Evaluation of the genetically modified corn resistance to fungal infection

Popescu Sorina*, Ioja-Boldura Oana Maria*

* Banat University of Agricultural Sciences and Veterinary Medicine Timisoara, Romania

*Corresponding author. Email: biotehnologii_usab@yahoo.com

Abstract The genus Fusarium has a global distribution and many species in the genus are phytopathogenic fungi infecting a wide range of crop plants including cereals such as maize, wheat, oat and barley. Insect pests of corn are often involved in the disease cycles of ear rot and stalk rot diseases. The European corn borer is particularly well known for its involvement in these diseases. Bt corn is a variant of maize, genetically modified to express the bacterial Bt toxin, which protect the plants against this pest, therefore reducing the intensity of Fusarium contamination. PCR based methods were successfully applied in the screening for identification of contamination with pathogenic fungi for the corn samples. 14 corn samples, GM and conventional were subjected to PCR screening in order to detect the presence of three Fusarium species. The presence of Fusarium proliferatum was not detected in the GM corn samples. All three tested Fusarium species, were found in the analyzed samples, in average conventional corn being the most affected.

Key words Bt corn, contamination, Fusarium ssp., detection, PCR

Insect pests of corn are often involved in the disease cycles of ear rot and stalk rot diseases. The European corn borer is particularly well known for its involvement in these diseases. The interaction between corn borer larvae and pathogenic fungi is developed in three steps: wounds on the plant made by corn borers are open infection sites for fungi; corn borer larvae act as vectors of pathogenic fungi (they carry the spores directly into the wounds); and the larvae cause stress on the plant that makes it more susceptible to infection and disease (Papst et al., 2005; Pazzi et al., 2006).

Because of these interactions, it is obvious that some disease control might be achieved through the control of European corn borers, either by insecticide or genetic engineering.

Bt corn is a variant of maize, genetically modified to express the bacterial Bt toxin, which protect the plants against European Corn Borer. Expressing the toxin was achieved by inserting a gene – cry IA (b) from the lepidoptera pathogen microorganism Bacillus thuringiensis into the corn genome.

Conventional characterization of toxigenic Fusarium species has been based mainly on morphological and cross-fertility criteria (Leslie et al. 2001), which are the most routinely performed all over the world. Nevertheless, recognition by morphological characters sometimes is not enough for accurate identification of fungal isolates at the species level. Furthermore, both morphological and mating type characterization are time consuming and require considerable expertise in Fusarium taxonomy and physiology (Leslie et al, 2007). As identification of Fusarium species is critical to predict the potential mycotoxic risk of the isolates, there is a need for accurate and complementary tools which permit a rapid, sensitive and reliable specific diagnosis of Fusarium species.

Therefore, effective measures to combat pests have a positive side-effect in reducing mycotoxin levels. Comparative analysis was used to evaluation of the studies dealing with the reduction of Fusarium mycotoxins in Bt maize. Nineteen out of 23 studies on Bt maize came to the conclusion that Bt maize is less contaminated with mycotoxins (FUM, DON, ZEA) than the conventional control variety in each case (Ostry et al., 2010).

The aim of this work was to confirm the genetically modification of the corn samples, to check their infection with the most common Fusarium species.

Materials and Methods

Biological materials

The corn samples were collected from farms where genetically modified (GM) crops are cultivated: Nadlac and Alunis – Arad county, Dudestii Noi, Timis county. From each location, the GM hybrid and its conventional correspondent were selected (Table 1).

From each sample, approximately 1 kg seed were collected, after harvest from each location.
The corn samples, conventional and genetically modified

<table>
<thead>
<tr>
<th></th>
<th>Conventional (hybrid)</th>
<th>GM (hybrid)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nadlac DKC 5783</td>
<td>DKC 5784</td>
<td></td>
</tr>
<tr>
<td>DKC 440</td>
<td>DKC 4442</td>
<td></td>
</tr>
<tr>
<td>DKC 3511</td>
<td>DKC 3512</td>
<td></td>
</tr>
<tr>
<td>Alunis DKC 5783</td>
<td>DKC 5784</td>
<td></td>
</tr>
<tr>
<td>DKC 440</td>
<td>DKC 4442</td>
<td></td>
</tr>
<tr>
<td>DKC 3511</td>
<td>DKC 3512</td>
<td></td>
</tr>
<tr>
<td>Dudestii Noi</td>
<td>DKC 3511</td>
<td>DKC 3512</td>
</tr>
</tbody>
</table>

Working methods

DNA extraction
The corn seeds (1000 pieces) were grinded and then, quantities of 100 mg from each sample were distributed in tubes.

The DNA was extracted using CTAB method. The DNA was quantified by spectrophotometer method and then it was analyzed.

DNA quality confirmation

The primers ZEIN 3 and ZEIN 4 specific to the maize zein gene was used to confirm the presence and quality of DNA extracted from the corn samples. If the extracted DNA is present, intact and amplifiable a band of 277 bp will be amplified (Querci et al. 2004).

The primers sequences:

ZEIN3: AGTGCGACCCATATCCAG and
ZEIN4: GACATTGTGGCATCATCATT

The content of the PCR mixture: PCR buffer 1x; MgCl2 2.5mM; dNTP 0.2mM; ZEIN3 0.5μM; ZEIN4 0.5μM; Taq DNA polymerase 0.025U. The amplification program was as follows: denaturation 95°C - 3 min; 50 cycles: denaturation 95°C - 1 min; Primer annealing 60°C - 1 min, DNA synthesis 72°C - 1 min; Final extension 72°C - 3 min.

The GM confirmation

The primers sequences:

mg1 5’ TAT CTC CAC TGA CGT AAG GGA TGA C 3’
and
mg2 5’ TGC CCT ATA ACA CCA ACA TGT GCT T 3’

The content of the PCR mixture: PCR buffer 1x; MgCl2 2.5mM; dNTP 0.2mM; primer mg1 0.5μM; primer mg2 0.5μM; Taq DNA polymerase 0.025U. The amplification program was as follows: denaturation 95°C - 10 min; 35 cycles: denaturation 95°C -45 sec; Primer annealing 60°C – 50 sec, DNA synthesis 72°C – 50 sec; Final extension 72°C – 3 min.

Fusarium identification

The primers used for had specific sequences (Sampietro et al., 2010).

Fusarium graminearum (450 bp amplicon)
Fgr-F: 5’-CTCCGGATATGTTGCCGCAA-3’
Fgr-R: 5’-GGTAGGATCCGACATGGCAA-3’
Fusarium culmorum (570 bp amplicon)
Fc-R: 5’-ATGGTGAACTCGTCGTGCC-3’
Fc-F: 5’-CCCTTCTTACGCATTCTCG-3’
Fusarium proliferatum (230 bp amplicon)
Fp3-F: 5’-CGGCCACCAGAGGATCGTG-3’
Fp4-R: 5’-AACAGCAGAATCCTCTGGTAC-3’
Fusarium verticillioides (700 bp amplicon)
Fps-F: 5’-CGCACGTATAGATGGACAAG-3’
Vert2-3’-CACCCGCAGAATCATCCATCAG-5’

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

Amplification reactions were performed in a Corbett RESEARCH Thermal Cycler, following the indications from literature.

Amplicons were analyzed by electrophoresis on 2% agarose gel (Promega, USA) and visualized in Ethidium Bromide (0.4 ng/ml) presence.

Results and Discussions

The DNA was extracted from all samples, conventional and genetically modified.

The primers ZEIN 3 and ZEIN 4, specific to the maize zein gene was used to confirm the presence and quality of DNA extracted from the maize samples.

The gel analysis confirmed that cross contamination was avoided in the DNA extraction and amplification procedures. All of the negative and positive controls were as expected and the amplified bands for the samples were similar (277 bp) to the positive control. All of the DNA samples were amplifiable and were further analyzed (Fig. 1).

Furthermore, for GMO screening, the 35 S promoter was identified as target sequence. For its specific detection, primers mg1 and mg2 were used (Fig. 2).
Fig. 1: The agarose gel analysis of the amplification products for corn samples, using the primers specific for zein gene: DKC 5783 Nadlac (C) – 1, DKC 440 Nadlac (C) – 2, DKC 3511 Nadlac (C) – 3, DKC 5783 Alunis (C) – 4, DKC 440 Alunis (C) – 5, DKC 3511 Alunis (C) – 6, DKC 3511 Alunis (Dudestii Noi (C) – 7, DKC 5784 Nadlac (MG) – 8, DKC 4442 Nadlac (MG) – 9, DKC 3512 Nadlac (MG) – 10, DKC 5784 Alunis (MG) – 11, DKC 4442 Alunis (MG) – 12, DKC 3512 Alunis (MG) – 13, DKC 3512 Alunis (Dudestii Noi (MG) – 14, Positive Control (CP), Negative Control (CN), Non Template Control (NTC), molecular marker (MM).

Fig. 2. The agarose gel analysis of the amplification products, using the primers specific for 35 S promoter A-DKC 5784 Nadlac (MG) – 1, DKC 4442 Nadlac (MG) – 2, DKC 3512 Nadlac (MG) – 3, DKC 5784 Alunis (MG) – 4, DKC 4442 Alunis (MG) – 5, DKC 3512 Alunis (MG) – 6, DKC 3512 Alunis (Dudestii Noi (MG) – 7, Positive Control (CP), Negative Control (CN), Non Template Control (NTC), molecular marker (MM); B-DKC 5783 Nadlac (C) – 1, DKC 440 Nadlac (C) – 2, DKC 3511 Nadlac (C) – 3, DKC 5783 Alunis (C) – 4, DKC 440 Alunis (C) – 5, DKC 3511 Alunis (C) – 6, DKC 3511 Alunis (Dudestii Noi (C) – 7, Positive Control (CP), Negative Control (CN), Non Template Control (NTC), molecular marker (MM).

The genetic modification was confirmed by the amplification with primers specific for 35 S promoter, a sequence used for GM detection. Further on, the DNA samples were amplified with the primers specific for different fungal species. First, the Fusarium graminearum specific primers were used (Fig. 3).
Fig. 3. The agarose gel analysis of the amplification products, using the primers specific for Fusarium graminearum: DKC 5784 Nadlac (MG)-1, DKC 4442 Nadlac (MG)-2, DKC 3512 Nadlac (MG)-3, DKC 5784 Alunis (MG)-4, DKC 4442 Alunis (MG)-5, DKC 3512 Alunis (MG)-6, DKC 3512 Alunis (Dudestii Noi (MG)-7, DKC 5783 Nadlac (C)-8, DKC 440 Nadlac (C)-9, DKC 3511 Nadlac (C)-10, DKC 5783 Alunis (C)-11, DKC 440 Alunis (C)-12, DKC 3511 Alunis (C)-13, DKC 3511 Alunis (Dudestii Noi (C)-14, Non Template Control (NTC), molecular marker (MM), Pozitive Control (CP).

It turned out that *Fusarium graminearum* infection was stronger in conventional corn, compared to GM. In Nadlac location, all of the analyzed conventional hybrids were infected, and only DKC 4442 from the GM ones. In Alunis location a very slightly infection was registered for both DKC 4442 and DKC 440 hybrids, in Dudestii Noi only the conventional hybrid was infected.

Further on, the *Fusarium culmorum* specific primers were used (Fig. 4).

Fig. 4. The agarose gel analysis of the amplification products, using the primers specific for Fusarium graminearum: DKC 5784 Nadlac (MG)-1, DKC 4442 Nadlac (MG)-2, DKC 3512 Nadlac (MG)-3, DKC 5784 Alunis (MG)-4, DKC 4442 Alunis (MG)-5, DKC 3512 Alunis (MG)-6, DKC 3512 Alunis (Dudestii Noi (MG)-7, DKC 5783 Nadlac (C)-8, DKC 440 Nadlac (C)-9, DKC 3511 Nadlac (C)-10, DKC 5783 Alunis (C)-11, DKC 440 Alunis (C)-12, DKC 3511 Alunis (C)-13, DKC 3511 Alunis (Dudestii Noi (C)-14, Pozitive Control (CP), Non Negative Control (NC), molecular marker (MM).

The band of 570 bp, specific for *Fusarium culmorum* was present in DKC 5784 Nadlac (MG), and DKC 3512 Alunis (MG) from the genetically modified samples and DKC 5783 Nadlac (C) and DKC 5783 Alunis, from the conventional ones. It seems that the hybrid 5783 (conv) with its GM correspondent 5784 were the most sensitive to Fusarium culmorum.

Next, the *Fusarium proliferatum* specific primers were used (Fig. 5).
The 230 bp amplicon, specific for *Fusarium proliferatum* was slightly present only in conventional samples, namely DKC 5783 Nadlac (C) DKC 3511 Nadlac (C) and DKC 3511 Alunis (C). In this case a higher sensitivity of DKC 3511 was pointed out.

**Conclusions**

1. PCR based methods were successfully applied in the screening for identification of contamination with pathogenic fungi for the corn samples.
2. The presence of transgenic corn was correctly determined, according to producers.
3. All three tested *Fusarium* species were found in the analyzed samples, in average conventional corn being the most affected.
4. The presence of *Fusarium proliferatum* was not detected in the GM corn samples.
5. This method proved to be suitable in screening of seed samples, in order to detect their contamination with pathogenic fungi.

**Acknowledgement.**

This work was published during the project “Szeged – Timisoara axis for the safe food and feed” SZETISA1, HURO/0901/147/2.2.2., financed by the Hungary-Romania Cross-Border Co-operation Programme 2007-2013, co-financed by the EU ERDF.

**References**

2. ISO 21569, 2005: Foodstuffs - Methods of analysis for the detection of genetically modified organisms and derived products - Qualitative nucleic acid based methods.