Detection of adventitious presence of genetically modified seeds in lots of non transgenic soybean[1]

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ABSTRACT – The difficulty on identifying, lack of segregation systems and absence of suitable standards for coexistence of non trangenic and transgenic soybean are contributing for contaminations that occur during productive system. The objective of this study was to evaluate the efficiency of two methods for detecting mixtures of seeds genetically modified (GM) into samples of non-GM soybean, in a way that seed lots can be assessed within the standards established by seed legislation. Two sizes of soybean samples (200 and 400 seeds), cv. BRSMG 810C (non-GM) and BRSMG 850GRR (GM), were assessed with four contamination levels (addition of GM seeds, for obtaining 0.0%, 0.5%, 1.0%, and 1.5% contamination), and two detection methods: immunoassay of lateral flux (ILF) and bioassay (pre-imbibition into 0.6% herbicide solution; 25 ºC; 16 h). The bioassay is efficient in detecting presence of GM seeds in seed samples of non-GM soybean, even for contamination lower than 1.0%, provided that seeds have high physiological quality. The ILF was positive, detecting the presence of target protein in contaminated samples, indicating test effectiveness. There was significant correlation between the two detection methods ($r = 0.82$; $p \leq 0.0001$). Sample size did not influence efficiency of the two methods in detecting presence of GM seeds.

Index terms: *Glycine max*, glyphosate, transgenic.

Detecção de presença adventícia de semente geneticamente modificada em lotes de soja não transgênica

RESUMO - A dificuldade de identificação, a falta de sistemas de segregação e a ausência de normas adequadas à coexistência de soja não transgênica e transgênica têm contribuído para que ocorram contaminações durante as etapas do processo produtivo. O objetivo deste trabalho foi verificar a eficiência de dois métodos de detecção de misturas de sementes geneticamente modificadas (GM) em amostras de soja não transgênica, para avaliar lotes de sementes quanto aos limites da legislação. Foram avaliados dois tamanhos de amostra (200 e 400 sementes), das cultivares BRSMG 810C (não transgênica) e BRSMG 850GRR (GM), com quatro níveis de contaminação (sementes GM adicionadas para obter contaminações de 0,0%, 0,5%, 1,0% e 1,5%), e dois métodos de detecção: imunoensaio de fluxo lateral (IFL) e bioensaio (pré-embebição em solução do herbicida a 0,6%; 25 ºC; 16 h). O bioensaio é eficiente na detecção da presença de sementes GM em amostras de semente não transgênica, mesmo para contaminação inferior a 1,0%, desde que apresentem alta qualidade fisiológica. O IFL foi positivo, detectando a presença da proteína alvo nas amostras com contaminação, indicando a eficiência do teste. Houve correlação significativa entre os dois métodos utilizados ($r = 0,82$; $p \leq 0,0001$). O tamanho da amostra não influenciou a eficiência dos dois métodos na detecção da presença de sementes GM.

Termos para indexação: *Glycine max*, glifosato, transgênicos.

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Introduction

In Brazil, the first cultivation officially authorized of genetically modified (GM) soybean, tolerant to glyphosate, and denominated RR (Roundup Ready), has occurred in the 2003/2004 crop season, in an area of 2.78 million hectares. In the next crop season (2004/2005), five million hectares were cultivated with GM cultivars, representing an increase of 66%, in relation to the previous crop season (James, 2008).

According to Galvão (2012), in 2011 Brazil sowed 30.3 million hectares with biotechnological field crops, divided among the three largest Brazilian crops: soybean, corn, and cotton; consolidating the second worldwide position with transgenic cultivars; with an increase of 19.3%, in relation to 2010. From this total, soybean was cultivated in 20.6 million hectares, with 82.7% of the area sowed with GM soybean, and only 17.3% of the total area sowed with non-GM soybean, thus substantially reducing the supply of this product.

According to information of Ministério da Agricultura, Pecuária e Abastecimento (Ministry of Agriculture, Livestock and Food Suppy), the soybean seed production area in the State of Minas Gerais, in the 2010/2011 crop season, was 104,211.62 hectares (personal communication). From this total area, 76% were intended to sowing of transgenic cultivars. Estimates of the Associação Brasileira de Sementes e Mudas (ABRASEM) (Brazilian Association of Seeds and Seedlings) for the 2011/2012 crop season indicate that the demand for transgenic soybean seeds is still more significant, and in mean figures, countrywide, the area sowed with GM soybean has already surpassed 80%.

Zito et al. (2011) have reported that the decrease in the area of non-GM soybean seed production is due to the difficulty, each time greater, in producing seed exempt of transgenic seeds. Although soybean is a cleistogamic autogamous plant, the exchange of genetic material among plants in the field occurs in variable rates, according to the distance of the transgenic pollen source and of the population of insects. The same authors cited that the ABRASEM conceptualizes the presence of small amounts of GM seeds within a non-GM soybean seed lot as adventitious seeds, once it is an eventual and non-intentional presence. Such difficulty, besides the increase on the demand for seeds of transgenic soybean cultivars, led some companies of seed production to opt in producing only seeds of soybean cultivars tolerant to glyphosate (RR).

The difficulty in identifying, the lack of segregation systems, and the absence of adequate standards to coexistence of non transgenic and transgenic soybean has contributed for the occurrence of contaminations during the different steps of production and commercialization processes; besides, imply in higher costs to seed companies and to producers who want to have their product recognized and certified as non-GM soybean seeds.

The are several commercial niches within the agribusiness and the need of means for detecting the main characteristics of a given cultivar becomes essential either for production of seeds with guaranteed genetic purity or for the certification of products (Pereira et al., 2007). The Genetic Breeding Program of the partnership of Embrapa, EPAMIG (Agricultural and Livestock Research Institution) from the State Government of Minas Gerais and Fundação Triângulo (Triangle Foundation), headquartered in municipality of Uberaba, State of Minas Gerais, is one of the few examples of these niches that maintain researches for development of non transgenic cultivars.

The demand for non transgenic soybean grains comes from the internal food industry, as well as from the external market, mainly from Europe. In internal market, the industry and commerce have followed the limit of 1% for presence of genetically modified organisms (GMO) in the foods; what is demanded by the law. The obligation of displaying on the label of food products the information that, in its composition, the product contains GMO is clearly specified on the Article No. 40, of the Bio-security Law (Law No. 11.105/2005).

Some studies, such as those of Miranda et al. (2005) and Miranda et al. (2006), have assessed the presence of GM soybean seeds in lots of non-GM soybean seeds at ratios of 0%, 1%, 3%, 5% and 8%; while Pereira et al. (2009) have found the presence of non-GM soybean seeds in seed lots of GM soybean.

Although further studies are still needed for the validation of methods for a more accurate detection of adventitious presence of genetically modified soybean seeds within seed lots of non-GM soybean; and that, in addition, meet the limits required by law. There are no studies in the literature concerning contamination percentages lower than 1%. In this sense, the establishment of adequate methodologies, safe and economical for the detection and identification of genetically modified soybean seeds within seed lots of non transgenic soybeans continues to be a challenge.

Considering the importance of the issue, it has to be considered the need for the adoption of appropriate and economically viable technologies to support genetic

improvement programs, in which thousands of soybean genotypes are annually evaluated. Given this, the objective of this study was to verify the effectiveness of two methods for the detection of soybean seed mixtures containing genetically modified Roundup Ready (RR) gene in seed samples of non-transgenic soybean to evaluate the seed lots, within the limits imposed by legislation.

Material and Methods

The study was carried out at Seed Analysis Laboratory and Molecular Biology of EPAMIG, Uberaba, State of Minas Gerais, Brazil. Samples of foundation seeds, cv. BRSMG 810C (non-GM) and cv. BRSMG 850GRR (GM), from genetic breeding program of the partnership Embrapa Soybean/EPAMIG/Triangle Foundation were used in the experiments.

Characterization of the samples as non transgenic soybean: for this procedure were utilized seeds from 840 plants of non-GM soybean, cv. BRSMG 810C, in process of foundation seed production. Soon after selection, these plants were tagged and immediately after, one leaf of each plant was removed, thus forming lots of 40 leaves, which were used for analysis and detection of the RR gene presence by using immunoassay of lateral flux (ILF). At harvest, circa 60 seeds were collected from each plant (to initiate their progeny of genetic seed); and more four seeds were removed and used for verifying the presence of the RR gene, by the ILF. The remaining seeds of each plant were grouped, constituting the origin seeds to carry out the present study. The four additional seeds removed from each plant constituted one subsample, forming 42 composed samples of 80 seeds each (with 20 plants providing four seeds each) for the 840 plants classified as non-GM, by the analysis of leaves.

To perform the experiment aiming at detecting contamination of GM seeds within non-GM soybean seed lots, two sizes of seed samples (200 seeds and 400 seeds) were used, with four contamination levels of GM seeds added to non-GM soybean samples for obtaining contamination ratios of 0.0%, 0.5%, 1.0%, and 1.5% (without the knowledge of the analyst).

To carry out the studies of detection, based in the presence of the CP4 EPSPS protein, two methods were used: bioassay (with pre-imbibition of seeds in a glyphosate herbicide solution) and immunoassay of lateral flux (ILF).

Pre-imbibition of seeds: four subsamples of 50 seeds each and eight subsamples of 50 samples each (depending on size of sample), were sown on germination paper (Germitest®) moistened with a herbicide solution, in a concentration of 0.6% Acid Equivalent (AE), and in a ratio of 2.5 times the mass of dry substrate; the set (substrate + seeds) were then turned into rolls. Immediately after, these rolls were then placed into a seed germinator, at 25 ºC, during 16 h, in the dark (Tillmann and West, 2004). To obtain the standard solution of the AE of the herbicide glyphosate, a formulation of the product Roundup® WG, containing 792.5 $g.kg^{-1}$ glyphosate ammonium salt, 720.0 $g.kg^{-1}$ of AE of N - Phosphonomethyl (glyphosate), and 207.5 g kg^{-1} of other ingredients) were used. After that period, the seeds were set to germinate; what was performed by placing the seeds, evenly distributed, on top of paper towels moistened with distilled water; with eight subsamples of 25 seeds each or 16 subsamples of 25 seeds each (depending on sample size), and then placed into a seed germinator, at 25 ºC, according to Rules for Seed Testing (Brasil, 2009). For the assessment of seedlings, the methodology described by Tillmann and West (2004) was used. Seedlings were sorted as normal (tolerant to glyphosate) and abnormal (non-tolerant to glyphosate, i.e., seedlings of non-GM soybean). Such assessment was performed at the fifth day after sowing; the percentage of seedlings infected by microorganisms was also assessed.

Length of seedlings: at the end of germination test, 15 abnormal seedlings were withdrawn from each subsample, measured from tip of the main root until insertion of cotyledons, with the aid of a ruler graduated in millimeters, and then five small seedlings (< 4 cm); five seedlings of median size (between 4 and 9 cm); and five larger seedlings (> 9 cm), were selected. The mean length of seedlings was obtained by the sum of all lengths, which was then divided by the number of seedlings measured. Results were expressed in $cm.seedling^{-1}$.

Immunoassay of lateral flux: four subsamples of 200 and 400 seeds each, for each treatment were assessed for presence of OGM, with 95% significance. The kit *Trait Rur - Soy Crops & Soybean testing*® was used according to instructions of manufacturer, with three replications per treatment. Dry soybean seeds were ground for 1 min. in a blender. Immediately thereafter, 250 mL of water were added, and the blender was turned on during 10 additional sec. To perform the test, 0.5 mL of the ground seeds extract (supernatant) was removed with the aid of a pipette and

placed into a 1.5 mL capacity test tube. Afterwards, the result was assessed by using the test strip. The presence the presence of two colorful bands on the test strip indicates that the test was positive for target-protein, while the presence of only one colorful band on the test strip indicates that the test was negative.

A completely randomized experimental design was used, with treatments arranged into a 2 x 4 factorial scheme {2 sample sizes (200 and 400 seeds) x 4 contamination levels by GM soybean (0.0%, 0.5%, 1.0% and 5%)}, with three replications. After testing normality of errors distribution (Shapiro-Wilk test) and homogeneity of variances, data were subjected to ANOVA and treatment means were compared by the Tukey test, at 5% probability. The Spearman's correlation analysis was applied and the correlation coefficient (r) between the two detection methods of GM soybean contamination was determined. For all the statistical analyses performed, the SAS software was used.

Results and Discussion

On the Table 1, results obtained for initial quality of the cultivars studied as well as the morphological characteristic of hilum color are shown. Seeds of the two cultivars under study {BRSMG 810C (non-GM) and BRSMG 850GRR (GM)} have presented physiological quality above the standard established for seed commercialization, i.e., 80% germination. Result obtained on detecting percentage of GM seeds in seed samples of soybean cultivar BRSMG 810C, sensitive to glyphosate, in function of sample size and percentage of contamination are shown of Table 2. The ILF test, also known as the fast test, which is performed by using the Trait Test kit, was positive and has indicated the presence of the target-protein in samples of seeds with contamination levels of 0.5%, 1.0%, and 1.5%. In the samples where no GM seed were added (0%), the result was negative, thus indicating efficiency of test.

The development of normal seedlings of the non-GM soybean, cv. BRSMG 810C, found in the germination test performed with pre-imbibition of seeds in paper towels moistened with the herbicide, was totally inhibited (Table 3); corroborating results obtained by (Tillmann and West, 2004; Cunha et al., 2005; Miranda et al., 2005; and Pereira et al., 2009). As higher the percentage of GM seeds was added in seed samples of non-GM soybean, reductions of normal seedling were detected; indicating the existence of seed mixtures and the feasibility of their detection by the seed analyst. The interaction between the factors: sample size and contamination level was not statistically significant. Sample size did not influence efficiency of the two methods used in detecting the adventitious presence of GM seeds; although it have been observed that detection was more precise (mean of 0.63%), when the sample size 1 (200 seeds) was assessed; as compared to sample size 2 (400 seeds) (Table 3).

When abnormal seedlings as well as infected seeds were assessed, statistically significant differences were found for the sample size variable (Table3). Higher percentage of abnormal seedlings was found when the larger size of sample (400 seeds) was used; while for the infected seeds, higher percentage was detected in the smaller size sample (200 seeds). These findings, however, are in function of contamination percentage and initial quality of the seeds used, once seeds of the GM soybean cultivar have presented lower germination than the non-GM soybean cultivar. These factors probably have caused interference on results. Such information corroborates results found by Miranda et al. (2005) who have stressed that the success of the bioassay depends on physiological quality of the seeds lots.

Table 1. Percentage of germination, mass of 100 seeds, and color of hilum obtained form seeds of two soybean cultivars: BRSMG 810C (non-GM) and BRSMG 850GRR (GM).

Cultivar	Germination (%)	Mass of 100 seeds (g)	Color of hilum
BRSMG 810C	91.5	16.4	Black
BRSMG 850GRR	80.5	16.6	Black

Table 2. Results of immunoassay of lateral flux, performed in soybean seeds, cv. BRSMG 810C, sensitive to glyphosate, in function of sample size and of percentage contamination with soybean seeds, cv. BRSMG 850GRR (GM) among seed samples of the non-GM cultivar.

Contamination percentage	Immunoassay of lateral flux	
	Sample size 1(*)	Sample size 2(*)
0.0	Negative	Negative
0.5	Positive	Positive
1.0	Positive	Positive
1.5	Positive	Positive

(*) Sample size 1 = 200 seeds; sample size 2 = 400 seeds.

Table 3. Percentage of normal and abnormal seedlings and seedlings infected by microorganisms, from seeds of the soybean cultivar BRSMG 810C (sensitive to glyphosate) obtained in the germination test performed with pre-imbibition of seed on paper towels moistened with the herbicide, in function of sample size and of percentage contamination with seeds of cultivar BRSMG 850GRR (genetically modified) among seeds of non-GM soybean seed samples.

Contamination percentage	Normal seedlings (%)			Abnormal seedlings (%)			Infected seedlings (%)		
	Size 1(*)	Size 2(*)	Mean	Size 1	Size 2	Mean	Size 1	Size	Mean
0.0	0.00	0.00	0.00 c	91.33	90.50	90.92 a	8.67	9.33	9.00 ab
0.5	0.33	0.33	0.33 bc	85.67	86.83	86.25 b	14.00	12.67	13.33 a
1.0	0.67	0.67	0.67 b	87.67	95.00	91.33 a	11.67	4.33	8.00 b
1.5	1.50	1.17	1.33 a	88.17	93.00	90.58 a	10.33	5.83	8.08 b
Mean	0.63 A	0.54 A	0.58	88.21 B	91.33 A	89.77	11.17 A	8.04 B	9.60
CV (%)		49.5			2.9			27.8	

(*)Sample size 1 = 200 seeds; sample size 2 = 400 seeds.

Means followed by the same capital letter on the line and small letter in the columns do not statistically differ between each other by the Tukey test, at 5% de probability.

Results of assessment on length of seedlings are presented on Table 4; and the morphology of seedlings is illustrated on Figure 1. As occurred in the germination test, interaction between the factors: size of samples and contamination levels was not significant. Variations on length of seedlings were observed, whose mean lengths were 2.9 cm (small seedlings), 6.5 cm (intermediary seedling size), and 9.5 cm (large seedlings); with a total mean length of 6.3 cm. Funguetto et al. (2004), and Miranda et al. (2005), have also obtained results of bioassays with solutions of glyphosate in which reduction on total length of seedlings occurred; and Pereira et al. (2009), have found reduction on occurrence of normal seedlings and secondary roots emission.

Table 4. Length of seedlings (small, intermediary, and large) and mean length of seedlings of the non-GM soybean cultivar BRSMG 810C (sensitive to glyphosate) using pre-imbibition of seeds on paper towels moistened with the herbicide, in function of sample size and of percentage contamination with seeds of the soybean cultivar BRSMG 850GRR (genetically modified), among seeds of non-GM soybean seed samples.

Contamination percentage	Seedling length (cm.seedling^{-1})											
	Small seedlings			Intermediary size seedlings			Large seedlings			Mean length of seedlings		
	Size 1(*)	Size 2(*)	Mean	Size 1	Size 2	Mean	Size 1	Size 2	Mean	Size 1	Size 2	Mean
0.0	2.9	3.1	3.0 a	5.5	6.8	6.1 b	9.1	11.0	10.1a	5.8	7.0	6.4 a
0.5	2.9	2.9	2.9 a	5.9	6.3	6.1 b	9.1	9.6	9.4a	6.0	6.3	6.1 a
1.0	2.9	2.8	2.9 a	6.2	7.2	6.7 ab	9.1	9.3	9.2a	6.1	6.4	6.3 a
1.5	2.7	3.1	2.9 a	7.0	6.9	7.0 a	8.9	9.5	9.2a	6.2	6.5	6.3 a
Mean	2.9 A	3.0 A	2.9	6.2 B	6.8 A	6.5	9.1 B	9.9 A	9.5	6.0 B	6.6 A	6.3
CV (%)		7,1			7,1			6,9			5,2	

(*) Sample size 1 = 200 seeds; sample size 2 = 400 seeds.

Means followed by the same capital letter on the line and small letter in the columns do not statistically differ between each other by the Tukey test, at 5% de probability.

In this study, with the pre-imbibition in the herbicide glyphosate, the seedlings of cultivar BRSMG 810C (sensitive to the herbicide) have presented several abnormalities that had already been found in other studies with different transgenic cultivars. In the literature are also found results on: reduction on length of primary root and its thickening (Funguetto et al., 2004; Pereira et al., 2009); paralyzation of development (Pereira et al., 2009); and lack of secondary roots (Funguetto et al., 2004; Cunha et al., 2005; Pereira et al., 2009).

The sudden shortening and narrowing of the hypocotyl (Figure 1) were also observed in this study. The cultivar BRSMG 850GRR (tolerant to glyphosate) presented a normal development of seedlings, with intactness on all the structures of hypocotyl; and with the primary and secondary roots perfectly preserved (Figure 1).

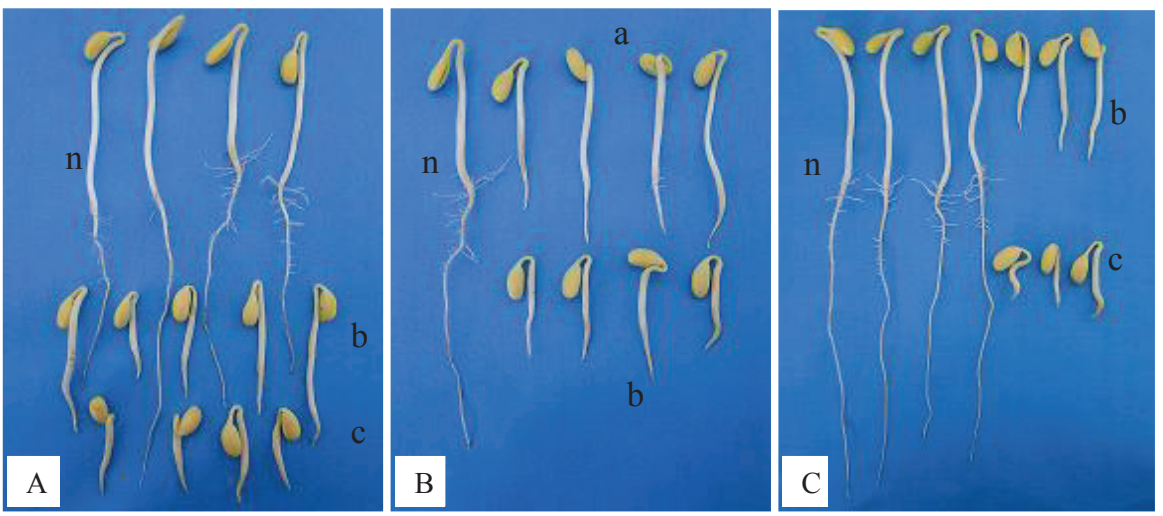

Figure 1. Illustration of size of soybean seedling obtained in the bioassay performed with pre-imbibition of seed. *Photo A*, upper part: n = normal seedlings {cultivar BRSMG 850GRR (genetically modified), tolerant to the herbicide}; below: b = medium size abnormal seedlings (4 to 9 cm); c = short abnormal seedlings (< 4 cm) – cultivar BRSMG 810C (susceptible to the herbicide). *Photo B,* left: n = normal seedling (BRSMG 850GRR); right: a = large size abnormal seedlings (> 9 cm); below: b = medium size abnormal seedlings (BRSMG 810C). *Photo C*, left: n = normal seedlings (BRSMG 850GRR); b = medium size abnormal seedlings; and c = short abnormal seedlings (BRSMG 810C).

By the analysis of Spearman's correlation, it was observed that there was high and statistically significant correlation between the two methods of detections used ($r = 0.82$; $p \leq 0.0001$). Such correlation, however, should have been equal to 1.0, or close to 1.0, to assure the high efficiency of the bioassay. In this study, the efficiency observed was 88.9%, once that from the 18 samples with contamination of seeds GM varying from 0.5% to 1,5%, the bioassay method has failed for two samples with 0.5% contamination with seeds GM (11.1); one of them in size of sample 1 (200 seeds) and another in size of sample 2 (400 seeds).

The bioassay method is efficient on detecting the presence of GM soybean seeds in seed samples of non-GM soybean, even for contamination below 1.0%, although without guaranteeing the efficiency of detection in 100% of cases. However, if both seed samples present high physiological quality, or if the adventitious seed presents a germination percentage similar or higher than the remaining seeds of the lot, the bioassay efficiency will be higher. In contrast, it is possible to infer that the adventitious seeds, which did not germinate during the germination test, likewise would not germinate in the field; what would increase the safety of the bioassay results.

Conclusions

The bioassay method is efficient in detecting the presence of seeds of GM soybean in seed samples of non-GM soybean, even for contamination level lower than 1.0%, once seeds of both genotypes present high and similar physiological quality;

The immunoassay of lateral flux is able to detect the presence of the target protein in samples of non-GM soybean seeds contaminated with GM soybean seeds, what indicates efficiency of the test;

There is statistically significant correlation between the detection methods.

The sample size does not influence efficiency of the two methods (immunoassay of lateral flux and bioassay) in detecting the adventitious presence of GM soybean seeds in samples of non-GM soybean seeds.

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