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Application of Polymerase Chain Reaction for High Sensitivity Detection of Roundup Ready™ Soybean Seeds and Grains in Varietal Mixtures

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Summary

Strong increase in the production of genetically modified organisms (GMOs) observed over the years has led to a consolidation of transgenic seed industries worldwide. The dichotomy between the evaluated risk and the perceived risk of transgenic use has defined their level of acceptability among different global societies. GMOs have been widely applied to agricultural commodities, among them the Roundup Ready™ (RR™) soybean line GTS 40-3-2 has become the most prevalent transgenic crop in the world. This variety was developed to confer plant tolerance against glyphosate-based agricultural herbicide Roundup Ready™. Issues related to detection and traceability of GMOs have gained worldwide interest due to their increasing global diffusion and the related socioeconomic and health implications. Also, due to the widespread use of GMOs in food production, labelling regulations have been established in some countries to protect the right of consumers and producers. Besides regulatory demand, consumer concern issues have resulted in the development of several methods of detecting and quantifying foods derived from genetically engineered crops and their raw materials. Polymerase chain reaction (PCR) has been proven to be the method of choice to detect the presence or absence of the introduced genes of GMOs at DNA level. The present paper aims to verify whether the PCR technique can detect RR™ soybean seeds among conventional ones to further certification as non-GM soybean seeds and grains. This analysis could be accomplished through the development of new methodology called 'intentional contamination' of soybean conventional seeds or grains with the respective RR™ soybeans. The results show that the PCR method can be applied with high sensitivity in order to certify conventional soybean seeds and grains.

Key words: certification of conventional soybean seeds and grains, detection of transgenic soybean, polymerase chain reaction (PCR), Roundup Ready™ (RR™) soybean

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Introduction

Genetically modified plants are living organisms that have their genetic characteristics unnaturally altered either by deletion, addition, replacement, or modification of at least one gene (*1*). Such changes seek to develop insect--resistant and/or herbicide-tolerant crops. Authorized for commercialization in the United States since 1994, GM crops, linked to promises of obtaining higher yields, have aroused the interest of farmers around the world. The crop for 2015 is estimated at 200 million hectares distributed among more than 40 countries (*2*), with approx. 70 % of the crop growing in the United States.

Currently, five companies (Monsanto, Novartis, Astra Zeneca, Aventis, and DuPont) are controlling almost the entire production of transgenic seeds in the world (*3*). RR™ soybean seeds stand out. The most cultivated transgenic line worldwide presents a genetic sequence that confers tolerance to glyphosate-based herbicides, a technology developed by the Monsanto Company.

Driven by the illegal introduction of RR™ soybean seeds in Brazil, the Federal Government has regulated the production, trade, and consumption of what will become the first transgenic event in the country and edited the Brazilian legislation in the 'Comuniqué n°. 54' of the National Technical Committee on Biosafety (CTNBio) (*4*). The government found no evidence of any risks either to the environment or to human and animal health resulting from the use of RR™ soybean GTS 40-30-2 (*5*). Besides the concern about the population's health, given the possible risks to health and environment, much of the controversy is related directly to the issues of convenience and socioeconomic opportunities, and national interest. It is therefore of a great importance to have some infrastructure, at world level, capable of monitoring the effects of genetically modified organisms in the long run, and to ensure the traceability of properly segregated chains. The availability of validated molecular and immunological methods for detecting and quantifying transgenic traces in seeds and processed foods is therefore a key element in the implementation of national policies on biosafety.

It is also vital to highlight the monopolistic power of biotechnology companies over the control of seed reproduction, since transgenesis brings with it a traceability system that allows a continuous and effective control over the payment of intellectual property rights, even of those unintentionally contaminated crops. Out of the 771 soybean cultivars registered so far in the National Register of Plant Varieties, an agency under the Ministry of Agriculture, Livestock, and Supply in Brazil, 269 are RR™ soybean cultivars (*6*). Because they are grown side by side, the probability of contamination is high. It is a prerogative of the national government to ensure the supply of contamination-free natural seeds. Seeds sustain the basis of agricultural chains, and the development of a reliable and affordable methodology as a controlling agent is of strategic importance (*6*). The genetically modified soybean seed shows no morphological differences when compared to the conventional varieties, so that its recognition is macroscopically difficult. Therefore, it is necessary to standardize a methodology that is highly sensitive and cost-effective aiming at its utilization by a larger number of producers and public agencies.

How to establish a method for detecting and quantifying the presence of GM seeds in the inspection samples of conventional seeds so as to ensure compliance with the existing standard? The need to monitor and verify the presence of transgenic seeds in conventional seed lots and food commodities (grain, bran, *etc.*) requires the use of proper analytical methods. They should be able to detect and/or quantify the gene sequence or DNA fragment inserted into the body or the proteins expressed by it. This study analyses whether the PCR methodology can be effective in detecting transgenic beans in samples consisting of conventional soybean meal contaminated with the known quantities of RR™ soybean. If so, what would be the lowest limit for such detection?

The present work aims at establishing a methodology that can be applied as a qualitative standard for seeds and soybeans marketed in Brazil, complying with the Brazilian legislation and those of foreign communities, as there is the need for certifying their quality by more specific methods than those employed at present in a manner that can be done not only by small producers but also by the major industries.

Material and Methods

Fig. 1 depicts a general outline of the different procedures employed in this work. The respective procedures for each major step are presented in alphabetical order.

Soybean seed samples

The sampling method used was according to Greiner and Konietzny (*7*) based on the standards of the International Seed Testing Association (*8*). For small-scale detection of RR™ soybeans mixed with larger amounts of conventional soybeans, an intentional contamination of soybeans in field samples and certified samples was carried out during the present work. Due to the risk of cross-contamination, each sample was prepared separately followed by rigorous cleaning and sanitizing procedures. To confirm the absence of cross-contamination, samples of certified conventional soybeans were analyzed in each prepared sample.

Soybean field samples

RR™ soybeans were purchased from Bunge S.A. (Ponta Grossa, Brazil), and conventional soybeans were purchased from Cocamar Agroindustrial Cooperative (Campo Mourão, Brazil). Certified GM soybeans were purchased from Pioneer Hi-Bred International, Inc. (Johnston, IA, USA) and used as positive control for transgenesis. As a negative control, seed samples certified by the Cocamar Agroindustrial Cooperative were used. The samples consisted of 100 g of non-GM soybeans contaminated with 1 to 14 RR™ soybean grains totalizing 14 seed samples contaminated with RR™ seeds. In the preparation of transgenic controls, 100 g of GM soybeans were used for the negative control sample, and 100 g of GM soybeans for the positive control. The samples were stored at 4 °C.

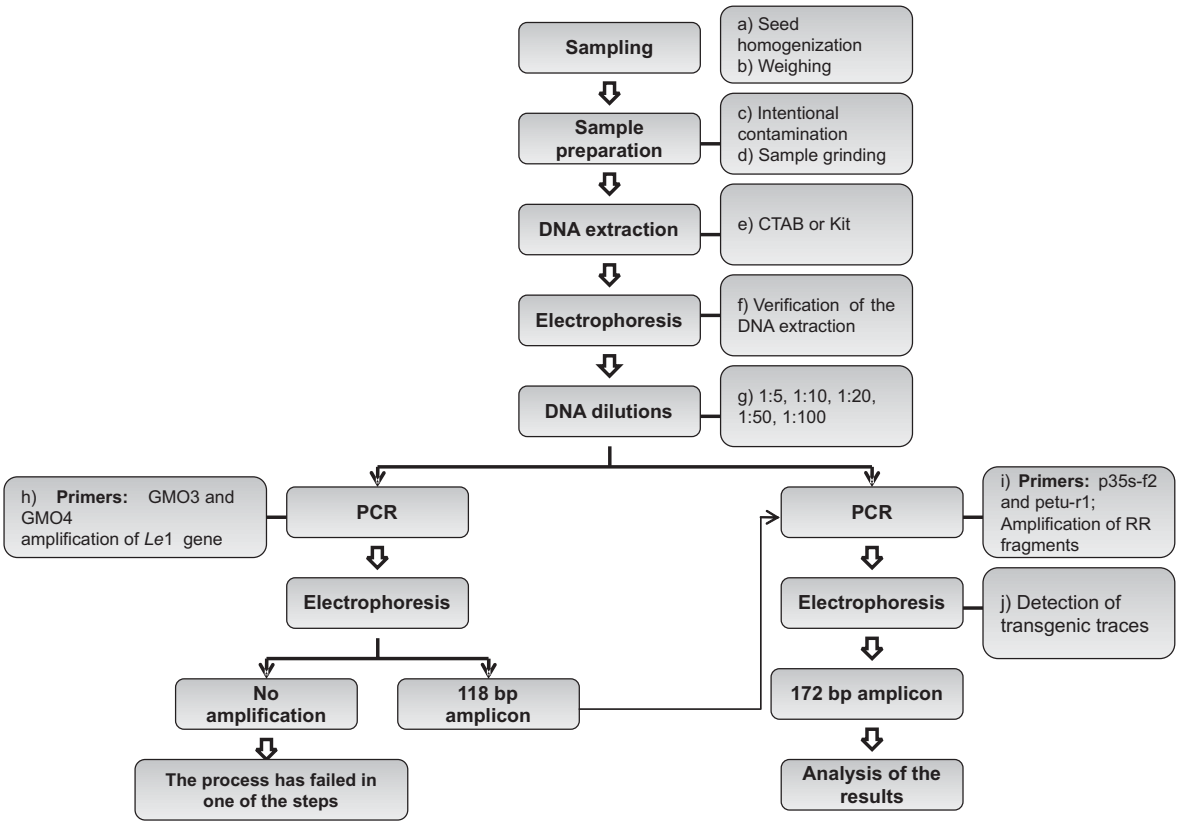

Fig. 1. General procedures adopted in the present work

Soybean reference samples

Pure seeds were kindly provided by the Agriculture and Supply Secretary of Parana State (SEAB, Curitiba, PR, Brazil). Certified RR™ soybean and first-generation conventional soybeans (C1) were used. The pure seed followed a different pattern of intentional contamination than the field samples. These were prepared in accordance with the upper and lower limits of the standards for the production and marketing of soybean seeds in Brazil, as determined by the Brazilian legislation (*9*). Samples were prepared by addding a number of seeds (2, 3, 5, 6, 9, 10, 15 or 20 RR™ soybean seeds) to 500 g of conventional seeds for intentional contamination of certified samples for subsequent analysis, which was performed in triplicate.

Sample preparation

The sampling procedure was performed in the laboratory of the Parana Company for Product Classification (CLASPAR, Curitiba, PR, Brazil) in accordance with the standards established by the Seed Testing International (*8*), as described below.

A sack of 10 kg of conventionally certified soybean seeds was poured into an appropriate container and homogenized manually. Then, small portions were collected from different locations in the container and grouped to obtain 500 g. The RR™ soybean seeds were homogenized in the same way. In the contamination step, a single seed of conventional soybean was replaced by a single seed of certified RR™ soybean, and thus successively for each sample. The seeds were crushed with a cleaned and sterile blender to obtain a homogeneous meal. Contaminations were carried out in triplicate for each sample.

Genomic DNA extraction

The samples were subjected to a cetyl trimethylammonium bromide (CTAB)-based DNA extraction method described by Greiner and Konietzny *(7)* with a few adaptations. A mass of 2 g of soybean meal was added to 10 mL of extraction buffer (100 mM Tris, 20 mM Na_2-EDTA, 1.4 M NaCl with 20 g/L of CTAB, pH=8) in a 50-mL tube. The solution was homogenized and incubated at 60 °C for 1 h followed by inversions every 5 min. Then the material was cooled down to room temperature and centrifuged at 4000×*g* for 15 min. The supernatant of each sample was transferred to a new 2-mL Eppendorf tube, centrifuged at 10 000×*g* for 10 min and the upper phase (water) was transferred into a new 2-mL tube. Then 2 volumes of 40 mM NaCl and 5 g/L of CTAB were added, incubated for 60 min at 25 °C and centrifuged at 10 000×*g* for 5 min. The precipitate was dissolved in 350 μL of 1.2 M NaCl, then 350 μL of chloroform were added, mixed carefully for about 30 s, and centrifuged at 10 000×*g* for 10 min. Afterwards, the upper phase (water) was transferred into a new 1.5-mL tube, 0.6 volumes of isopropanol were added, centrifuged at 10 000×*g* for 10 min, then the supernatant was removed completely, and 500 μL of 70 % ethanol were added to the pellet and mixed. The mixture was centrifuged at 10 000×*g* for 10 min and the supernatant was removed completely. DNA was air dried for 30 min up to 1 h, dissolved in 100 μL of ddH_2O and stored at 4 or –20 °C.

The current literature suggests that DNA can be further purified using a DNA extraction kit (7). Thus, a different DNA extraction procedure was done with the commercial DNA*Extractor Clean* kit (Eurofins, GeneScan, Des Moines, IA, USA) for the extraction of DNA from food, feed and grain (Cat. nos.: 5224700610 and 5224700810), as follows: 100 g of the material were mechanically grounded with a cleaned and sterile blender and 2 g were transferred to a 50-mL tube, followed by the addition of 10 mL of lysis buffer (100 mM Tris-Cl, pH=8.0, and 100 mM EDTA) and 10 μL of proteinase K (20 mg/mL). The samples were incubated at 60 °C for 2 h under constant manual agitation and then centrifuged at 4000×*g* for 10 min. Then, 800 μL of the supernatant were added into a 1.5-mL Eppendorf tube. A volume of 600 μL of chloroform was added and the tubes were mixed by vortexing. A volume of 600 μL of the aqueous supernatant was transferred into a fresh 1.5-mL Eppendorf tube. After that, 2 μL of the glycogen solution with 480 μL of 80 % isopropanol were added into the tube and mixed thoroughly. The samples were incubated for 20 min at room temperature and centrifuged at 10 000×*g* for 15 min. The supernatant was discarded and 500 μL of 75 % ethanol were added. A step of vortexing and then centrifugation (at 10 000×*g* for 5 min) were done. Subsequently, the supernatant was discarded, the tubes were centrifuged again (at 10 000×*g* for 1 min) and the DNA pellet was dissolved with 100 μL of sterile water and kept in a freezer at –20 °C.

The DNA samples extracted by both methods were analyzed by electrophoresis in 0.8 % agarose gels, stained with ethidium bromide (0.5 mg/mL), followed by photo documentation.

Qualitative PCR detection of transgenic and lectin sequences

The sequences of oligonucleotide primers used are shown in Table 1. A series of dilutions (1:5, 1:10, 1:20, 1:50 and 1:100) were performed from the original DNA samples with ultrapure sterile water. Amplification reactions were carried out with Platinum™ Taq DNA polymerase (Invitrogen, Carlsbad, CA, USA) in a final volume of 25 μL, containing the following components (in μL): ddH_2O 13.4, 10× PCR buffer 2.5, magnesium chloride (1.5 mM) 1.0, primer A (5 μM) 1.0, primer B (5 μM) 1.0, dNTP solution (5.0 mM) 1.0, Platinum® Taq (5 U/μL) 0.1, and 5 μL of the diluted DNA. In the PCR mix intending to amplify the lectin gene, primer A and primer B corresponded to GMO3 and GMO4, respectively. In the PCR mix intending to amplify the RR™, primer A and primer B corresponded to p35s-f2 and petu-r1, respectively.

The iCycler Thermal Cycler (Bio-Rad Laboratories, Inc, Berkeley, CA, USA) was used for lectin sequencing with the cycling programme consisting of: denaturation at 95 °C for 10 min, 35 cycles, and then at 95 °C for 30 s, annealing at 60 °C for 30 s, elongation at 72 °C for 30 s, final elongation at 72 °C for 3 min. The cycling program for RR™ soybean detection was as follows: denaturation at 95 °C for 10 min, 35 cycles, and then at 95 °C for 30 s, annealing at 60 °C for 30 s, elongation at 72 °C for 30 s and final elongation at 72 °C for 3 min.

The electrophoretic analysis was performed in 1.6 % agarose gel in 1×TBE buffer followed by ethidium bromide staining and photo documentation. After the electrophoretic analysis, samples with bands corresponding to the lectin gene were detected and amplified again (from previously performed dilutions) using primers corresponding to the RR™ gene.

A PCR negative control with 5 μL of ultrapure sterile water was used to check the absence of contaminants in reagents. A PCR positive control with the target DNA sequence was done to check whether the PCR reagents were in good conditions.

Results and Discussion

Quality of extracted DNA

In general, the extracted DNA of the soybean samples (field and certified samples) by the CTAB method presented good quality and quantity (Fig. 2). The extraction by this method showed amplification of the RR™ gene for all field samples (Coca 1 to Coca 14) and for transgenesis control. The results with certified samples also showed amplification for all (SS1, SS2, SS3, SS5, SS6, SS9, SS10, SS15, SS20, SST, SSØ and Ø) samples and also for transgenesis control, and no amplification for the extraction controls, which refers to the absence of DNA from the sample, where SST is totally transgenic sample, or without mixture of conventional soybean seeds; SSØ is the negative control (conventional certified soybean seeds), used to verify possible cross-contamination that could occur during each of the steps of the analysis; and Ø is the sample of conventional soybean seeds. It is important to determine whether the blender (or other equipment) used for crushing seeds was contaminated or not with transgenic seeds. The results showed that none of the Ø samples was positive for the RR™ transgenic soybean gene.

In order to compare DNA extraction methods, the samples of soybean seeds were also submitted to an extraction with a commercial DNA extraction kit from Eurofins GeneScan. However, no DNA bands were detected in the samples extracted by this method, even repeating the extraction procedure (Fig. 3). Therefore, in this study, DNA extraction by the CTAB method proved to be more

Table 1. Primers used for qualitative PCR and their respective band lengths

	Primer	Sequence	Length
Conventional soybeans (lectin specific)	GMO3	5′-GCC CTC TAC TCC ACC CCC ATC C-3′	118 bp
	GMO4	5′-GCC CAT CTG CAA GCC TTT TTG TG-3′	
RR™ soybeans	p35s-f2	5′-TGA TGT GAT ATC TCC ACT GAC G-3′	172 bp
	petu-r1	5′-TGT ATC CCT TGA GCC ATG TTG T-3′	

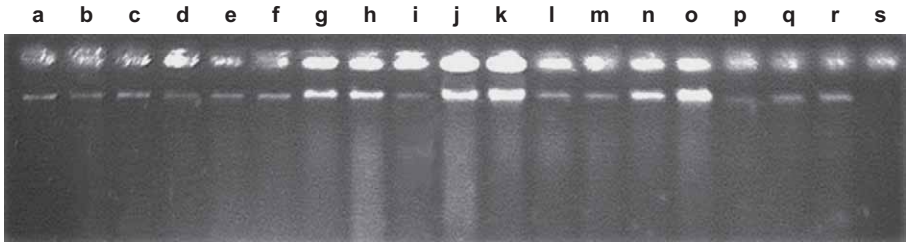

Fig. 2. DNA samples extracted by CTAB method corresponding to the field samples and analyzed by agarose gel electrophoresis at 0.8 %. From a to r: genomic DNA of the samples; s: negative extraction control

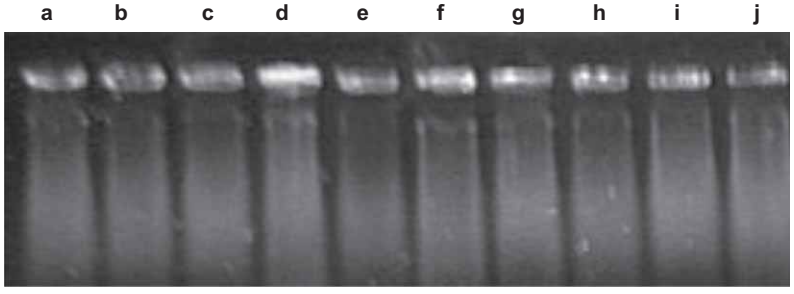

Fig. 3. DNA samples of soybean seeds extracted using the Eurofins GeneScan kit. From a to j: genomic DNA of the samples

effective according to the images visualized by agarose gel electrophoresis (Fig. 4).

These results are corroborated by the data obtained by Moriuchi *et al*. (*10*), who claim that CTAB is an efficient protocol for extracting genomic DNA from plant tissues. For extracting DNA from processed food products, methods for the removal of a series of PCR inhibitors such as polysaccharides, polyphenols and proteins are needed (*11*). However, the grains and seeds do not contain so many inhibitted or contaminated compounds, and so the standard method can be used for an effective DNA extraction (*11*). In processed foods, DNA extraction produces different sizes of gene fragments, indicating different levels of DNA degradation according to the processing steps (*12*).

The DNA extraction method used in the present work has already been applied in previous studies for the purification of raw materials and also of processed foods containing soybean (*11*,*12*). Most of the published scientific articles evaluate more than one protocol of DNA extraction and show that CTAB presents effective results (*7*, *10–15*). The success of DNA amplification methods depends largely on the DNA extraction protocols, which should provide high quality and yield of DNA (*11*).

Qualitative PCR detection

Lectin gene amplifications

PCR amplification of the soy-specific lectin gene was designed to identify whether there was amplification inhibition of Taq polymerase enzyme by some compound in the reaction mixture. Both field samples and artificially contaminated samples showed a specific band of 118 base pairs (bp) confirming the quality of both amplification and DNA extraction procedures (Fig. 5) and the results of amplification are discussed below.

Various controls are necessary to monitor the DNA detection and amplification process. There are positive controls, using primers that amplify a fragment that is, in any case, contained in the plant under study. They are designed to control the quality of DNA preparation and the adequacy of chemical parameters in general for DNA amplification. A detection failure, *i.e.* if no PCR product is generated, means that there are inhibiting factors in the reaction, the amount of DNA is insufficient or excessive, or even that the parameters used for the reaction to occur are not adequate (choice of primers, time, and temperature for each PCR step, the number of cycles of DNA amplification) (*16*).

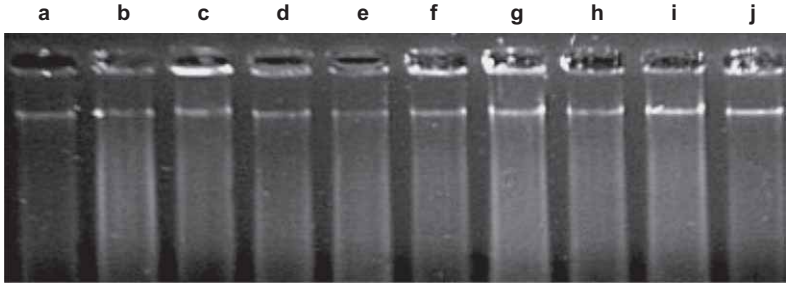

Fig. 4. DNA samples of soybean seeds extracted by the CTAB method. From a to j: genomic DNA of the samples

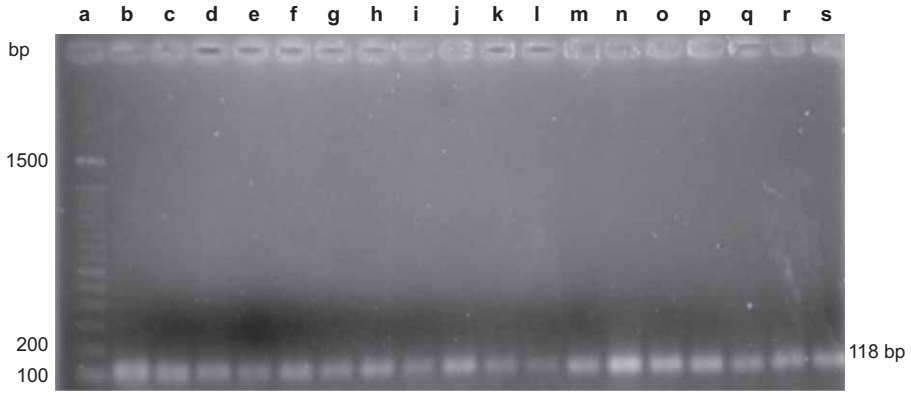

Fig. 5. PCR electrophoresis of the lectin gene from the concentrated solution of the DNA extracted from various samples. a: Molecular marker from 100 to 1500 bp, from b to s: PCR products for lectin of certified seeds

A method for detection of specific species, such as the soy lectin gene (*Le*1), is necessary to control the reaction (*17*). This control can be done by using a pair of primers GM03 and GM04, both for conventional soybeans and for genetically modified soybeans. The efficacy of the reaction amplifies a DNA product of 118 bp (*18*).

The negative control, whose PCR mixture tube does not contain DNA, is designed to check the possibility of any transgenic contamination in the laboratory. If a band is generated, this means that the procedures for preventing contamination of materials and/or solutions used for DNA extraction and amplification must be revised and improved. In case of a successful trial, the negative control will not present any band (*16*). Other authors have also used gene amplification of the soy lectin gene with the same primers as control (*7,12,14,18*).

Amplifications of RR™ soy gene

Except for the negative controls, all analyzed samples showed a specific band for the RR™ gene, whose size is 172 bp, confirming them as transgenic samples regardless of the amount of transgenic DNA in the sample (Fig. 6). Different dilutions were performed to determine which of them showed the highest intensity of the band relative to transgenesis, and it was possible to observe that the results differed among the samples, oscillating from the most contaminated to the least contaminated sample (Table 2).

The amplifications carried out to check whether the PCR molecular technique was able to detect the presence of contaminating transgenic material in different concentrations among conventional soybeans proved to be effective (Fig. 7). Unlike the results obtained by amplification of the RR™ gene for field samples, the certified samples showed no amplification when diluted in the same proportions (Table 3). This possibly indicates that DNA quantity was not sufficient for gene amplification. However, in the samples contaminated with 20 seeds of RR™ soybeans, characteristic bands of this gene in different dilutions were observed. Similarly, the positive control sample for transgenesis also showed bands

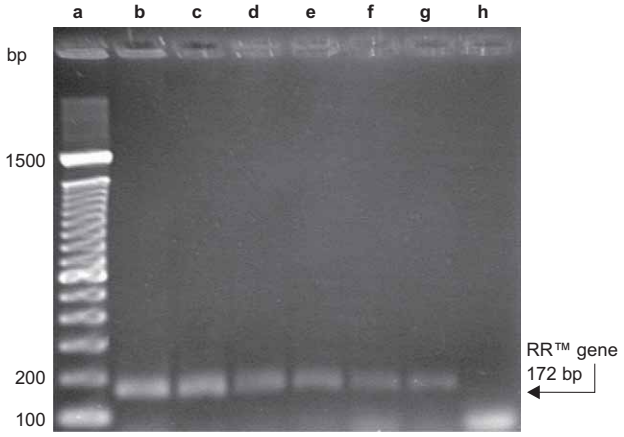

Fig. 6. Amplification of the RR™ gene obtained from the concentrated extracted DNA of one of the positive samples at different dilutions. a: Molecular marker from 100 to 1500 bp; b: RR™ PCR product of the concentrated solution; c: RR™ PCR product of the 1:5 DNA dilution; d: RR™ PCR product of the 1:10 DNA dilution; e: RR™ PCR product of the 1:20 DNA dilution; f: RR™ PCR product of the 1:50 DNA dilution; g: RR™ PCR product of the 1:100 DNA dilution; h: negative control of the reaction

Table 2. Results of amplifications performed in the field samples corresponding to the RR™ gene

Sample	DNA dilution					
	1	1:5	1:10	1:20	1:50	1:100
Coca 1	+	–	–	–	–	–
Coca 2	+	+	+	+	+	–
Coca 3	+	+	+	+	+	–
Coca 4	+	+	+	+	+	–
Coca 5	+	+	+	+	+	+
Coca 6	+	+	+	+	+	+
Coca 7	+	+	+	+	+	+
Coca 8	+	+	+	+	+	+
Coca 9	+	+	+	+	+	+
Coca 10	+	+	+	+	+	+
Coca 11	+	+	+	+	+	+
Coca 12	+	+	+	+	+	+
Coca 13	+	+	+	+	+	+
Coca 14	+	+	+	+	+	+
Transgenesis control	+	+	+	+	+	+
Extraction control	–	–	–	–	–	–

+ corresponds to the amplification of the expected gene, – represents the absence of amplification

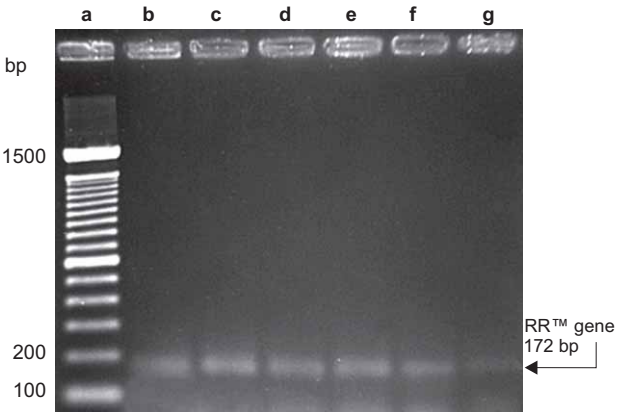

Fig. 7. Amplification of the RR™ gene obtained from the respective samples of concentrated purified DNA of an intentionally contaminated sample. a: Molecular marker from 100 bp to 1 μg/μL, b: RR™ PCR product of the initial concentrated solution, c: RR™ PCR product of the 1:5 DNA dilution, d: RR™ PCR product of the 1:10 DNA dilution; e: RR™ PCR product of the 1:20 DNA dilution, f: RR™ PCR product of the 1:50 DNA dilution, g: RR™ PCR product of the 1:100 DNA dilution

Table 3. Results of amplifications performed in the certified samples corresponding to the RR™ gene

Code of sample	DNA dilution					
	1	1:5	1:10	1:20	1:50	1:100
SS2	+	–	–	–	–	–
SS3	+	–	–	–	–	–
SS5	+	–	–	–	–	–
SS6	+	–	–	–	–	–
SS9	+	–	–	–	–	–
SS10	+	–	–	–	–	–
SS15	+	–	–	–	–	–
SS20	+	+	+	+	+	+
SST	+	+	+	+	+	+
SSØ	–	–	–	–	–	–
Ø	–	–	–	–	–	–
Transgenesis control	+	+	+	+	+	+
Extraction control	–	–	–	–	–	–

+ corresponds to the amplification of the expected gene, – represents the absence of amplification

in all dilutions, but with higher intensity. This is explained by the fact that it was a genuine transgenic sample.

Different pairs of primers for analyzing the RR™ soybeans using PCR have been developed and published (*17*). They represent categories that differ according to their target and level of specificity.

The pair of primers used in the present work, p35s--f2 and petu-r1, amplifies a fragment of 172 bp, which comprises the artificial link between the 35s promoter and a portion of the petunia gene. The gene fragment is typical of RR™ soybeans (*16*).

Studies using these primers have been published (*7,14*) and our choice was based on the effectiveness of these previous results. Other authors have used different primers to amplify a fragment corresponding to the RR™ gene. However, depending on the primer pair used, the fragment will differ according to the target gene and amplified fragment size (*10–13,15,19–25*).

Influence of sample DNA dilutions in PCR reactions

The DNA dilutions of each sample from the DNA initial solutions prepared before amplification were used to analyze the most suitable dilution and conditions for analyzing the amplification results, both of the lectin gene and of the RR™ gene.

The amplifications carried out in the field and certified samples corresponding to the lectin gene show that there is gene amplification in all dilutions of the samples (except for negative controls), thus confirming the quality of the DNA extracted by the chosen CTAB method. More specifically, the amplification of the expected gene performed with the field samples (Coca 1 to Coca 14) corresponding to the lectin gene was positive in all tested dilutions (1, 1:5, 1:10, 1:20, 1:50 and 1:100) including the transgenesis control, and no amplification of the extraction control was observed. The results of amplification of the expected gene performed in certified seeds (SS2, SS3, SS5, SS6, SS9, SS10, SS15, SS20, SST, SSØ and Ø) were positive for all samples and also for transgenesis control in the evaluated DNA dilutions (1, 1:5, 1:10, 1:20, 1:50 and 1:100), while no amplification was observed in the extraction control sample.

Table 2, which presents the results of amplifications performed in the field samples (grains) corresponding to the RR™ gene, clearly demonstrates the importance of dilutions. Thus, the initial solution (which is more concentrated and referred to as 1) was satisfactory for gene amplification. Therefore, in the same way that the intentional contamination increased, the dilutions should also be increased, presenting gene fragments that could be identified by PCR amplification of the respective gene.

The difference observed in the results presented in Table 3 is explained by the fact that the samples are pure, *i.e.* certified seeds have fewer contaminants than field grains, and so, besides the transgenesis control, only the sample with more transgenic material resulted in the amplification in all performed dilutions.

Comparison of PCR with other qualitative methods

Many of the genetically modified crops approved for consumption contain newly introduced genes that are expressed in transgenic plants and, therefore, may be detected by DNA analysis and by protein-based methods (*26*). According to Holst-Jensen *et al.* (*17*) and Poms *et al.* (*27*), PCR is known to be a very sensitive method, especially when compared to the protein-based methods, such as the enzyme-linked immunoassay (ELISA).

Thus, we conclude that PCR proves to be the most accurate technique applicable to seeds and commercial products, without being affected by the level of food processing. Moreover, until now no other technique of analysis has reached the same level of specificity as the one offered by PCR (*28*).

Importance of the analysis of processed food and raw materials

Moriuchi *et al.* (*10*) argue that most products on the food market are voluntarily labelled as non-GM by their respective manufacturers. The authors analyzed semi-processed, processed soybeans, and raw material using the CTAB method for DNA extraction. However, the results of the present study show that the lectin and GM soy genes were amplified in all products. As such, the authors conclude that there is insufficient inspection of food labelling standards, suggesting thereby the need to check whether the non-GM products correspond to their label. The method validated here could be useful in food inspection and regulation, not only in detecting GMOs in food, but also in detecting food adulteration (*10*).

According to Zhou *et al.* (*23*), the monitoring of foods containing GMOs in their composition is of great relevance due to their spreading out around the world in a short period of time, since their long-term risk has not been validated yet. There should be a concern not only about the consumers' rights but also about a sensitization over the characteristics of foods and the creation of an effective regulation system for planting GM crops and for controlling the food market.

Similar monitoring studies have been carried out in other countries and/or regions (*15,17,23,24,29–31*). It can be seen that most of them include monitoring of foods derived from raw materials that might contain modified genes in their composition. However, in order to be able to analyze the seeds from the culture planting to cultivation, and then harvest and distribution, independently of their processing stage, it is relevant to point out the monitoring of the seeds, which forms the basis of the industrial processing, as proposed in the present paper. In Brazil, tracing studies were performed to detect GMO in RR™ soybean used in processed foods (*7,12,14,32,33*). Nevertheless, none of them has evaluated the classification of seeds. The present work is directed at the analysis of soybean seeds that could be used even as raw material in Brazil, especially in Parana State, and aims to maintain the competitiveness of agricultural commodities produced from non-genetically modified seeds on domestic and international market. However, the development, and especially commercialization of GM crops do not cease to grow, constituting a fierce technological race among the leading companies in the GM seed market.

Considering the advance of genetically modified crops in Brazil, it is important to use an efficient detection technique, relatively quick and cost-effective, the results of which can be reproduced in laboratories under different conditions. The PCR fits perfectly this prerequisite, proving to be an ideal method for tracing grains and soybean seeds, transgenic or not, provided that the standards of reaction quality control be guaranteed. The development of a standard quantitative methodology for analyzing transgenic residues present in samples of conventional soybean seeds is of utmost relevance *(34)*. Another aspect to be pointed out is the quantitative analysis of GM food, since the upper limits of GMOs constitute the basis for compulsory labelling. This is the next step of our work.

Conclusion

The results obtained in this work show that the PCR method can be applied with high sensitivity in order to certify conventional soybean seeds and grains. Monitoring the seeds from the culture planting, through cultivation, until harvest and distribution, independently of their processing stage, is a relevant segment that forms the basis of the industrial processing chain.

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