Article

Comparison of DNA isolation protocols from soybean

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Abstract

The low efficiency of some nucleic acid extraction protocols and the high cost of commercial products, derives in the comparison between methods. In the present work three DNA extraction methods were compared from soybean, to obtain nucleic acids of adequate concentration and quality for PCR amplification. The protocols studied included the methods with 1% and 3% CTAB solutions, with 1% sarcosine and with phenol/chloroform. The experiments were carried out in the DNA and Genomics laboratory of the National Genetic Resources Center-INIFAP. The yield, purity, integrity and functionality of the obtained nucleic acids were evaluated. In all methods, adequate DNA yield was achieved, however, the required purity of the material was only obtained with the phenol/chloroform solution. With the methods of CTAB at 1% and 3% and sarcosine, PCR inhibiting substances were observed, while, with phenol/chloroform, the values of the $A_{260/280}$ ratio were in a range of 1.96 to 2.00 and the $A_{260/230}$ ratio in a range of 1.75 to 2.44, with significant differences ($p< 0.0001$) with the rest of the methods, in addition, the DNA was of high molecular weight and the rbcL gene was amplified by PCR in all the samples. The use of the phenol/chloroform protocol allowed to obtain from soybean, nucleic acids of adequate concentration and quality for PCR amplification.

Keywords: DNA extraction, DNA quality, nucleic acids.

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Introduction

The high variety of secondary components and metabolites that exist between plant species and even genotypes avoid the existence of a single standardized universal method for the isolation of nucleic acids in plants (Friar, 2005).

To achieve the amplification of white regions, it is necessary to start with DNA of adequate quantity and quality (Rocha-Salavarrieta, 2002; Mafra *et al*., 2008; Sharma *et al*., 2013). During the choice of the plant DNA isolation method, one must consider a) the type of material from which it is split (seed, leaf, stem or others); b) type of DNA (genomic or organelle); and c) the type of analysis that will be carried out (Rocha-Salavarrieta, 2002).

Reports of methodologies in fresh foliar tissues are more common than in seeds (Sharma *et al*., 2013) and several methods have been published to reduce the limitations they face during the isolation of nucleic acids (Saghai-Maroof *et al*., 1984; Doyle and Doyle, 1990; Lopes *et al*., 1995; López-Mora *et al*., 2011; Pérez-Urquiza *et al*., 2013; Huang *et al*., 2013; Youssef *et al*., 2015).

However, due to the limited efficiency of some methods of obtaining DNA (López-Mora *et al*., 2011), work comparison protocols are performed to obtain nucleic acids in good yield, purity, integrity and functionality (Demeke and Jenkins, 2010). These four aspects are determined by the amount, the degree of degradation and the molecular size of the nucleic acids and the presence of contaminating substances that inhibit PCR (Elsanhoty *et al*., 2011; Sharma *et al*., 2013).

Regarding soy, there are reports of methods applied in food and beverages (Mafra *et al*., 2008) and from the seeds themselves (Demeke *et al*., 2009; Sharma *et al*., 2013). Although commercial methods have also been used because of their speed (Chandu *et al*., 2016), the main disadvantage is the increase in cost (Demeke and Jenkins, 2010).

The concentration of DNA can be estimated by spectrophotometry by measuring at 260 nm wavelength, knowing that a unit of optical density at 260 nm corresponds to 50 μg of double-stranded DNA (Rocha-Salavarrieta, 2002; López-Mora *et al*., 2011).

On the other hand, purity is measured with the parameters of the $A_{260/280}$ and $A_{260/230}$ ratios. In the $A_{260/280}$ ratio, the range of expected values is greater than 1.7 to 2 and represents the amount of DNA in relation to the proteins contained in the sample (López-Mora *et al*., 2011); while, in the $A_{260/230}$ ratio, the values are expected to be close to 2 and indicate the presence of polysaccharides and polyphenols that were not removed during the DNA purification process (Demeke *et al*., 2009; Demeke and Jenkins, 2010), but it is important to remove them because they can inhibit the action of Taq polymerase during the PCR amplification process (Friar, 2005; Sharma *et al*., 2013).

Integrity is the measure of the size of the molecular weight of the DNA obtained that can be determined by comparison with a reference molecular weight marker, usually lambda DNA with a size 48 Kb; through, of the electrophoresis technique (Sanger *et al*., 1982).

Finally, the functionality is determined by the amplification capacity of a locus or several loci. Currently, many of the methodologies used in molecular analysis are based on PCR, in such a way that ensuring that the material obtained is free of PCR inhibitors is a measure of functionality. In the case of plants to determine the presence or absence of inhibitors rbcL or matK genes can be used (Levin *et al*., 2003).

In the present work three DNA isolation protocols from soybean were compared to obtain nucleic acids of adequate concentration and quality to perform PCR amplification.

Materials and methods

Materials and pulverization of the seed

The experiments were carried out in the National Center for Genetic Resources, National Institute of Forestry, Agriculture and Livestock Research, within the DNA and Genomics laboratory. Six samples of ten soybeans were included for the study.

Nucleic acid extraction

The seeds are mechanically pulverized in TissueLyser II equipment Qiagen brand. Then, the powder was transferred to 2 mL microcentrifuge tubes and stored at -80 °C until DNA extraction.

Three protocols were evaluated to determine the adequate method of obtaining DNA: a) conventional method with buffer solution with CTAB (Saghai-Maroof *et al*., 1984) with modifications; b) method with buffer solution with 1% sarcosine (Lopes *et al*., 1995) with modifications; and c) method with phenol/chloroform solution (Pérez-Urquiza *et al*., 2013) with modifications.

Conventional method with buffer solution with CTAB

This protocol was evaluated with two different concentrations of CTAB, at 1% and 3%. The procedure was the same in both cases, starting with 50 mg of pulverized seed in a 2 mL tube with 1 mL of extraction buffer solution (100 mM TRIS-HCl, pH 7.5, 700 mM NaCl, 50 mM EDTA, pH 8, 2-betamercaptoethanol (BME) 140 mM, 1% polyvinylpyrrolidone and 1% or 3% CTAB) previously heated to 65 °C.

The tubes were shaken vigorously with a vortex mixer and incubated at 65 °C for 90 minutes with slow and constant agitation. Then, they were left at room temperature (TA) for five minutes, 700 µL of chloroform-octanol solution (24:1) were added and mixed by inversion gently for 10 min. They were centrifuged at 4 500 x g for 15 min at TA; the aqueous phase was transferred to a new tube containing 700 µL of chloroform-octanol solution (24:1) and this step was repeated. The supernatant was transferred to a new tube with 10 µL of RNAse (10 mg mL^{-1}), mixed by inversion and incubated at 37 °C for 30 min.

Subsequently, 800 µL of cold isopropanol was added and mixed by inversion until the DNA was precipitated. The tubes were centrifuged at 4 500 x g for 10 min at TA for pellet formation, decanted and 1 mL of solution containing 10 mM ammonium acetate and 76% ethanol was added, left at TA for 20 min, centrifuged at 4 500 x g for 10 min at TA, the tubes were decanted and a solution was washed with 10 mM ammonium acetate and 0.25 mM EDTA. Subsequently, the samples were allowed to dry and 200 µL of TE buffer solution (10 mM TRIS and 1 mM EDTA, pH 8) were added until the DNA was completely dissolved.

Method with buffer solution with 1% sarcosine

250 mg of the seed spray was transferred into a 2 mL microcentrifuge tube, then, 1 mL of extraction buffer solution was added (100 mM TRIS-HCl, pH 8.5, 100 mM NaCl, 20 mM EDTA, pH 8, N-laurilsarcosine 1%), were mixed allowing the solution to have complete contact with the powder and were left at TA for 10 min.

Then, the samples were mixed with 600 µL of phenol and centrifuged at 10 000 x g for 15 min. The supernatant was transferred to a microcentrifuge of tube new 2 mL, containing 30 µL of RNase (10 mg mL^{-1}), allowed to incubate at 37 °C for 120 min. Again, 600 µL of phenol was added and centrifuged at 10 000 x g for 10 min. The supernatant was transferred to a fresh tube with 600 µL of chloroform-isoamyl alcohol solution (24:1), mixed vigorously and the samples were centrifuged at 10 000 x g for 10 min.

Subsequently, the supernatant was recovered in a new tube with 600 µL of cold isopropanol, mixed gently by inversion until the precipitated DNA was observed, centrifuged at 10 000 x g for 5 min to obtain pellets, then the supernatant was discarded and through of capillary absorption with absorbent paper removed as much of the supernatant as possible.

Next, the pellet was purified with two washes, the first with 70% ethanol and the second with 100% ethanol to remove contaminating proteins and carbohydrates. The samples were centrifuged at 10 000 x g for 5 min, the ethanol was discarded and left at TA until the ethanol was completely evaporated. Finally, the DNA pellet was dissolved in 100 µL of TE buffer solution (10 mM TRIS and 1 mM EDTA, pH 8).

Method with phenol/chloroform solution

In a tube with 250 mg of powdered tissue, 1.6 mL of extraction buffer solution (50 mM tris base, 5 mM EDTA and 30 g/L sodium dodecylsulfate, pH 8) and 20 µL of proteinase K (10 mg mL^{-1}) were added. The tubes were allowed to incubate at 65 °C in slow and constant agitation for 90 min. Subsequently, they were left at TA for five minutes and 20 µL of RNAse (10 mg mL^{-1}) was added.

The samples were centrifuged at 5 000 x g for 30 min at TA. The supernatant was recovered in a tube with 800 µL of phenol, mixed vigorously and centrifuged at 10 000 x g for 5 min. Then, the aqueous phase was recovered in a tube with 800 µL of phenol/chloroform-isoamyl alcohol solution (25:24:1) and mixed vigorously. Subsequently, they were centrifuged at 10 000 x g for 10 min, the aqueous phase was recovered and this step was repeated to obtain a clean interface.

Each tube with supernatant was mixed with 800 µL of chloroform-isoamyl alcohol solution (24:1) and centrifuged at 10 000 x g for 5 min; the supernatant was recovered and mixed by inversion with 30 µL of 3 M potassium acetate solution (pH 5.2) and 750 µL of 96% ethanol. The samples were incubated at -20 °C for 30 min. Then, they were centrifuged at 10 000 x g for 5 min and the supernatant was discarded taking care not to pour the pellet and trying to eliminate as much liquid by capillary absorption with a paper towel.

Next, 1 mL of 70% ethanol was added and the tubes were shaken until the pellet was peeled off to discard the salts, centrifuged at 5 000 x g for 5 min, then the supernatant was discarded and the samples were left at TA until evaporated the ethanol completely. The DNA was dissolved in 100 µL of TE buffer solution (10 mM TRIS and 1 mM EDTA, pH 8).

The DNA obtained through the three protocols was stored under refrigeration at 4 ºC until the analysis of concentration and quality.

Yield of DNA extraction methods

The yield of the methods was determined by the ratio of the DNA concentration and the amount of pulverized seed used. The quantification of nucleic acids was performed by spectrophotometry in a NanoDrop 2000 TM (Thermo Scientific); through, of the measurement of optical density at 260 nm wavelength. Prior to the measurement, the samples were removed from refrigeration and incubated at TA for 10 min.

Purity of nucleic acids

The purity of the nucleic acids was determined by the parameters of the $A_{260/280}$ and $A_{260/230}$ ratios. Both were estimated by spectrophotometry on a NanoDrop 2000^{TM} (Thermo Scientific) equipment; through, optical density measurements at 230, 260 and 280 nm wavelength.

Integrity of nucleic acids

In order to verify the molecular weight of the genomic DNA, electrophoresis was performed on a 1% agarose gel stained with GelRed (Biotium®). In the gel, 30 ng of DNA from each sample was applied and 3 µL of λ DNA (Promega®) without cutting (10 ng μL^{-1}) was used as a reference marker. The electrophoretic shift was performed at 100 volts for 45 min. The gels were visualized with UV light in a transilluminator device and captured the image by digital photography.

Functionality of DNA

The functionality was verified with the amplification of the rbcL gene by PCR. The reaction mixture was prepared with RedTaq 0.8X (Sigma-Aldrich®), 0.35 µM sense primer, 0.35 µM antisense primer and 10 ng of DNA. The sequences of the primers previously described were used (Levin *et al*., 2003). The amplification conditions were: initial denaturation at 95 ºC for 10 min, 40 cycles of denaturation at 95 ºC for 40 s, hybridization at 55 ºC for 40 s and extension at 72 ºC for 40 s, final extension at 72 ºC for 1 min and maintenance at 4 ºC (Levin *et al*., 2003). The PCR products were visualized by electrophoretic shift at 100 volts for 60 min on a 2% agarose gel stained

with GelRed (Biotium®). It is 5 µL of the amplified product was applied and a 100 bp ladder (Promega®) was used as reference marker. Then, the working dilution was prepared at 10 ng μL^{-1} with a volume of 100 µL.

Statistical analysis

The design of the experiment applied was blocks completely randomized with six repetitions, the blocks being the soybean seeds. Three response variables were studied: a) yield; b) $A_{260/280}$ ratio; and c) $A_{260/230}$ ratio. The statistical analysis was carried out with the use of the SAS program, Version 9.3 of the SAS system for Windows (SAS Institute Inc., Cary, NC, USA). The ANOVA analysis of three quantitative variables and the comparison of the means by means of the Tukey test with a level of significance of 0.05 was performed.

Results and discussion

Yield of DNA extraction methods

The highest yield was obtained with the protocols that used CTAB solution at 3% and 1%, followed by the protocols with solutions of sarcosine and phenol/chloroform (Table 1). In all the methods, a good amount of DNA was obtained, although it is not the only parameter to be considered for the realization of later molecular studies.

Table 1. Average yield and purity values of nucleic acids obtained from soybeans *Glycine max* (L.) Merrill. with the extraction protocols evaluated.

Extraction protocol	Yield (mg g^{-1})		Purity			
			260/280		260/230	
	µ	s	µ	s	µ	s
Solution with 1% CTAB	5.65	2.578	1.22	0.095	2.41	0.254
Solution with 3% CTAB	11.93	4.058	1.14	0.027	0.62	0.46
Solution with 1% sarcosine	0.76	0.165	0.76	0.08	1.6	0.328
Phenol/chloroform solution	0.07	0.016	1.97	0.016	2.14	0.247

µ= mean; s= standard deviation.

Although with the phenol/chloroform protocol, lower DNA yield was obtained (152 to 263 µg mL^{-1}) with an average of 186 µg mL^{-1}, this concentration is sufficient when the purpose is to perform marker amplification by PCR, as has been reported in other studies in which a smaller amount of DNA has also been obtained from soybean with the CTAB method (Sisea and Pamfil, 2007; King *et al*., 2014).

In the results of the Anova, significant differences were observed among the protocols used, $p > 0.0001$ (Table 2), while, in the comparison of means, three different groups were observed: a) 3% CTAB solution; b) 1% CTAB solution; and c) solutions of sarcosine and phenol/chloroform (Table 3).

Table 2. Mean squares and significance of the variables yield and purity of DNA from soybean seeds *Glycine max* (L.) Merrill.

Source of variation	GL	Average square of yield		Average squares of Purity			
				$A_{260/280}$		$A_{260/230}$	
Protocol	3	180	***	1.5	***	3.7	***
Error	15	5.1		0		0.1	
CV (%)	-	49.2		3.8		19.4	

Gl= degrees of freedom; CV= coefficient of variation; ***= significant with $p \leq 0.0001$.

Table 3. Comparison of means with the Tukey test of the yield variables and relationships $A_{260/280}$ and $A_{260/230}$.

Grupos		Yield		Purity			
				$A_{260/280}$		$A_{260/230}$	
		Mean	Protocol	Mean	Protocol	Mean	Protocol
	A	11.93	CTAB 3%	2.09	Sarcosine	2.41	CTAB 1%
B	A					2.14	Phenol/chloroform
B		5.65	CTAB 1%	1.97	Phenol/chloroform	1.6	Sarcosine
	C	0.76	Sarcosine	1.22	CTAB 1%	0.62	CTAB 3%
	C	0.08	Phenol/chloroform	1.14	CTAB 3%		

Means with the same letter do not have significant differences ($\alpha = 0.05$).

The highly significant differences in yield between protocols can be due to the fact that other substances are also detected at 260 nm wavelength, such as single-stranded DNA, phenols, RNA and nucleotides, which are not distinguishable from DNA double chain (Demeke and Jenkins, 2010). This coincides with the values of the lower $A_{260/280}$ ratio observed with the use of the protocols with CTAB and sarcosine solutions (Table 1), which makes it difficult to accurately determine the amount of nucleic acids (López-Mora *et al*., 2011).

Purity of isolated nucleic acids

In addition to obtaining the sufficient amount of DNA to carry out the desired analyzes, it is of high importance to take purity into account. The $A_{260/280}$ ratio allows us to know the amount of nucleic acids in relation to the amount of proteins present in the sample.

With the use of phenol/chloroform solution, all samples obtained values in the $A_{260/280}$ ratio of high purity (1.97 on average), while with the use of solutions of CTAB at 1% and 3% and sarcosine, the values indicate purity of low quality DNA (Table 1). Similar results were obtained by Kamiya and Kiguchi (2003) who, from soybeans, obtained DNA of good quality ($A_{260/280}$ from 1.74 to 1.81) with the use of phenol/chloroform/isoamyl alcohol.

Likewise, with the solution with phenol/chloroform, the values of the $A_{260/230}$ ratio obtained had an average value of 2.14. In contrast, with the use of solutions of 1% and 3% CTAB and sarcosine, the values suggest DNA samples with low quality purity, as shown in Table 1. According to previous reports, low values in this relationship indicate the presence of PCR

inhibitors, such as polysaccharides (Demeke and Jenkins, 2010), which may be due to the content of sucrose, fructose, raffinose and stachyose, which correspond to 25% of the composition of the soybean seeds (De Luna-Jimenez, 2007) increasing up to 48% in some varieties (Zhang *et al*., 2017), and they are not completely eliminated during the isolation process. With the use of CTAB, chloroform and the enzymes α-amylase, pectinase, cellulase and hemicellulase the sugars can be removed (Demeke and Jenkins, 2010).

In the analysis of variance, highly significant differences were observed in the $A_{260/280}$ and $A_{260/230}$ ratios, among the protocols studied, $p< 0.0001$ (Table 2). Through the comparison of means, in the $A_{260/280}$ ratio, three groups are obtained: a) sarcosine solution; b) phenol/chloroform solution; and c) solutions with 1% and 3% CTAB; while, in the $A_{260/230}$ relation, the protocols are grouped as follows: a) solutions of CTAB at 1% and phenol/chloroform; b) phenol/chloroform and sarcosine solutions; and c) 3% CTAB solution (Table 3).

Integrity of isolated nucleic acids

In order to determine if the samples obtained have high molecular weight and may be useful for long-term preservation, electrophoretic running on 1% agarose gel was performed. In the electrophoresis of nucleic acids obtained with 1% CTAB (Figure 1a) and phenol/chloroform (Figure 1c), bands indicating high molecular weight DNA were obtained, according to the λ DNA marker, whereas, with sarcosine, no DNA was detected. they observe bands in the gel (Figure 1b). In the case of the samples obtained with the protocol using the 3% CTAB solution, it was not considered useful to perform the electrophoresis because there were low values in the $A_{260/280}$ ratio (range of 1.11 to 1.17), which indicate poor quality of nucleic acids.

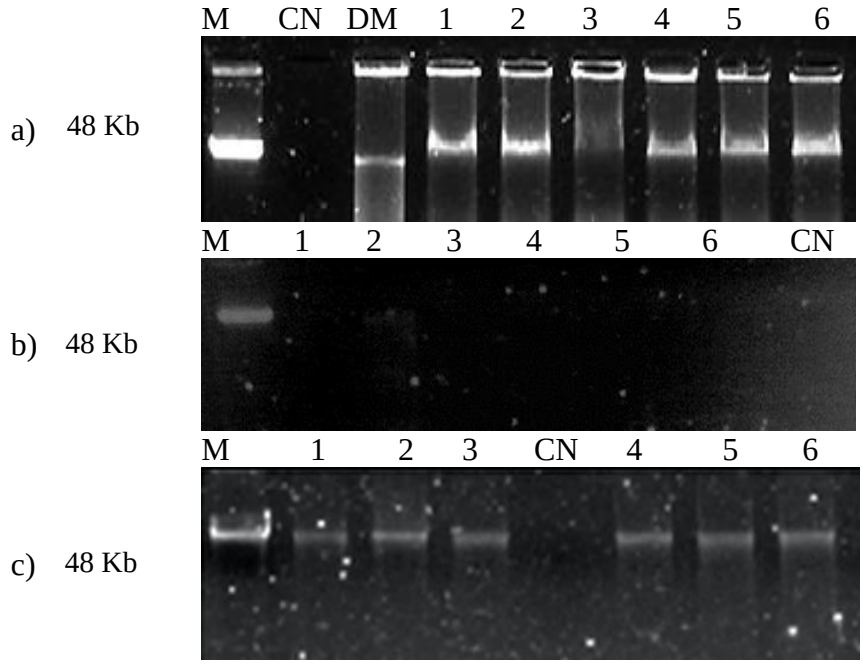

Figure 1. The 1% agarose gel electrophoresis of the nucleic acids obtained with three extraction protocols. a) DNA samples isolated with 1% CTAB solution; b) DNA samples isolated with 1% sarcosine solution; and c) DNA samples isolated with phenol/chloroform solution. M= reference marker λ DNA; CN= negative control; DM, corn DNA; 1-6= Soybean DNA.

During the integrity analysis of nucleic acids, it is expected to observe bands on the gel with certain intensity and high molecular weight revealed by the use of a reference marker. The presence of scavenging or spots is indicative of degraded DNA, fragmented or presence of contaminating RNA (Figure 1). In the present work, bands of intensity similar to the reference marker were observed when phenol/chloroform was used, lower intensity with CTAB and in sarcosine no bands were observed, despite the fact that high readings were presented at A_{260nm}, which may be due to products contaminants that are detected at the same wavelength.

The results of the present study are similar to those reported by Kamiya and Kiguchi (2003) who obtained DNA with good integrity from soybean seeds with the use of phenol/chloroform/isoamyl alcohol solution.

Functionality of DNA

The functionality was evaluated only with the samples obtained with the phenol/chloroform solution protocol, because the results of DNA yield, purity and integrity tests were in accordance with the expected parameters. In the electrophoretic shift of Figure 2, the amplification bands of the rbcL gene are observed in the six DNA samples, which suggests the absence of PCR inhibitory substances.

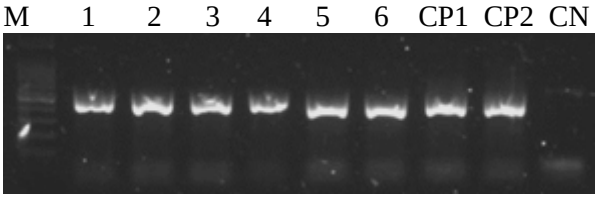

Figure 2. The 2% agarose gel electrophoresis of the PCR-amplified products of the rbcL gene. M, ladder reference marker 100 bp; 1-6 amplicons with soy DNA; CP1 and CP2, positive controls 1 and 2, respectively, with maize DNA; CN, negative control.

Although, the CTAB method produced a greater amount of DNA and is the most widely used in plant species (Demeke *et al*., 2009), in the present study, better quality was obtained with the phenol/chloroform method. Unlike the results found in the present study, the CTAB method was effective to obtain high quality DNA (Pinto *et al*., 2011) and even with soybean cotyledon tissue (Al-Amery *et al*., 2016).

With the results of the present work, the laboratories will be able to optimize resources with the implementation of the appropriate method for obtaining nucleic acids from soybean seeds, but also, it can be applied in seeds of several plant species.

Conclusions

Obtaining high concentrations of DNA with some particular methodology is not necessarily indicative that DNA is useful for further analysis as PCR amplification.

The phenol/chloroform solution method is suitable for obtaining DNA in appropriate amount, purity, integrity and functionality to perform PCR amplifications.

The CTAB and sarcosine methods showed low values in the $A_{260/280}$ ratios indicating the presence of proteins, degraded or single-stranded DNA or RNA, and in $A_{260/230}$ representing the presence of carbohydrates or polyphenol inhibitors of PCR.

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