Monitoring of GM Soybean in High Categories of Seed on the Croatian Seed Market

Renata HANZER [1(✉)]
Dijana OCVIRK [1]
Sanja ŠPOLJARIĆ MARKOVIĆ [1]
Hrvoje FULGOSI [2]

Summary

Croatia has a long tradition in seed production for domestic use and for export. As an EU candidate country Croatia has harmonized its legislation with EU directives and therefore no GM soybean is authorized for cultivation. So far there is no record of introduction of GM soybean into Croatian environment; however there are earlier records of growing genetically modified soybean in the region. Aim of this study is to monitor current position in domestic seed market regarding genetically modified soybeans through substantial PCR screening for three lines: GTS 40-3-2 (Roundup Ready, RRS), A2704-12 (Liberty Link , LL) and MON89788 soybean (RR2). Screening was performed on high category soybean seed that was certified for Croatian market in season 2010/2011. Total of 18 different varieties of soybean seed were tested and all resulted negative for the presence of the tested GM lines. This is the first such screening of Croatian seed market.

Key words

genetically modified soybean, polymerase chain reaction, Croatian seed market, prebasic and basic seed

[1] Croatian Centre for Agriculture, Food and Rural Affairs, Institute for Seed and Seedlings, Seed Testing Laboratory, Usorska 19, Brijest, 31000 Osijek, Croatia
✉ e-mail: renata.hanzer@hcphs.hr
[2] Ruđer Bošković Institute, Department of Molecular Biology, Bijenička 54, 10000 Zagreb, Croatia

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Introduction

Genetically modified soybean represents the most widely spread genetically modified crop around the globe, occupying nearly 70 million hectares worldwide. More than half of all produced Soybean is genetically modified and the most cultivated GM soybean is Monsanto's Roundup Ready Soybean (RRS). Although there is no cultivation in Europe, still EU annually imports approximately 40 million tons of soybean and product from countries that grow GM soybean (James, 2011; GMO Compass, 2010). Croatia as an EU candidate country has harmonized its legislation with EU directives and therefore no GM soybean is authorized for cultivation. So far there is no record of introduction of GM soybean into Croatian environment.

According to the Croatian bureau of statistics in year 2011 the total of 59,000 ha was planted with this crop in Croatia. Over the past years, there is a constant increase in soybean production, which suggests that this crop is becoming more important for local agriculture. There are several approaches to detection of GM plants, which are divided in two main categories: protein based methods, which detect novel proteins, and DNA based methods which detect presence of transgenic DNA in the tested material. Each method has its own specificity and limitations. Still, DNA based methods, mainly PCR methods, are worldwide considered as the method of choice. Detection of GM products using PCR comprises specifically designed oligonucleotides which are characteristic for inserted gene fragment and thus highly specialized for detection of GM materials (Stawe, 2002; Lin et al., 2001).

Croatia has a long tradition in seed production for domestic use and for export, and it is keen to ensure its position in demanding global seed market. The aim of this study is to screen high categories of soybean seed certified for trade on Croatian market for the presence of genetically modified soybean.

Materials and methods

Plant material

The study was conducted on all lots of high categories soybean seed (Glycine max), prebasic and basic, that were certified for Croatian market in season 2010/2011. Size of each tested sample was approximately 3000 kernels per sample (approximately 1000 g of sample). Sampling procedure was done according to the Commission Recommendation on technical guidance for sampling and detection of genetically modified organisms and material produced from genetically modified organisms as or in products in the context of Regulation (EC No 1830/2003) and according to ISTA guidelines on seed sampling (ISTA, 1999).

The total of 18 different varieties of Soybean were tested, namely: Hrvatica, Zlata, Buga, Ruzica, Tena, Julijana, Sanda, Zora, Ika, Vita, Lucija, Korana, Ana, Sanja, Zagrepcanka, Dora, Marija, and Gallec. As positive and negative controls certified reference materials were used; for Roundup Ready soybean (RRS) dry soybean powders were used containing 0%, 0.1%, 0.5%, 2% and 10% of RRS, produced by Institute for Reference Material and Measurements (IRMM, Belgium); for MON89788 dry soybean powder containing 100% of MON89788, produced by American Oil Chemists' Society (AOCS). For taxon specific PCR as a negative control a dry maize powder of MON810 (0%), produced by IRMM, Belgium, was used.

DNA extraction and quantification

Seed samples were grinded and homogenized, and DNA was extracted from 200 mg of seed powder, two repetition of each sample. DNA extraction and purification was performed according to a CTAB. Method was validated by the working group of the German Federal Health Board (ISO 21571:2005; Quercy et al., 2006a). Quality of extracted DNA was tested by agarose gel electrophoresis and using a spectrophotometer (Biophotometer, Eppendorf). The concentration of raw DNA extracted was normalized to 50 ng/µl.

PCR method

To establish presence of amplifiable soybean DNA, a taxon specific PCR was performed, using primers GMO3 and GMO4, which amplify DNA fragment specific for soybean lectin gene (Hübner et al., 1999; Meyer, 1999). Detection of genetically modified soybean was performed employing primers designed to amplify 35S promoter, and NOS terminator that are most commonly used regulatory elements of inserted cassettes (Lipp et al., 1999; Quercy et al., 2006; ISO 21569:2005). In addition samples were tested for the presence of MON89788 event, which insert does not contain those elements, by using event specific Real Time PCR reaction (Quercy et al., 2010). All used oligonucleotide primers and probes are listed in Table 1.

Table 1. Oligonucleotide primers and probes

Primer	Sequence 5' to 3'	Gene	Amplicon (bp)	Reference
GMO3	GCCCTCTACTCCACCCCCATCC	lectin	118	ISO 21569:2005; Collection of official methods under Article 35
GMO4	GCCCATCTGCAAGCCTTTTTGTG			
p35S-cf3	CCACGTCTTCAAAGCAAGTGG	P-35S	195	ISO 21569:2005, Lipp et al., 2001
p35S-cr4	TCCTCTCCAAATGAAATGAACTTCC			
HA-nos 118-f	GCATGACGTTATTTATGAGATGGG	T-NOS	118	ISO 21569:2005, Lipp et al., 2001
HA-nos 118-r	GACACCGCGCGCGATAATTTATCC			
MON89788-F	TCC CGC TCT AGC GCT TCA A	MON89788	139	Charles Delobel et al., 2008
MON89788-R	TCG AGC AGG ACC TGC AGA A			
MON 89788-P	FAM CTG AAG GCG GGA AAC GAC AAT CTG TAMRA			
lec F	CCA GCT TCG CCG CTT CCT TC	lectin	74	Charles Delobel et al., 2008
lec R	GAA GGC AAG CCC ATC TGC AAG CC			
lec P	FAM CTT CAC CTT CTA TGC CCC TGA CAC TAMRA			

Table 2. Conditions for PCR analysis

Step	Lectin	P-35S	T-NOS	MON89788
Decontamination (UNG)	–	–	–	50°C 2'
Initial denaturation	95°C 3'	95°C 3'	95°C 10'	95°C 10'
Denaturation	95°C 30"	95°C 25"	95°C 25"	95°C 15"
Annealing	63°C 30"	62°C 30"	62°C 30"	60°C 60"
Extension	72°C 30"	72°C 45"	72°C 45"	
Number of cycles	40	50	50	45
Final extension	72°C 3'	72°C 7'	72°C 7'	-

For lectin gene, 35S promoter and NOS terminator the amplifications were carried out using Applied Biosystems Verity Thermal Cycler, and for the MON89788 event specific analysis Applied Biosystems 7300 Real Time PCR was used. Conditions of thermocycling reactions for the performed analysis are listed in the Table 2.

Reaction volume for PCR amplification was 25µl and it contained: 14.365 µl sterile water, 2.5 µl 10xPCR buffer, 1.25 µl 50mM $MgCl_2$, 0.5 µl 4mM dNTP, 0.63 µl of each primer, 0.125µl Taq DNA polymerase (Invitrogen) and 5 µl of 50ng/µl DNA solution. The Real Time PCR reaction contained the following: 6.5 µl sterile water, 12.5 µl TaqMan Universal PCR master mix, 0.375 µl of each primer, 0.25 µl TaqMan probe (Applied Biosystems) and 5 µl of 50ng/µl DNA solution.

Positive, negative and non template controls accompanied every series of reactions. Amplification was verified by electrophoresis in 2% agarose gel stained with SYBRsafe (Invitrogen). Size of amplicons is given in Table 1. Results of the Real Time PCR analysis were read directly from the amplification plots.

Results and discussion

The results of these study show that used method for DNA extraction and purification produced amplifiable soybean DNA, which was confirmed by PCR analysis using primers for lectin gene and presented as 118 bp amplicon on 2% agarose gel (Fig. 1).

Specificity of this reaction was confirmed using positive and negative control. The amplification of taxon specific gene is necessary to demonstrate that DNA is of good quality and of sufficient purity. Successful amplification of taxon specific marker indicates absence of PCR inhibitors and thus eliminates possibility of false negative result (Nikolić et al., 2008).

Currently three GM Soybean lines are authorized for placing on the market as food and feed in EU as well as in Croatia: MON40-3-2 (Roundup Ready, RRS), A2704-12 (Liberty Link, LL) and MON89788 (RR2) (The Government of the Republic of Croatia, 2005). 35S promoter regulates the gene expression of RRS and LL. Amplicon size 195 bp indicates presence of 35S promoter, thus indicating transgenic material in the sample. RRS is also regulated by NOS terminator which is not present in LL and positive reaction distinguishes those two events. Second generation of Roundup ready soybean (RR2) does not contain any of those two regulatory elements and therefore additional reaction is performed using MON89788 event specific primers. Such testing strategy allows efficient detection of all three events and also suggests their identification (Table 3). To assure identification of each individual GMO, an event specific analysis is needed.

Sensitivity of the performed test was demonstrated using certified reference materials containing different fraction of GM soybean (0.1% to 10%). The results of this study show for all tested samples negative reaction of 35S promoter (Fig. 2), NOS terminator and MON89788 event in the all tested soybeans.

Although no GM soybean is authorized for cultivation in EU at the moment there is nothing to state that the situation may not change in the future. Earlier records of growing genetically modified soybean in the region encourage us to determine current situation in Croatia. According to Nikolic et al. (2008) in

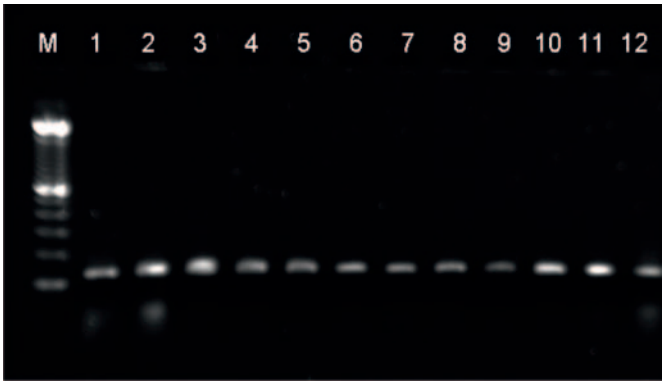

Figure 1. Agarose gel electrophoresis for lectin PCR products (LineM: 100 bp DNA Ladder, Line 1-12: DNA from soybean samples)

Table 3. Testing strategy table for different GMO targets

	35S promoter	NOS terminator	MON89788
Roundup Ready	+	+	–
Liberty Link	+	–	–
MON89788	–	–	+

Figure 2. PCR products obtained for the 35S promoter (Line M: DNA Ladder, Line 1-10: DNA from samples, Line 11: non template control, Line 12: negative control, Line 13: positive control)

the Vojvodina province in year 2006 from 7142 tested seed samples totally 44 were positive and in year 2007 GMO soybean was confirmed in 108 out of 7171 analyzed samples.

Conclusions

In season 2010/2011 on Croatian market totally 20 seed lots of high category soybean seed were certified (18 different varieties). All samples were collected and tested for the presence of specific GM elements: 35S promoter, NOS terminator and additionally soybean line MON89788 which does not contain these elements. The results of this study demonstrated absence of all tested GM elements in soybean samples and thus suggested that Croatian seed market is still free of genetically modified soybean lines. To confirm these results it is necessary to perform screening in forthcoming seasons of certification.

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