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ISSN print: 1224-5984
ISSN online: 2248-3942

Rom Biotechnol Lett. 2021; 26(1): 2223-2229
doi: 10.25083/rbl/26.1/2223.2229

Received for publication, August, 24, 2020
Accepted, October, 20, 2020

Original paper

Detection of genetically modified organisms in soy feed and food in Romania

FLORICA BARBUCEANU[1,2], IULIA ZYBACZYNSKI[2], DIANA IONELA DUMITRESCU[2], GABRIEL PREDOI[1], STELIAN BARAITAREANU[1]

[1]University of Agronomical Sciences and Veterinary Medicine of Bucharest, Splaiul Independentei 105, District 5, 050097, Bucharest, Romania
[2]Institute for Diagnosis and Animal Health, 63 Dr Staicovici Nicolae Str, District 5, 050557, Bucharest, Romania

Abstract The paper describes the diagnostic methods used in Romania to detect the genetically modified organisms in soy feed and food and the results recorded between 2013 and 2019. The retrospective analyses of surveillance and diagnostic methods (qualitative and quantitative PCR techniques) developed for the genetically modified organisms detection in soy feed and food and of the transgenic Roundup Ready soybean line event 40-3-2 in Romania covered a total of 687 tests between 2013 and 2019 and revealed 32/131 GM events in 2013, 25/142 GM events in 2014, 24/117 GM events in 2015, 16/115 GM events in 2016, 6/97 GM events in 2017, 8/53 GM events in 2018, and 2/32 GM events in 2019.

Keywords GMO, genetically modified organisms, Roundup Ready, Glycine max, PCR.

To cite this article: BARBUCEANU F, ZYBACZYNSKI I, DUMITRESCU DI, PREDOI G, BARAITAREANU S. Detection of genetically modified organisms in soy feed and food in Romania. *Rom Biotechnol Lett*. 2021; 26(1): 2223-2229. DOI: 10.25083/rbl/26.1/2223.2229

*Corresponding author: FLORICA BARBUCEANU, Doctor of Veterinary Medicine, PhD, Associate Professor, Department of Preclinical Sciences, Faculty of Veterinary Medicine, University of Agronomical Sciences and Veterinary Medicine of Bucharest, Splaiul Independentei 105, sector 5, 050097, Bucharest, Romania, Tel/Fax: +4021- 318.04.69 / +4021- 318.04.98
Institute for Diagnosis and Animal Health, 63 Dr Staicovici Nicolae Str, sector 5, 050557, Bucharest, Romania, Tel/Fax: +40374322000/ +4021-4113394
E-mail: florica.barbuceanu@idah.ro

Introduction

World Health Organization defined GMO as "[…] *organisms (i.e. plants, animals or microorganisms) in which the genetic material (DNA) has been altered in a way that does not occur naturally by mating and/or natural recombination*." (OIE, 2014 [1]). The possible adverse effects of the genetic modified crops on human health and environmental safety are mostly focused on doubts but they are scientifically probable, and presented in good faith (ZHANG & al, 2016 [2]). Despite of the possibility of the unintended effects, the benefits of the genetic modified crops are not negligible mainly for countries where the food security challenges are linked with the crisis of water paucity and reduced rainfall [DADGARNEJAD & al, 2017 [3]).

The European Union made the legislation to ensure the safety of feed and food before the placing on the market of any GMO, in which the labelling of food products sold in EU market made with GMO is mandatory (EU, 2001[4]; EU, 2003 [5]; EU, 2003 [6]; EU, 2009 [7]; EU, 2011 [8]; EU, 2015 [9]).

Soy is an important source of food, reach in protein and oil, whose production has grown continuously around the world (FRIEDMAN & BRANDON, 2001 [10]; KORTH, 2008 [11]; JAMES, 2011 [12]).

40-3-2 is a variety of genetically modified soy, also known as Roundup Ready (RR), grown in many countries (CROPLIFE, 2017 [13]) due to the ability to not be destroyed by action of the herbicide glyphosate (PADGETTE & al, 1995 [14]). The genetically modified soybean line Roundup Ready GTS 40-3-2 was developed by Monsanto Company and was based on recombinant DNA technology, which targeted the gene synthesizing 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), which was isolated from the CP4 strain of Agrobacterium tumefaciens. Several soy products with GMO events have been recorded in the last years (Table 1), and the detection and identification of genetically modified soy in food and feed become an important activity of national and international authorities.

Common technique used for the detection and identification of genetically modified soy in food and feed analyses is polymerase chain reaction (LAU & al, 2004 [15]; SIERADZKI & al, 2008 [16]). The Romanian Reference Laboratory for Genetically Modified Organisms is hosted by the Molecular Biology Service of the Institute of Diagnosis and Animal Health.

The aim of study was the retrospective analyses of surveillance and diagnostic methods (qualitative and quantitative PCR techniques) developed for the genetically modified organisms' detection in soy feed and food and of the transgenic Roundup Ready soybean line event 40-3-2 in Romania.

Table 1. GMO soy (Glycine max) products with or without the 35S promoter and / or the NOS terminator

Product code	GMO event	P35S		T-nos	
		T	V	T	V
260-05 (=G94-1, G94-19, G168)	DD-026005-3	+		+	
305423	DP-305423-1	-	*	-	*
356043	DP-356043-5	+	+	-	*
A2704-12	ACS-GM005-3	+	+	-	-
A2704-21, A5547-35	ACS-GM004-2	+		-	
A5547-127 (LibertyLink)	ACS-GM006-4	+	+	-	-
BPS-CV127-9	BPS-CV127-9	-	-	-	-
DAS-44406-6	DAS-44406-6	-	-	-	-
DAS-68416-4	DAS-68416-4	-	-	-	-
DAS-81419-2	DAS-81419-2	-	-	-	-
FG72	MST-FG072-2	-	-	+	+
GTS 40-3-2 (Roundup Ready)	MON-04032-6	+	+	+	+
GU262 (LibertyLink)	ACS-GM003-1	+		-	
MON87701	MON-87701-2	-	-	-	-
MON87705	MON-87705-6	-	-	-	-
MON87708	MON-87708-9	-	-	-	-
MON87712	MON-87712-4	+		-	
MON87751	MON-87751-7	-		-	
MON87769	MON-87769-7	-	-	-	-
MON89788	MON-89788-1	-	*	-	*
SYHT0H2	SYN-000H2-5	+	+	+	+
W62, W98 (Liberty Link)	ACS-GM001-8	+		+	

"-" the event does not contain the screening element, "+" the event contains the screening element, "T" present theoretically (based on information from the EUginius database), "V" verified using reference materials or other controls, "* " Unexpected results

Experimental part

Materials and methods

The methodology of this study followed all European Union, European Committee for Standardization and Romanian competent authorities' recommendations in the field of GMOs testing (EU, 2001 [4]; EU, 2003 [5]; EU, 2003 [6]; EU, 2009 [7]; EU, 2011 [8]; EU, 2015 [9]; EN/TS, 2006 [17]; ISO, 2006 [18]).

Food and feed samples

DNA isolation and purification were carried out between 2013 and 2019 from various raw or processed food and feed with soy (Table 2) from Romanian market in accord with general requirements and definitions and sampling strategies of analysis for the detection of genetically modified organisms and derived products (EN/TS, 2006 [17]; ISO, 2006 [18]).

Table 2. Soy food and feed samples analysed between 2013 and 2018

MATRIX	2013	2014	2015	2016	2017	2018	2019
Soya beans	18	34	39	10	19	8	2
Feed with soy	11	2	7	16	0	5	0
Food with soya	102	106	71	89	78	40	30
Total	**131**	**142**	**117**	**115**	**97**	**53**	**32**

DNA extraction, quantification and purity

For each analytical sample, DNA extraction was performed in duplicate in accord with standardised methods of analysis for the detection of genetically modified organisms and derived products (ISO, 2005 [19]; ISO/TS, 2005 [20]).

Samples were grinded with Maxi Grinder Solo (Genomic Industry, France) and Grindomix GM 200 (Retsch, Germany) in particles with size below 500 microns and the DNA extraction were performed with NucleoSpin Food kit (Macherey-Nagel, Germany) or GENESpin kit (Eurofins GeneScan, Germany) by using 200 mg sample/test. The extraction was carried out according to the manufacturer instructions.

Briefly, the steps of the DNA extraction with NucleoSpin Food kit (Macherey-Nagel, Germany) were (1) Incubation with lysis buffer, (2) Digestion of endogenous nucleases and other impurities in the presence of Proteinase K; (3) Binding DNA to the silica membrane of NucleoSpin Food tubes; (4) Washing DNA, purifying salts, proteins and other cellular impurities; and (5) Recovering of the purified DNA with elution buffer. Purified DNA is free of contaminants and enzyme inhibitors, being usable in all types of genetically modified DNA detection and quantification reactions. DNA concentration and purity were determined spectrophotometrically. After completing the extraction steps, 0.1-10 µg nucleic acids / 200 mg sample were obtained, the amount depending on the type of matrix analysed.

Briefly, the steps of the DNA extraction with GeneSpin kit (Eurofins GeneScan, Germany) were (1) Homogenization of the sample and lysis of cells: 550 µl CF lysis buffer, preheated to 65°C, is homogenised with 200 mg of sample in 2 ml centrifuge tubes; (2) Adjusting the DNA binding conditions with 200 µl ethanol; (3) DNA binding: The sample is loaded into the filters combined with the collection tubes; (4) Washing and drying with CQW washing buffer and working solution of C5 (C5 buffer + 80 ml ethanol); (5) DNA elution with CE elution buffer, preheated to 70°C: two elution steps are performed using 2x100 µl of elution buffer, the filters were incubated for 5 minutes at 18-25°C and centrifuged 1 min, at ~13000 rpm.

Qualitative PCR detection methods used to identify GMOs

The test procedure comprises identifying the presence of plant-specific DNA and identifying genetically modified material by PCR methods for detecting genetic elements that can be found in most GMOs (35S promoter and / or NOS terminator). The identification of these genetic elements has the disadvantage that they can be present naturally in the host organism, due to the presence of viruses (Cauliflower Mosaic Virus) and/or bacteria (*Agrobacterium tumefaciens*) that contain these elements.

Qualitative nucleic acid-based methods used in this study were in accord with the standardised methods of analysis for the detection of genetically modified organisms and derived products [ISO, 2005 [20]; ISO, 2005 [21]).

Taxon-specific PCR system: identification of the soybean plant-specific sequence Glycine max L. (lectin)

DNA identification specific to the GTS 40-3-2 line was performed using either a dedicated kit "GMO Ident Roundup Ready Soy" (Eurofins GeneScan, Germany) with TaqDNA polymerase (AmpliTaqTM Gold DNA Polymerase) 5UI / µl, or the in-house method with primers and probes.

Identification of GTS 40-3-2 line-specific DNA (qualitative detection methods) using the "GMO Ident Roundup Ready Soy" kit (Eurofins GeneScan, Germany) was made in accordance with the manufacturer's recommendations. The kit allows the identification by amplifying a specific 128 bp sequence of the lectin (Le1) gene, naturally present in the genome of the soybean plant, indicating the presence of plant-specific DNA and the lacking of inhibitory compounds in the isolated DNA solution.

20 µl Master Mix + 5 µl DNA (50-100 ng) / H_2O were pipetted on ice / cooling block avoiding the generation of air bubbles following pipetting, centrifuged in the cold for a few seconds and placed on the thermocycler according to the operating parameters described in Table 3.

Table 3. The thermocycler operating parameters for the soybean specific DNA identification with GMO Ident Roundup Ready Soy Kit (Eurofins Genescan, Germany)

Step	Temperature (°C)	Time	Number of cycles
Initial Denaturation	94	10 min	1x
Denaturation	94	25 sec	50x
Annealing	62	30 sec	
Extension	72	45 sec	
Final Extension	72	3 min	1x
Hold	4	∞	-

For the in-house method, reagents were mixed according to the protocol detailed in Table 4 with the soybean specific sequence forward primer GMO3: 5'-GCC CTC TAC TCC ACC CCC ATC C -3', and reverse primer GMO4: 5'-GCC CAT CTG CAA GCC TTT TTG TG -3'.

Table 4. PCR reaction mixture for the soybean specific DNA identification with the in-house method

Reagent	**Final concentration of the mix**	**Volume per reaction (μl)**
Nuclease-free H_2O	-	28.75
Buffer 5X (without $MgCl_2$)	1x	10
$MgCl_2$ (25 mM)	1.5 mM	3
dNTPs (10 mM)	0.4 mM	1
GMO_3 (20μM)	0.4 μM	1
GMO_4 (20μM)	0.4 μM	1
Taq DNA polymerase (5UI/μl)	0.625 U	0.25
TOTAL (μl)		45

45 μl Master mix GMO3/GMO4 + 5 μl ADN (50-200 ng) / H2O were pipetted on ice / cooling block avoiding the generation of air bubbles following pipetting, centrifuged in the cold for a few seconds and placed on the thermocycler according to the operating parameters described in Table 5.

Table 5. The thermocycler operating parameters for the soybean specific DNA identification with the in-house method

Step	**Temperature (°C)**	**Time**	**Number of cycles**
Initial Denaturation	95	5 min.	
Denaturation	95	30 sec.	40 x
Annealing	63	30 sec.	
Extension	72	30 sec.	
Final Extension	72	3 min	
Hold	4	∞	

PCR system specific to the genetic construct: identification of the sequence representing the junction region between the CaMV 35S promoter and the CTP4 (Chloroplast Transit Peptide) sequence derived from Petunia hybrida

PCR primers for the sequence representing the junction region between the CaMV 35S promoter and the CTP4 sequence are primer forward 35s-f2: 5'-TgA TgT GAT ATC TCC ACT gAC g-3'and primer reverse petu-r1: 5'-TgT ATC CCT TgA gCC ATg TTg T -3'. Reagents were mixed according to the protocol detailed in Table 6.

Table 6. PCR reaction mixture for the identification of the sequence representing the junction region between the CaMV 35S promoter and the CTP4 (chloroplast transit peptide)

Reagent	**Final concentration of the mix**	**Volume per reaction (μl)**
Nuclease-free H2O	-	13.375
Buffer 10 (without MgCl2)	1x	2.5
MgCl2 (25 mM)	1.5 mM	2
dNTPs (10 mM)	0.4 mM	1
35s-f2 (20 μM)	0.4 μM	0.5
petu-r1 (20 μM)	0.4 μM	0.5
Taq DNA polymerase (5UI/μl)	0.625 U	0.125
TOTAL (μl)		20

20 μl Master Mix 35s-f2/petu-r1 + 5 μl ADN (50-200 ng) / H2O, were pipetted on ice / cooling block avoiding the generation of air bubbles following pipetting, centrifuged in the cold for a few seconds and placed on the thermocycler according to the operating parameters described in Table 7.

Table 7. The thermocycler operating parameters for the identification of the sequence representing the junction region between the CaMV 35S promoter and the CTP4 (chloroplast transit peptide)

Step	Temperature (°C)	Time	Number of cycles
Initial Denaturation	95	5 min.	
Denaturation	95	30 sec.	40 x
Annealing	60	30 sec.	
Extension	72	25 sec.	
Final Extension	72	3 min	
Hold	4	∞	

Agarose gel electrophoresis of PCR products

The results were assessed by agarose gel electrophoresis (2.5 %, w/v for GMO Ident Roundup Ready Soy Kit and 1.5 %, w/v; TAE system for in-house method) set at 100V for 25-30 min. After completion of electrophoresis, the gel is analysed in the video documentation system. The specific bands obtained have a size of 118 bp for the lectin gene, respectively 128 bp for the P35S-CTP4 genetic construct in the case of the method using a dedicated kit and 172 bp for the GTS 40-3-2 line-specific sequence for the in-house method (Fig. 1).

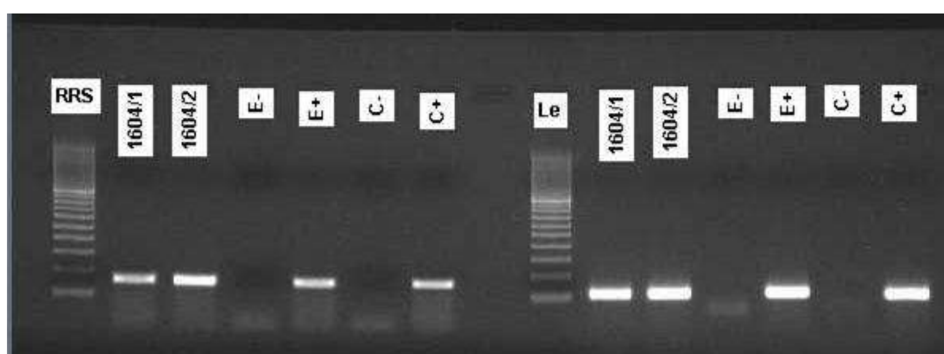

Figure 1. Identification of the specific-DNA to line 40-3-2 and the specific-sequence to the soybean plant Glycine max L. (lectin) by conventional PCR technique. RRS= 40-3-2 (Roundup Ready Soy); Le= lectin; 1604/1= soya beans/1; 1604/2=soya beans; E-= control negative extraction; E+= control positive extraction; C-= Control negative target DNA; C+= control positive target DNA.

PCR technique for GTS 40-3-2 line-specific DNA quantification

Quantitative nucleic acid-based methods used in this study were in accord with the standardised methods of analysis for the detection of genetically modified organisms and derived products [ISO, 2005 [20]; ISO, 2005 [22]).

Method was carried out with the GMO Quant (HR) Roundup Ready Soy kit (Eurofins GeneScan, Germany) as per the manufacturer's recommendations with an Applied Biosystems 7900 HT Fast Real-Time PCR System (Invitrogen, SUA) (Fig. 2), at which the following operating parameters have been set: decontamination (UNG) 50ºC – 2 min; activation of DNA polymerase 95ºC – 10 min; amplification 95ºC – 15 sec, 60ºC – 1 min, and number of cycles 45. The kit uses ready-to-use reagents Master Mix (HR) GSE-P-07.42 RRS-HT-P/C and Master Mix (HR) Soy lectin 1. The concentration of DNA solutions was adjusted at ~ 40 ng / µl (~ 200 ng DNA / reaction) and at a final volume / reaction of 25 µl.

20 µl Master Mix + 5 µl DNA (200 ng) / H2O were pipetted on ice / cooling block avoiding the generation of air bubbles following pipetting, centrifuged in the cold 5 sec at 3000 rpm and placed on the thermocycler.

Results and Discussions

This study covered the surveillance and monitoring of 40-3-2 events performed by the National Reference Laboratory for Genetically Modified Organisms, hosted by the Molecular Biology Service of the Institute of Diagnosis and Animal Health, between 2013 and 2017 (Table 8).

In 2013, 40-3-2 events were recorded in 32 of 131 soya matrix samples (24.42%), of which the soya beans samples were 4 below the 0.9% threshold and 3 above 0.9% threshold, the feed with soy samples were 1 below the 0.9% threshold and 4 above 0.9% threshold, and the food with soya samples were 10 below the 0.9% threshold and 10 above 0.9% threshold.

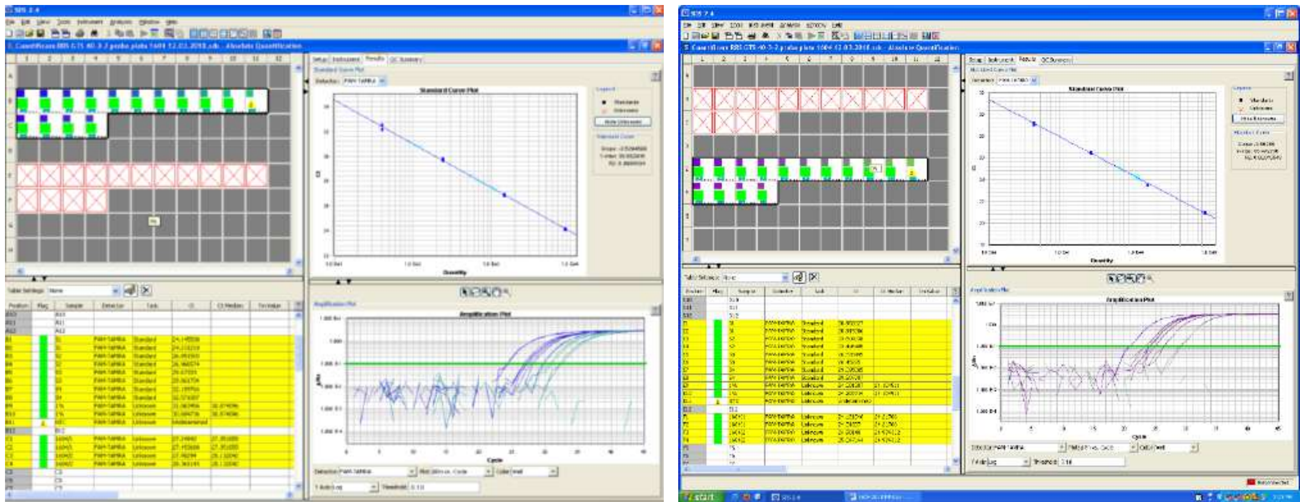

Figure 2. Quantification of DNA specific to line 40-3-2 in both systems (GMO and Species-Ref.) with the GMO Quant (HR) Roundup Ready Soy kit (Eurofins GeneScan, Germany) in Applied Biosystems 7900 HT Fast Real-Time PCR System (Invitrogen, SUA).

Table 8. Determination of the 40-3-2 events in feed and food with soy between 2013 and 2019, in Romania

MATRIX	Year / Event value	2013	2014	2015	2016	2017	2018	2019
Soya beans	<0.9%	4	6	4	2	-	-	1
	>0.9%	3	8	13	2	6	2	1
Feed with soy	<0.9%	1	-	-	-	-	-	-
	>0.9%	4	2	2	9	-	2	-
Food with soya	<0.9%	10	9	5	3	-	4	-
	>0.9%	10	-	-	-	-	-	-
Total		**32**	**25**	**24**	**16**	**6**	**8**	**2**

The tests performed in 2014 revealed 25 events in 142 soya matrix samples (17.61%), of which the soya beans samples were 6 below the 0.9% threshold and 8 above 0.9% threshold, the feed with soy samples were 2 above 0.9% threshold, and the food with soya samples were 9 below the 0.9% threshold.

In 2015, 24 events were recorded in 117 tests, of which the soya beans samples were 4 below the 0.9% threshold and 13 above 0.9% threshold, the feed with soy samples were 2 above 0.9% threshold, and the food with soya samples were 5 below the 0.9% threshold.

In 2016, 16 events were recorded in 115 tests, of which the soya beans samples were 2 below the 0.9% threshold and 2 above 0.9% threshold, the feed with soy samples were 9 above 0.9% threshold, and the food with soya samples were 3 below the 0.9% threshold.

In 2017 were tested 97 samples and were recorded only 6 events above 0.9% threshold in soya beans samples.

In 2018 were recorded 8 events in 53 tests, of which the soya beans samples were 2 above 0.9% threshold, the feed with soy samples were 2 above 0.9% threshold, and the food with soya samples were 4 below the 0.9% threshold.

In 2019 were performed 32 tests and only 2 events were recorded in soya bean samples, one below the 0.9% threshold and one above 0.9% threshold.

The surveillance of feed and food in various European and non-European markets proved the circulation of GMO in the last decades (JAMES, 2011; MANDACI & al, 2014 [23]; SAFAEI & al, 2020). Some non-European studies, reported over 95% of soy analysed samples positive at the detection of 35S promoter and NOS terminator sequences (SAFAEI & al, 2020), while other reported that all of the tested food samples contained less than 0.1% of 5-enol-pyruvyl shikimat-3-phosphate synthase (EPSPS) gene (MANDACI & al, 2014 [23]).

Conclusions

The genetically modified organisms' detection in soy feed and food detection is a complex methodology that start with the isolation, purification and quality control of isolated DNA and continue with qualitative PCR methods of the taxon detection (identification of the sequence specific to the Glycine max L.) and the genetic construct detection (identification of the sequence representing the junction region between the CaMV 35S promoter and the CTP4), and is finalised with a real-time-PCR technique for GTS 40-3-2 line-specific DNA quantification.

The retrospective analyses revealed 32/131 GM events in 2013, 25/142 GM events in 2014, 24/117 GM events in 2015, 16/115 GM events in 2016, 6/97 GM events

in 2017, 8/53 GM events in 2018, and 2/32 GM events in 2019.

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