиме

Гљиве из рода Fusarium, познате као токсигене врсте, чести су паразити и контаминацији пивског јечма. У овом раду испитиван је састав врста рода Fusarium, пореклом из пивског јечма, као и њихов потенцијал у синтези зеаракаленона.


Из узорака јечма изоловано је 7 Fusarium врста, и то: F. acuminatum, F.avenaceum, F.culusorum, F. equiseti, F. poae, F. sporotrichioides и F. tricinctum. Найјастицењена је била врста F. poae (10,26%). У културама инокулисаног зрна јечма са F.avenaceum (SSK6 и SSK12), F.culusorum (SSK8), F. tricinctum (SSK1), F. sporotrichioides (SSK7 и SSK12) и F. poae (SSK5, SSK9 и SSK10) утврђена је конценрација зеаракаленона од 120,0 до 430,0 µg · kg⁻¹. F. equiseti (SSK2) и F. poae (SSK6) нису показале способност синтезе овог токсичног метаболита.