RESEARCH NOTE

Alternative methods for detecting soybean seeds genetically modified for resistance to herbicide glyphosate[1]

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ABSTRACT – The objective of this study was to verify application of two methodologies: substrate moistened with herbicide solution (SM) and immersion of seeds in herbicide solution (IH) for detecting soybean seeds genetically modified. For this, non-transgenic and transgenic soybean seeds, harvested in the 2008/2009 crop seasons were used. The treatments with substrate moistened were: SM1) 0.03% herbicide solution, at 25 ºC, with evaluation in the sixth day (hs -0.03% -25 ºC, 6th d); SM2) HS -0.03% -35 ºC, 5th d; SM3) HS -0.03% - 40 ºC, 5th d; and SM4) hs -0.06% -5 ºC, 5th d. In the methodology of immersion of seeds the following treatments were performed: IH1) seed immersion in a 0.6% herbicide solution, at 25 ºC, for 1 h, (si -0.06% -25 ºC, 1 h; IH2) si -0.06% - 35 ºC, 30 min.; IH3) si -0.06% -40 ºC, 30 min.; IH4) si -0.12% -35 ºC, 30 min.; and IH5) si -0.12% -40 ºC, 30 min. Bioassays allow detecting soybean seeds tolerant to glyphosate herbicide within five days. The seeds of non-genetically modified and genetically modified soybean cultivars may be easily distinguished through the treatments SM2 and SM4 of the moistened substrate methodology; and treatments IH3, IH4, and IH5 of seed immersion methodology. Both methodologies are easily feasible, practical, and applicable in seed analysis laboratories, once do not require special equipments.

Index terms: *Glycine max*, varietal purity, seed mixtures.

Métodos alternativos para a detecção de sementes de soja geneticamente modificadas para resistência ao herbicida glifosato

RESUMO – Esta pesquisa teve por objetivos verificar o efeito da aplicação de duas metodologias: substrato umedecido com solução herbicida (SU) e imersão das sementes em solução herbicida (IS), na detecção de sementes de soja geneticamente modificadas. Para isso, foram utilizadas sementes de soja convencional e transgênicas, colhidas na safra de 2008/2009. Os tratamentos com substrato umedecido foram: SU1) solução herbicida a 0,03%, a 25 ºC, com avaliação no sexto dia (SH-0,03% -25 ºC, 6º d); SU2) SH -0,03% -35 ºC, 5º d; SU3) SH -0,03% -40 ºC, 5º d; e SU4) SH -0,06% -35 ºC, 5º d. Na metodologia de imersão das sementes foram realizados os seguintes tratamentos: IS1) solução herbicida a 0,06% por 1 hora a 25 ºC (SH -0,03% -1 h -25 ºC; IS2) SH -0,06% -30 min. -35 ºC; IS3) SH -0,06% -30 min. -40 ºC; IS4) SH -0,12% -30 min. -35 ºC; e IS5) SH -0,12% -30 min. -40 ºC. Os bioensaios permitem detectar sementes de soja tolerante ao glifosato, dentro de cinco dias. As sementes das cultivares convencionais e das geneticamente modificadas podem ser separadas pelos tratamentos SU2 e SU4 da metodologia substrato umedecido com solução herbicida; e IS3, IS4 e IS5 da metodologia imersão. As duas metodologias pesquisadas são de fácil montagem, são práticas e aplicáveis em laboratórios de analise sementes, uma vez que não exigem equipamentos especiais.

Termos para indexação**:** *Glycine max*, pureza varietal, mistura de sementes.

Introduction

The cultivation area of genetically modified plants presents accentuated worldwide expansion. The transgenic soybean is present on chemical composition of innumerous products daily consumed by majority of world population and presents a cultivated area that undergoes accentuated expansion at each crop season.

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Considering the increase in production and commercialization of genetically modified products, increases the importance in identifying and quantifying seeds of plants genetically modified within seed lots of conventional cultivars or seeds of non-transgenic plants within seed lots of genetically modified plants.

In this sense, research works have been perfected in the search of methods of differentiation and detection of presence of seeds of soybean cultivar genetically modified (GM) from the cultivars non-genetically modified (NGM) , and among them should be emphasized the studies carried out by Torres et al. (2003), Funguetto et al. (2004), Menezes et al. (2004), Tillmann and West (2004), Cunha et al. (2005), Bertagnolli et al. (2006), and Bervald et al. (2010), where bioassays were performed to try to clarify the issue.

Torres et al. (2003) have studied germination of GM seeds and NGM seeds, using the method of germination on sheets of blotter paper (Germitest®), moistened with solutions of water + 0.50, 100, and 200 mL of glyphosate and have found that seeds of transgenic soybean cultivars, tolerant to the herbicide, germinated normally after the application of the solutions, and between seven to 10 days have developed a normal root system; while among the seeds of cultivars non-transgenic soybean have occurred interruptions on growth of primary root and on emission of secondary roots.

Funguetto et al. (2004) have performed three bioassays based on the germination test. For the first bioassay, the seeds were subjected to pre-imbibition in water, during 16 h, and subsequently maintained in contact with substrate moistened with herbicide solution; for the second bioassay, the seeds were not subjected to pre-imbibition and the substrate was maintained permanently moistened with the herbicide solution; and for the third bioassay, the seeds were immersed into the herbicide solutions; since in each one of the three bioassays different concentrations of the herbicide were used. The authors have verified that the bioassays have allowed detecting seeds tolerant to herbicide glyphosate in concentrations of 0.030 of the formulation of 480 g $.L^{-1}$ of a.i. and of pre-imbibition of seeds during 16 h in a solution containing 0.4 and 0.6 of glyphosate of the formulation of 480 g $.L^{-1}$ of a.i., which are recommended for routine use in seed analysis laboratories. The authors have still verified that glyphosate causes abnormalities on NGM soybean plants such as: thickening and gradual inhibition of primary root development and emission of secondary roots, among other abnormalities.

Tillmann and West (2004), working in two bioassays with eight soybean genotypes, four of them GM and four of them NGM, one with seeds soaked in herbicide solutions in concentrations between 0.0 and 1.5 a.i., at 25 ºC, during 16 h; and another with the seeds subjected to herbicidal concentrations between 0.0 and 4.8 a.i., at 30 ºC, during 1 h, have verified that both methods were efficient in identifying soybean genotypes resistant to the herbicide. They have also verified that the use of Germitest® paper, pre-moistened with herbicide solution, in the 0.6% concentration, were the most appropriated in identifying the seeds of GM cultivars (bioassay 1); and that the imbibition of seeds in herbicide solution (bioassay 2) has shown that the concentration of 0.12% a.i. was also highly efficient in detecting seeds of GM soybean genotypes.

However, Cunha et al. (2005) in testing several methods for detecting seeds of GM cultivars, such as: seed pre-imbibition; seeds germinated in substrate moistened with the herbicide glyphosate; immersed in solution containing glyphosate and/or spraying seedlings with such solution; kit Trait Test; and detection by PCR, have verified that all methodologies used have allowed differentiating seed lots containing GM and NGM cultivars within five days, through germination percentage; excepting the spray, which allowed differentiation only after eight days. The same authors have stressed that PCR method is a fairly sensitive and trustable technique; however requiring sophisticated equipment and high cost reagents.

Testing biochemical methods, for detecting seeds of GM and NGM cultivars, Menezes et al. (2004) have found that it is possible differentiating the GM soybean cultivars from the NGM soybean cultivars through colorimetric reaction activity of peroxidase enzyme.

Using hydroponic system with herbicide solution, Bertagnolli et al. (2006) have found that this system allows detecting soybean seeds of glyphosate resistant cultivars in only five days; and Miranda et al. (2006), in a bioassays conducted under greenhouse conditions, have sown soybean seeds on a sand substrate, and after germination, they have treated the seedlings with solution of herbicide glyphosate, verifying that the treatment was efficient, practical and of easy conduction.

However, a larger volume of information becomes each time more necessary, aiming perfecting the procedures of the bioassays conduction, whose result expected is reduction of minimum time able to allow assessment of GM soybean plants, although without compromising its efficiency. Therefore, the objective of this study was to contribute with increase of number of these information, by means of assessment of substrate moistened with herbicide solutions, as well as of seed immersion into the herbicide solutions, on detecting GM soybean seeds; adopting variations on the concentration of the solutions, in addition to combinations of temperature x assessment time.

Material and Methods

For performing this study, were used seeds of transgenic soybean cultivars resistant to herbicide glyphosate, henceforth

identified as Cultivar A, Cultivar B, Cultivar C, and Cultivar D; and seeds of the conventional soybean cultivar E, harvested in the 2008/2009 crop season. Before starting experiment, a seed characterization was performed by means of test of germination on paper rolls, using four subsamples of 25 seeds each, which were maintained at 25 ºC, during six days, and then assessed according to Rules for Seed Testing (Brasil, 2009), in which the following results were obtained and expressed in percentage of normal seedling : Cultivar A (80%); Cultivar B (89%); Cultivar C (93%); Cultivar D (81%); and Cultivar E (89%).

Bioassays

Substrate moistened with herbicide solution (SM)

- Treatment 1: substrate moistened with a 0.03% herbicide solution, incubated at 25 ºC, and assessed in the sixth days (control);
- Treatment 2: substrate moistened with a 0.03% herbicide solution, incubated at 35 ºC, and assessed in the fifth day;
- Treatment 3: substrate moistened with a 0.03% herbicide solution, incubated at 40 ºC, and assessed in the fifth day;
- Treatment 4: substrate moistened with a 0.06% herbicide solution, incubated at 35 ºC, and assessed in the fifth day.

Seed immersion in herbicide solution (IH)

- Treatment 1: immersion of seeds in a 0.06% herbicide solution, incubated at 25 ºC, during 1h (control);
-Treatment 2: immersion of seeds in a 0.06% herbicide solution, incubated at 35 ºC, during 30 min.;
-Treatment 3: immersion of seeds in a 0.06% herbicide solution, incubated at 40 ºC, during 30 min.;
-Treatment 4: immersion of seeds in a 0.12% herbicide solution, incubated at 35 ºC, during 30 min.;
-Treatment 5: immersion of seeds in a 0.12% herbicide solution, incubated at 40 ºC, during 30 min.

For obtaining solution at 0.06% active ingredient (a.i.) of herbicide glyphosate, was used a formulation with 480 $g.L^{-1}$ of isopropylamine salt of N(phosphometyl) glycine (glyphosate), being 360 $g.L^{-1}$ a.i. of glyphosate and 684 $g.L^{-1}$ of inert ingredients, following information of Tillmann and West (2004). The remaining concentrations used in the treatments cited within this study followed the same procedure for obtaining the standard solution to 0.6% of the herbicide.

Assessments

On assessing substrate moistened with herbicide solution, the same procedures used for performing the germination tests were used; however, substituting the water used to moisturize the Germitest® paper by solutions of the herbicide in different concentrations, in which seeds were maintained for different periods and temperatures, as previously cited.

For assessing procedures of seed immersion in herbicide solution, samples of 100 seeds each, were placed into plastic cups (100 mL capacity), containing 50 mL of herbicide solution, determined for each treatment, and according to combinations of time x temperature previously defined Miranda et al. (2005) . Subsequently, these seed were subjected to germination test, using the same procedures described for initial characterization of seeds.

Statistical procedures

A completely randomized experimental design was used with four replications for each treatment. Means were compared by Tukey test, at 5% probability and transformed in arcsin sqrt x/100 (Banzatto and Kronka, 1995). Data were analyzed by software STAT, version 2.0.

Results and Discussion

All five cultivars used in the experiment have presented germination above the minimum value demanded by the Ministry of Agriculture, Livestock and Supply, which is 80%, for being suitable for seed lots commercialization (Embrapa, 2008).

Results achieved (Tables 1 and 2); have shown that it is possible to detect presence of GM seeds, by the difference on germination obtained by the methodology used in the bioassay. By these results, it can be observed that in presence of herbicide glyphosate, the development of normal seedlings was completely inhibited and that the seeds simply initiated the germination process; nevertheless, without reaching either the length or the root system formation adequate and sufficient as to be considered normal seedlings. In all treatments, even the lowest concentrations of the herbicide solutions were able to separate and indentify seeds of conventional soybean. However, the seeds of GM cultivars were able to produce normal and well developed seedlings, even in the presence of herbicide glyphosate.

This occurs on NGM seeds, due to the fact that the herbicide glyphosate inhibits the enzyme 5-enolpiruvilshiquimato-3-phosphate synthase (EPSPS) and prevents that the plant produces essential amino acids for synthesis of protein and also of some secondary metabolites (Kruse et al., 2000).

Glyphosate causes abnormalities on NGM soybean seedlings, causing formations of tapered, smooth, and firm roots, besides reduction on length of primary root and aerial parts of the plant, and inhibition of secondary roots emission; while the GM soybean seedlings display primary root and aerial parts well developed and abundant, when treated with the herbicide (Bertagnolli et al., 2006). Cultivars GM have

the gene for resistance to glyphosate, thus allowing formation and development of normal seedlings; and for this reason, the soybean seedlings originating from GM seeds remain unchanged after treatment with glyphosate, contrasting with death, or growth reduction, of seedlings observed in treatment of conventional soybean with glyphosate, to which it is sensitive (Padgette et al., 1995).

Through results obtained, using the SM method, the seeds from the four cultivars GM (A, B, C, and D) have presented statistical significance ($p < 0.01$), indicating that among the four treatments with herbicide solutions, the treatment 2 (SM 0.03%; 30 °C; 5th d) and the treatment 4 (SM 0.06%; 30 °C; 5th d) have had equal performance to treatment-standard 1(SM 0.03%; 25 °C; 6th d); and that the treatment 3 (SM 0.03%; 35 °C; 5th d) has presented a very drastic result, reaching the point in which no normal seedling has been identified. The treatment 3 has presented the best performance in the highest temperature (35 °C), in relation to remaining treatments. It can also be verified that the dosage of herbicide in the treatment has not exerted influence on results, once the double of a dose was used in the treatment 4; nevertheless its performance was equal to those obtained in treatments 1 and 2 (Table 1).

Table 1. Mean values of germination assessed by number of normal and abnormal seedlings and dead seeds, in function of different concentrations of the glyphosate herbicide obtained for five soybean cultivars (A, B, C, D, and E) genetically modified (GM) and non-genetically modified (NGM), sown in substrate moistened with the herbicide solutions at different temperatures and incubation times (days).

Cultivars and identification	Treatment concentration; Temperature; incubation time (d)	Normal seedling	Abnormal seedling	Dead seed
		----------%----------		
E (NGM)	T1 0,03%; 25 °C; 6 d	0	83.94 a*	6.05 a*
	T2 0,03%;30 °C; 5 d	0	78.75 a	11.24 a
	T3 0,03%;35 °C; 5 d	0	80.93 a	9.06 a
	T4 0,06%; 30 °C; 5 d	0	81.49 a	8.50 a
A (GM)	T1 0,03%; 25 °C; 6 d	44.44 a[1]	41.25 b	13.02 a
	T2 0,03%; 30 °C; 5 d	49.70 a	36.63 b	10.28 a
	T3 0,03% 35 °C; 5 d	0.57 b	80.93 a	9.06 a
	T4 0,06%; 30 °C; 5 d	40.92 a	47.88 b	4.53 a
B (GM)	T1 0,03%; 25 °C; 6 d	77.79 a	11.24 b	3.31 b
	T2 0,03%; 30 °C; 5 d	73.82 a	16.17 b	0.57 b
	T3 0,03%; 35 °C; 5 d	0.57 b	71.69 a	18.30 a
	T4 0,06%; 30 °C; 5 d	71.69 a	18.30 b	0.57 b
C (GM)	T1 0,03%; 25 °C; 6 d	78.75 a	11.24 b	0.57 a
	T2 0,03%; 30 °C; 5 d	70.69 a	18.34 b	3.31 a
	T3 0,03%; 35 °C; 5 d	0.57 b	79.98 a	10.01 a
	T4 0,06%; 30 °C; 5 d	69.99 a	16.16 b	10.01 a
D (GM)	T1 0,03%; 25 °C; 6 d	65.80 a	24.19 b	0.57 b
	T2 0,03%; 30 °C; 5 d	66.94 a	23.05 b	0.57 b
	T3 0,03%; 35 °C; 5 d	0.57 b	79.98 a	10.01 a
	T4 0,06%; 30 °C; 5 d	58.08 a	26.51 b	15.90 a

*Means followed by the same letter in the columns, within each treatment, do not statistically differ between each other by Tukey test, at 5% probability.
[1]Data transformed by arcsin sqrt x/100.

All four treatments of germination test (different levels of glyphosate, temperature, and time of exposure) of conventional soybean cultivars presented a response that impaired normal development of seedling; what was perceived through very low percentages or even nil of normal seedlings. For the GM soybean cultivars, the treatment 1 considered a standard-methodology (Miranda et al., 2005), and the treatments 2 and 4 were those that presented the highest values for normal seedling; indicating that could be used in assessing mixtures of seeds in lots of NGM and GM seeds.

By interpreting results achieved within this study it can be inferred that: after applying treatment 2 and/or treatment 4, the same result of the standard-treatment (treatment 1), as well as the same precision on results would be obtained; besides the advantage of the one-day gain in the assessment. When it comes to seed-producing companies, whose workflow in the seed laboratory is large during crop harvesting, the advantage in releasing a seed lot in the shortest time is an important differentiator in sales operations,

transport logistics, delivery, and sowing.

For results obtained by IH method, the GM soybean cultivars (seed lots A, C, and D) have presented response for normal seedlings equal to standard-methodology, i.e., were not able to differentiate between treatments with glyphosate showing that alternative methodologies (treatments T2, T3, T4, and T5) can be used without loss in precision; on the contrary, responses can be faster. On the lot B results were statistically significant ($p < 0.05$); however, treatment T2 should be excluded, due to the high number of normal seedlings. The other treatments have had responses close to the standard-treatment 1 (Table 2).

Table 2. Mean values of germination assessed by number of normal and abnormal seedlings and dead seeds, in function of different concentrations of the glyphosate herbicide obtained for five soybean cultivars (A, B, C, D, and E) genetically modified (GM) and non-genetically modified (NGM), after seed immersion in the herbicide solutions at different temperatures and times of immersion (minutes).

Cultivars and identification	Treatment concentration; Temperature; time of immersion (min.)	Normal seedling	Abnormal seedling	Dead seed
		----------%----------		
E (NGM)	T1 0,06%; 25 °C; 60 min.	0	56.57 a*	32.84 a*
	T2 0,06%; 35 °C; 30 min.	0	60.79 a	29.20 a
	T3 0,06%; 40 °C; 30 min.	0	64.21 a	25.78 a
	T4 0,12%; 35 °C; 30 min.	0	56.37 a	33.62 a
	T5 0,12%; 40 °C; 30 min.	0	70.34 a	19.65 a
A (GM)	T1 0,06%; 25 °C; 60 min.	46.74 a[1]	34.80 a	21.92 a
	T2 0,06%; 35 °C; 30 min.	58.82 a	20.80 b	22.00 a
	T3 0,06%; 40 °C; 30 min.	52.20 a	28.91 ab	19.65 a
	T4 0,12%; 35 °C; 30 min.	50.80 a	25.67 ab	27.10 a
	T5 0,12%; 40 °C; 30 min.	52.20 a	32.92 ab	13.98 a
B (GM)	T1 0,06%; 25 °C; 60 min.	60.11 b	20.35 a	19.52 a
	T2 0,06%; 35 °C; 30 min.	76.28 a	10.01 a	6.05 a
	T3 0,06%; 40 °C; 30 min.	73.27 a	14.94 a	6.05 a
	T4 0,12%; 35 °C; 30 min.	71.65 ab	12.76 a	12.76 a
	T5 0,12%; 40 °C; 30 min.	64.99 ab	18.34 a	13.9 a
C (GM)	T1 0,06%; 25 °C; 60 min.	53.15 a	25.46 a	23.7 a
	T2 0,06%; 35 °C; 30 min.	57.54 a	18.96 a	24.9 a
	T3 0,06%; 40 °C; 30 min.	66.01 a	15.20 a	15.1 a
	T4 0,12%; 35 °C; 30 min.	60.07 a	16.78 a	21.1 a
	T5 0,12%; 40 °C; 30 min.	60.77 a	24.18 a	12.0 a
D (GM)	T1 0,06%; 25 ºC; 60 min.	20.17 c	15.55 b	13.8 a
	T2 0,06%; 35 °C; 30 min.	60.07 a	22.00 ab	16.3 a
	T3 0,06%; 40 °C; 30 min.	47.30 b	36.62 a	15.5 a
	T4 0,12%; 35 °C; 30 min.	55.07 ab	30.36 ab	14.4 a
	T5 0,12%; 40 °C; 30 min.	46.72 b	36.74 a	18.8 a

*Means followed by the same letter in the columns, within each treatment, do not statistically differ between each other by Tukey test, at 5% probability.
[1]Data transformed by arcsin sqrt x/100.

It can also be observed that in the bioassay SM, glyphosate has presented the highest mean in the treatment with the herbicide in concentration of 0.03, at 30 °C, with assessment at the fifth day; and in the bioassay IH, the highest mean has occurred in herbicide concentration of 0.06, at 35 °C, for 30 min., with assessment at the fifth day. The means obtained in bioassay IH were higher than those obtained in bioassay SM.

Conclusions

The Bioassays tested allow detecting soybean seeds tolerant to herbicide glyphosate within five days.

The seeds of conventional soybean cultivars can be distinguished from seeds of genetically modified soybean cultivars when sown in substrate moistened with solution of herbicide glyphosate at the concentrations of 0.03% or 0.06% and incubated at 30 ºC, during five days.

The immersion of seeds in solution of herbicide glyphosate in the concentrations of 0,06%, at 40 °C, during 30 min.; 0,12%, at 35 °C, during 30 min.; and 0,12%, at 40 °C, during 30 min., also allow distinguishing seeds of conventional soybean cultivars from seeds of genetically modified soybean cultivars contained within the same seed lot.

The both methodologies are easily feasible, practical, and applicable in seed analysis laboratories, once do not require special equipments.

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