

## DETECTION OF GENETICALLY MODIFIED CROPS IN ANIMAL FEED IN SERBIA

Gordana Zdjelar<sup>1\*</sup>, Zorica Nikolić<sup>1</sup>, Ivana Vasiljević<sup>2</sup>, Dušica Jovičić<sup>1</sup>, Maja Ignjatov<sup>1</sup>,  
Dragana Milošević<sup>1</sup>, Gordana Tamindžić<sup>1</sup>

<sup>1</sup>Institute of Field and Vegetable Crops, Novi Sad, Serbia

<sup>2</sup>A BIO TECH LAB Ltd., Sremska Kamenica, Serbia

\*Corresponding Author. E-mail: gordana.zdjelar@nsseme.com

Institute of Field and Vegetable Crops, Laboratory for Seed Testing, Maksima Gorkog 30, 21000 Novi Sad, Serbia

### ABSTRACT

The survey was conducted on a total of 100 non-labelled samples of feed and feed mixture containing maize, soybean and rapeseed, originating from countries with different legislation systems. Screening of all samples was performed using primers for Cauliflower Mosaic Virus 35S (CaMV35S) promoter, primers for the *Agrobacterium tumefaciens* nopaline synthase (NOS) terminator and event-specific primers for GT73 rapeseed. Roundup Ready soybean was found in 26 samples, with the amount of GM soybean above the limit of 0.9% in 9 of them. There was one maize seed sample positive for the presence of MON810 maize and no rapeseed meal samples contained GM rapeseed. The results found in this study clearly showed that imported maize and soybean and complete mixtures intended for animal feed on the Serbian market contain GMO. Monitoring plans are required to control the distribution of non-labelled feeds containing GMO in the Serbian market.

**Key words:** GMO, PCR, real time PCR, soybean, maize, rapeseed.

### INTRODUCTION

Genetically modified (GM) plants represent an increasingly significant portion of the crops available on the feed market. The largest producers and exporters of GM crops for animal feed are the USA, Brazil, Argentina and Canada. The global area on which GM crops are cultivated is concentrated and constantly increasing in developed countries. Twenty nine countries, out of which eight are members of the European Union (EU), planted commercialised GM crops in 2011. In the last fifteen years the global area of GM crops increased from 1.7 million hectares in 1996 to 160 million hectares in 2011. Year-to-year growth measured either in absolute hectares or by percent, was higher in developing countries than in industrial countries (James, 2011).

Since the first GM plant was introduced, GM crops have become an integral part of agricultural production. The number of GM plant species, that are commercially available for food/feed production, is constantly increasing. GM soybean is widely used in

animal feed and represents the dominant GM crop in the world occupying almost 50% of the global biotech crop area. After soybean, the most important GM crop is maize, followed by cotton and canola (James, 2011).

Although the global area with GM crops has been steadily increasing, European Union continues to be a region where the commercial cultivation of GM crops is very limited. Regulation 1829/2003 on genetically modified food and feed and Regulation 1830/2003 concerning the traceability and labelling of genetically modified organisms and the traceability of food and feed products produced from genetically modified organisms have been in operation since April 18<sup>th</sup>, 2004. Since the entry into force of Regulation (EC) 1829/2003 on genetically modified food and feed, the labelling threshold for GMO content in food has been lowered from 1% to 0.9%. The same threshold will apply for feed (Regulations (EC) No. 1829/2003, 1830/2003). However, products such as milk, meat, and eggs, which are derived from livestock fed with transgenic feeds, are exempt from EU-labelling laws.

According to the Law on GMO, Serbia strictly prohibits all imports, production and commercial growing of GM crops or products containing GMO (Official Gazette of RS, 2009). All shipments of soybeans, maize, rapeseed, sugar beet and rice and their products entering Serbia must be tested for GMO content, and are allowed to be imported only if they are GMO-free. However, the contamination of 0.9% and 0.1% of seed is permitted in the agricultural products of plant origin.

Polymerase chain reaction (PCR) is one of the most commonly used methods for screening because of its high sensitivity and reliability. It is based on the detection of genetic elements present in most of the GMOs like Cauliflower Mosaic Virus 35S (CaMV35S) promoter and/or the *Agrobacterium tumefaciens* nopaline synthase (NOS) terminator. They are used as universal markers in the analysis of 95% of all GM plants. Since the 0.9% is the threshold for labelling of accidentally and technically unavoidable admixture, it is necessary to determine the exact percentage of GMOs in food/feed. Real-time PCR is a useful technique for obtaining more precise and numerical information determining the exact amount of specific nucleic acid sequences (Querci et al., 2010).

Given that Serbia's own resources are insufficient to respond to the needs of animal nutrition, there is a need to import certain quantities of animal feed and supplements for compound feedstuffs (enzymes, additives etc.). Hereby a number of GM events could enter Serbia mostly as a raw material to be used directly as feed or in feed/food industry. The Roundup Ready soybean was already found at the Serbian fields (Nikolić et al., 2009a) as well as in food (Nikolić et al., 2009b; Zdjelar et al., 2013) and feed samples (Miljuš-Djukić et al., 2010; Nikolić et al., 2010a). In order to detect the presence of GMO maize in the Serbian market, a triplex PCR was applied to maize grains and processed maize samples during the three years period (Nikolić and Vujaković, 2011).

First case-specific monitoring of herbicide-tolerant rapeseed events RT73, Rf3 and T45 on the Serbian market was carried out in 2009 by Nikolić et al. (2010b).

The aim of the study was to determine how often GM feed materials and complete feed mixtures with GM crops are used in the Serbian feed market, to identify the sources of GMO (feed from domestic market or imported) and to determine types of modifications.

## MATERIAL AND METHODS

### Samples

The survey was conducted on a total of 100 non-labelled samples of feed and feed mixture collected in the year 2009 and 2010. The samples were originally from countries with different GMO labelling legislation systems, such as USA, Argentina, and Brazil, countries from EU and from domestic market. The basic raw materials in tested samples were maize, soybean and rapeseed (Table 1).

### DNA extraction

DNeasy Plant Mini Kit (Qiagen, Germany) was used for DNA extraction, according to the manufacturer's manual, in duplicate. The quality and quantity of the extracted DNA was checked with a UV/VIS spectrophotometer (Evolution 100, Thermo Scientific, USA). The  $A_{260}/A_{280}$  of extracted DNA ranged from 1.7-2.0.

### Qualitative PCR

Screening of all samples was performed using primers for CaMV35S promoter (Metabion, Germany), which amplifies a product size of 123bp and primers for NOS terminator, which amplifies a product size of 118bp (Lipp et al., 2001). Since GT73 canola has no CaMV35S promoter and NOS terminator, detection of this modification was performed according to Demeke et al. (2002) using event-specific primers. As a quality control of DNA and PCR efficiency reference genes specific for soybean and maize (lectin, zein) (Meyer et al., 1996; Studer et al., 1997)

and universal primers for plant chloroplast DNA for rapeseed (Taberlet et al., 1991) were used. The CRM consisting of 0%, 0.1%, 0.5%, 1%, 2%, and 5% dried RR soybean and MON810 maize powder (IRMM, Belgium), 100% canola leaf DNA T45 (AOCS, USA), Rf3 (AOCS, USA) and canola seed RT73 (AOCS, USA) were used as a positive control. The sensitivity of PCR reaction was 0.1%. Samples showing the presence of GMOs were analyzed using construct-specific primers for RR soybean (Jankiewicz et al., 1999) and event-specific primers for MON810 maize (Holck et al., 2002) in order to determine the type of GMO present.

The PCR was carried out using premix of 2x PCR Master Mix, (Fermentas, Lithuania) containing 4mM MgCl<sub>2</sub>, 0.4mM dNTP, 0.05units/μl Taq DNA Polymerase (recombinant). PCR was performed in a final volume of 25 μl of PCR mix containing 0.2pmol/μl primers and approx. 50 ng DNA was used.

Amplifications were carried out in a Eppendorf Mastercycler Gradient (Eppendorf, Germany) under the following programs: denaturation at 95°C for 10min followed by 40 cycles of 95°C for 30 sec, 58°C for 30 sec and 72°C for 30 sec and the final extension was carried out at 72°C for 3 min. Each extract was amplified in duplicate assays including positive controls, negative control and no-template control (blank).

The amplification fragments were determined using electrophoresis on 2% agarose gel containing ethidium bromide (0.5g/mL). The expected size of the amplified fragments was estimated by comparison with FastRuler DNA Ladder, Low Range (Fermentas, Lithuania). The agarose gel was visualized using UV transilluminator, and the images were captured with DOC II PRINT system (Vilber Lourmat, USA).

### Quantitative Real-time PCR

Quantitative analysis of GM was performed on 7500 Real Time PCR System

(Applied Biosystems, USA). The CRM consisting of dried RR soybean powder or dried MON810 maize powder with 0%, 0.1%, 0.5%, 1%, 2%, and 5% (IRMM, Belgium) were used. Commercial kits for detection of CaMV35S promoter were used for the analysis (GMO Soy 35S TaqMan Detection Kit and GMO Maize 35S TaqMan Detection Kit, Applied Biosystems, USA) according to the manufacturer's manual.

## RESULTS AND DISCUSSION

The presence of genetic modification was analysed in a total 100 samples of feed and compound feed mixture (Table 1).

The basic raw materials in all tested samples were maize, soybean and rapeseed. All samples were screened for the presence of CaMV35S promoter and NOS terminator. Additionally, rapeseed meal samples were analysed for GT73 modification using event-specific primers. CaMV35S promoter and NOS terminator are used as universal molecular markers for analysis of 95% of all authorized GM plants in EU.

In order to evaluate DNA quality and PCR efficacy, and to reduce the risk of false negatives, host specific internal target gene was tested in all assays. It is necessary to exclude possibility of false negative results due to possible inhibitor presence or poor quality of extracted DNA. A positive signal for the presence of CaMV35S promoter and/or NOS terminator is not always sufficient (Ovesná et al., 2010). Plants may be naturally infected with the Cauliflower mosaic virus and *A. tumefaciens* what could lead to a false positive result (Wolf et al., 2000; Chaouachi et al., 2008).

Therefore, a positive result of CaMV35S will suggest that, probably, the transgene sequence is present. In such cases further PCR tests should be performed with event-specific or construct-specific primers designed to amplify the specific transgenic DNA (Jinxia et al., 2011).

Table 1. Results of GMO analysis of non-labelled feed samples collected in the territory of Vojvodina in 2009 and 2010

Sample type	Total of samples	Qualitative analysis		Quantitative analysis	
		Positive	Negative	<0,9%	>0,9%
<b>SOYBEAN</b>					
Seed	10	4	6	3	1
Soybean grits	4	1	3	1	0
Soybean meal	11	5	6	2	3
Total	25	10	15	6	4
<b>MAIZE</b>					
Seed	20	1	19	1	0
Total	20	1	19	1	0
<b>RAPESEED</b>					
Rapeseed meal	15	0	15	0	0
Total	15	0	15	0	0
<b>COMPOUND FEED MIXTURE</b>					
Feed mixture	40	16	24	11	5
Total	40	16	24	11	5
<b>TOTAL</b>	<b>100</b>	<b>27</b>	<b>73</b>	<b>18</b>	<b>9</b>

In total, 27 samples of different matrices were positive for the presence of CaMV35S promoter (Table 1). There was only one maize seed sample positive for CaMV35S promoter, but with absence of NOS terminator. Analysis with event-specific primers showed the presence of MON810 modification in that maize sample (Figure 1). The amount of GM maize in analyzed sample was below the limit of 0.9%. In order to detect the presence of GM maize in the Serbian market, in previous three year study, a triplex PCR was applied to maize grains and processed maize samples. The number of positive samples varied from 12% in 2006 year, to 14.5% in 2008, with the amount of GM between 0.1 and 0.9% and only two maize seed samples with more than 0.9%, in 2006 (Nikolić and Vujaković, 2011). The similar study conducted in the three year's period in Poland also showed presence of MON810 maize (Kwiatk et al., 2007). Our result was expected because the major part of maize used in Serbia for animal feeding is grown in Serbia or in EU countries. MON810 is the only GM maize approved for cultivation in EU, namely in Czech Republic, Poland, Portugal, Romania, Slovakia and Spain.

However, it can be expected that the cultivation of the GM maize will increase in the following years (James, 2011).

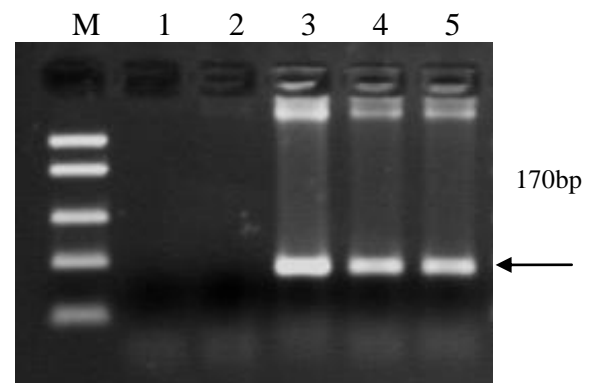


Figure 1. Agarose gel electrophoresis of PCR products from maize samples for analysis of the MON810 maize. M-Low Range DNA Ladder, 50-1500 bp; 1. Blank-no template control; 2. Negative control; 3. CRM MON810 maize 0.5%; 4.-5. sample positive for MON810

More than 80% of globally planted soybean is genetically modified Roundup Ready (RR) soybean of which approximately 85% is processed into soybean meal, a significant and cheap source of protein for animal feeds (James, 2011). Although RR

soybean is still not approved for cultivation in the EU, this GM event continues to be very important due to imports. The primer pair, described by Jankiewicz et al. (1999), p35s-f2/petu-r1 is specific for the genetic modification construct in RR soybean and amplifies a 172bp segment. PCR reaction of 26 samples positive for the presence of both universal GM markers, using these construct-specific primers, showed the presence of RR soybean (Figure 2). The percentage of GM soybean ranged between 0.11% and 5%. In 9 samples (1 soybean seed, 4 soybean meals and 5 complete feed mixtures) the amount of GM soybean was above the limit of 0.9% (Table 1). The majority of imported soybean meals, that were found to be positive for the presence of RR soybean, were of Brazilian origin. The Roundup Ready soybean was already found at the Serbian fields (Nikolić et al., 2009a) as well as in food (Nikolić et al., 2009b; Zdjelar et al., 2013) samples. Among 40 examined samples of feed in Serbia in 2009, more than one third showed mostly the presence of GM soybean, GM maize or both of the modified species, with a presence of GM above the

limit of 0.9% (Miljuš-Djukić et al., 2010). Another study of occurrence of RR soybean in feed products in 2010 showed the GMO content over 0.9% in 14 out of 36 positive samples (18%). The percentage of positive samples in different categories of feed samples varied from 38% in soybean meal samples to 55% in feed for calves and cattle (Nikolić et al., 2010a). Similar results were reported by Gryson et al. (2007) who showed that 7 out of 32 GM feed products analyzed in Belgium had GMO contents more than 0.9%. The three year's study conducted in Poland showed that the GM soybean is commonly used in animal feed (Kwiatek et al., 2007). Costa and Martinelli (2007) also reported frequent presence of GM soybean, which was not declared on the label. Survey of feed samples in Lithuania carried out in 2007 by Paulauskas et al. (2008) showed that 18, 4 and 5 samples from soybean, poultry, and cattle feeds, respectively, contained GM materials. This contamination was also a result of imports, mostly from South America, where Brazil is one of the largest producers of Roundup Ready soybean meals.

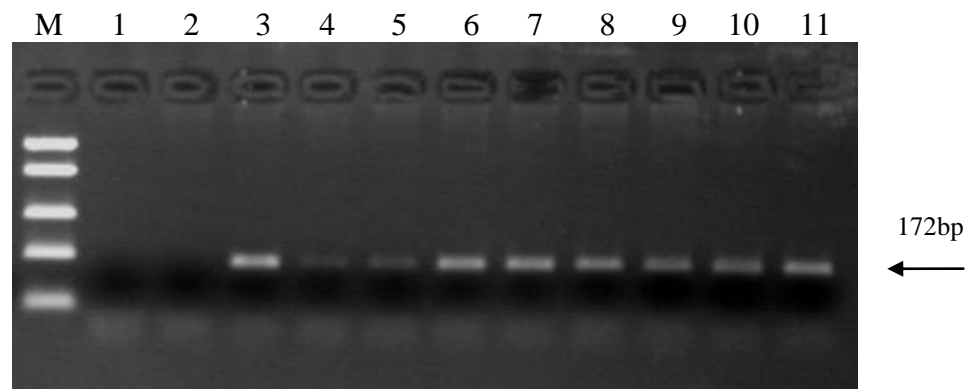


Figure 2. Agarose gel electrophoresis of PCR products from soybean samples for analysis of the Roundup Ready (RR) soybean. M-Low Range DNA Ladder, 50-1500 bp; 1. Blank-no template control; 2. Negative control; 3. CRM RR soybean 1%; 4.-11. samples positive for RR

Rapeseed is the second most important source of vegetable oil, after the soybean. After seed crashing and oil extraction, remains the meal that is used as a protein-rich component in animal feed (Devos et al., 2004). In Serbia, as in many countries in the EU, no commercial cultivation of GM rapeseed takes place, but their import is expected to increase, and their unforeseen,

intended or accidental cultivation may eventually occur. First case-specific monitoring of herbicide-tolerant rapeseed events RT73, Rf3 and T45 on the Serbian market was carried out in 2009 by Nikolić et al. (2010b). They found seven positive feed samples and no positive seed sample. Two samples positive for P35S promoter were positive for event T45 and two out of five Rf3

positive samples contained both Rf3 and RT73 events. In our study among 15 analyzed rapeseed meal samples none contained GM rapeseed, which is an expected result since all samples originated from EU where control and labelling of products is mandatory.

## CONCLUSIONS

The results found in this study clearly showed that imported maize and soybean and complete feed mixtures intended for animal feed on the Serbian market contain GMO. Especially compound feed mixtures contained high degree of RR soybean. Those products were without any declaration of the percentage of GM material on the labels of the package. On the other hand, all local products were GMO-free.

Detection of GMOs will become more complex in the following years. The number of new commercially available transformation events is increasing. Furthermore, the detection of unauthorized GMO remains a problem mainly due unavailability of sequence information. It would be recommendable to conduct the monitoring for the presence of GM plants continuously, at proper time intervals. Monitoring plans are required to control the distribution of non-labelled feeds containing GMO in the Serbian market.

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