Fungal infection evaluation for different ecological products

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Abstract In the last period there are controversial opinions regarding the fungal infection in the ecological crops grown without synthetic fertilizers and pesticides. The aim of this work was to identify the presence of fungal infection in different ecological and conventional food products based on PCR methods. These methods were previously used in the screening processes for identification of contamination with pathogenic fungi. 6 ecological food products and three conventional ones were subjected to PCR screening in order to detect the presence of two Fusarium species. The *Fusarium culmorum* was not detected in the analyzed samples. *Fusarium graminearum* was detected in both, ecological and conventional food products, mainly in the unprocessed ones.

Ecologic agriculture, the term used in Romania is similar with the terms organic agriculture or organic farming used in other Member States (http://www.madr.ro/ro/agricultura-ecologica.html).

This agricultural system has seen a constant development due to its contribution to safe food production, more suitable for human metabolism, in complete correlation with natural resources protection and biodiversity conservation. It is part of a broad spectrum of methodologies that support environment based on the minimization of external inputs, avoiding the use of synthetic fertilizers and pesticides.

In the last period there are scientists who consider that organic food and feed is more heavily contaminated with mycotoxins than conventional and genetically modified foods because the organic production does not use chemical fungicides, therefore they are more likely to be infected with different fungi. But the United Nations Food and Agriculture Organization (FAO) states that there are no evidence to prove that the organic products leads to a greater risk of mycotoxin contamination (http://www.issis.org.uk/IMIOF.php).

The aim of this work was to identify the presence of fungal infection in different ecological and conventional products.

In Romania, as well as worldwide, Fusarium head blight (FHB), also known as scab, is a devastating disease of small grains causing serious yield losses and low grain quality, with the ability to produce mycotoxin. The main causal agents of FHB in Europe are *Fusarium graminearum* and *Fusarium culmorum*, but *Fusarium proliferatum* or *Fusarium verticillioides* could be also present. Although FHB may cause wheat yield losses, the interest in FHB is primarily fuelled by the ability of Fusarium species to produce mycotoxins (*Bennett and Klich*, 2003).

For fungal identification, the molecular techniques were used, based on the DNA sequence for each Fusarium specie (*Sampietro* et al., 2010). A rapid, sensitive and reliable specific diagnosis was used, namely polymerase chain amplification (*Leslie* et al., 2007). As identification of Fusarium species is critical to predict the potential mycotoxigenic risk of contamination the tools which permit an accurate and complementary system for fungal identification are of great importance.

Materials and Methods

**Biological materials:** Ecological products purchased from special stores: wheat bio, barley bio, rye flakes, durum wheat pasta, biscuits Maria bio, sunflower seeds and conventional products: corn flour, wheat flour and musli – integral cereals

**Working methods**

**DNA extraction** was done based on CTAB method (*ISO 21571, 2005*) because it is proper for a large category of matrices. 100 mg ground material was mixed with 300 μl sterile distilled water. 700 μl CTAB buffer (CTAB -20g/l; NaCl- 1,4 M; Tris-HCl - 0,1 M; Na2EDTA- 20 mM; pH 8) was added together with 20 μl RNase solution (10 mg/ml) and the mixture was incubated at 65 °C for 30 min.

The samples were centrifuged at 12,000xg for 10 min and the supernatant was transferred to a tube with 500 μl chloroform, vortexed and centrifuged at 12,000 xg for 15 min. The upper layer was transferred to a new tube and 2 volumes of CTAB precipitation solution (CTAB – 5g/l; NaCl – 0,04M) were added. The samples were incubated at room
temperature for 60 min and centrifuged at 12,000×g for 5 min. The pellet was dissolved in 350 μl NaCl 1.2M and 350 μl chloroform was added. The samples were mixed by vortex and centrifuged at 12,000 ×g for 10 min. The upper layer was precipitated with 0.6 volumes of isopropanol, incubated at room temperature for 20 min and centrifuged at 12,000 ×g for 10 min. The pellet was washed in 70% ethanol, vacuum dried and resuspended in 100 μl sterile ultrapure water.

The DNA concentration was evaluated using the spectrophotometric method, with the NanoDrop 8000 UV-Vis Spectrophotometer.

**PCR amplification**

The primers specific for *Fusarium graminearum* and *Fusarium culmorum* with the following sequences were used: *Fusarium graminearum* Fgr F: 5'–CTCCGGATATGTGTCCTAA-3' and Fgr R: 5'-GGTAGGTATCCGACATGGCAA-3' and *Fusarium culmorum* Fe-R: 5'-ATGGTGAACTCGTCGTGGC-3' and Fe-F: 5'-CCCTCTTACGCCTTTACGC-3'

(Chandler et al., 2003; Lopez-Errasquin et al., 2007)

PCR reagents were as follows: Go Taq Green Master Mix PCR kit from Promega 2X 12.5 µl, 20 pmol of each primer, 1µl DNA template, adjusted with distillate water to 25µl. The amplification conditions followed the literature data (Sampietro et al., 2010).

The PCR programme for each pair of primers was as follows: *Fusarium graminearum*: Denaturation 95°C, 3 min, 25 cycles: Denaturation 95°C, 30 sec, Primer annealing 53°C, 30 sec, DNA synthesis 72°C, 30 sec and the Final extension 72°C, 3 min. *Fusarium culmorum*: Denaturation 95°C, 3 min, 40 cycles: Denaturation 95°C, 30 sec, Primer annealing 60°C, 30 sec, DNA synthesis 72°C, 30 sec and the Final extension 72°C, 3 min.

The amplification products were analyzed by agarose gel electrophoresis and visualized by UV radiation in ethidium bromide presence.

**Results and Discussions**

In the first step, all of the food products were grinded to obtain homogeneous samples, which were transferred in tubes – 100 mg/tube. For the wheat samples the specified quantities were weighed and transferred to tubes.

For each samples, the DNA was extracted and the concentration was determined using the spectrophotometric method. In the same time, the ratio A 260/280 was determined, as a measure of the DNA quantity (Table 1).

**Concentration and quality for the DNA samples, extracted from different food products**

<table>
<thead>
<tr>
<th>The analyzed product</th>
<th>Concentration ng/µl</th>
<th>A260/280</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Ecological products</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 Wheat bio</td>
<td>233.5</td>
<td>2.06</td>
</tr>
<tr>
<td>2 Barley bio</td>
<td>484.6</td>
<td>2.07</td>
</tr>
<tr>
<td>3 Rye flakes</td>
<td>173.0</td>
<td>1.82</td>
</tr>
<tr>
<td>4 Durum wheat pasta</td>
<td>301.4</td>
<td>1.99</td>
</tr>
<tr>
<td>5 Biscuits Maria bio</td>
<td>50.7</td>
<td>1.91</td>
</tr>
<tr>
<td>6 Sunflower seeds</td>
<td>763.6</td>
<td>0.81</td>
</tr>
<tr>
<td><strong>Conventional products</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7 Corn flour</td>
<td>312.5</td>
<td>2.04</td>
</tr>
<tr>
<td>8 Wheat flour</td>
<td>684.4</td>
<td>1.98</td>
</tr>
<tr>
<td>9 Musli – integral cereals</td>
<td>548.7</td>
<td>2.05</td>
</tr>
</tbody>
</table>
We analyzed in parallel samples purchased from specialized shops selling organic products and also products collected from households, from chemically untreated crops.

The analysis of the qualitative and quantitative results pointed out that the matrices have a greater importance than the products origin. The DNA extraction was possible for all of the analyzed samples, the minimum concentration being 50 ng/µl.

The highest concentration was obtained for sunflower seeds (763.6 ng/µl) and the lowest for biscuits. It turned out that the advanced processing and the complex composition of biscuits was the cause of the low DNA concentration. Nevertheless, the DNA concentration was enough for the future evaluations.

For the wheat pasta, products which are also highly processed, the DNA concentration was higher – 301.4 ng/µl.

In the same time the ratio between the optical densities measured at 260 nm and 280 nm were determined, as a measured of the DNA quality. It is obvious that, in general the quality of the DNA samples was proper, the OD 260/280 report being around 1.8-1.9. The only exception is the sunflower seeds, with a value of 0.81. It was considered that, for sunflower seeds the high quantity of fatty acids negatively influenced the DNA purification.

Further on, all of the DNA samples were amplified with the specific primers for the ribulose-1,5-bisphosphat carboxilase gene (RuBisCo), a reference gene, present in all of the plant tissues. When the presence of this gene is visualized in a DNA sample extracted from a plant product, it turn out that it is amplifiable, with a proper quality.

The amplification products were analyzed by agarose gel electrophoresis (Fig. 1). For all of the analyzed samples the amplified fragments had the expected length. For the sample extracted from sunflower seeds the band intensity was reduced, due to the high level of inhibitors, impurities which were identified when the DNA purity was spectrophotometrical evaluated.

![Fig. 1: The agarose gel electrophoresis of the amplification products specific for the gene ribulose-1,5-bisphosphat carboxilase (RuBisCo), for different food products: lane 1- wheat bio, lane 2- barley bio, lane 3- rye flakes, lane 4- durum wheat pasta, lane 5- biscuits Maria bio, lane 6- sunflower seeds, lane 7,8- corn flour, lanes 9,10- wheat flour, lanes 11,12- muesli – integral cereals, lane 13- positive control, 14 - PCR marker, Promega](image)

All of the analysed samples was successfully amplified with the primers specific for a plant gene, therefore the next step was developed for the fungal detection.

Therefore, the detection of *Fusarium graminearum* was performed, because this fungus had the larger area of infection in Romania (Fig 2). The gel analysis pointed out the *Fusarium graminearum* presence in the wheat bio and barley bio samples.

Beside the expected fragment (450 bp), bands with different dimensions were visualized due to unspecific amplifications. For the durum wheat pasta and biscuits, bands with low intensities were visualized, but the fragment specific for *Fusarium graminearum* was not present. It is possible that during heat treatment processes the fungal DNA to be destroyed. As positive control, the fungal DNA, extracted from pure culture of *Fusarium graminearum*. 

For the sunflower seeds sample the primers specific for *Fusarium graminearum* didn’t amplified the extracted DNA. The conventional samples – wheat and maize flour generated a large number of bands both, the specific one and other unspecific. For the product musli integral cereals the presence of *Fusarium graminearum* was not pointed out.

All the extracted samples were amplified with the primers specific for *Fusarium culmorum*, but none of the samples were positive.

**Conclusions**

The method used for DNA extraction was appropriate for different types of matrices. The DNA concentration was at least 50 ng/µl and their quality was good, the OD260/280 ratio being 1.8-1.9. The only exception was the sunflower seeds, when the high amount of fatty acids did not allow a proper purification.

All the analyzed DNA samples were amplified with the primers specific for a plant gene (ribulose-1,5-bisfosfat carboxilase – RuBiSCo), therefore they were amplifiable.

*Fusarium graminearum* was present in the bio wheat and barley samples. Besides the expected band (450 bp) other unspecific bands were visualized, due to unspecific amplifications. For the wheat pasta and biscuits a low intensity amplification was visualized, but the band specific for *Fusarium graminearum* was not present. It is possible the fungal DNA to be degraded during heat treatment processes. For the DNA sample extracted from sunflower seeds, the primers specific for *Fusarium graminearum* didn’t amplified.

The conventional wheat and maize samples generated a large number of bands, both specific for *Fusarium graminearum* and other unspecific. It turned out that the Muesli product integral cereals was not infected with *Fusarium graminearum*.

The second fungal specie – *Fusarium culmorum* was not identified in any sample.

**Acknowledgement**

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**References**

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