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Optimization of DNA Isolation Protocol for Soybean Leaf

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Abstract*—Soybean is an important food legume crop with high nutritional value. A DNA isolation protocol that is optimized for soybean could be useful in molecular studies of soybean. Here we present the optimization of the DNA isolation protocol for fresh plant tissue by Doyle and Doyle (1990) on the quality and quantity of isolated soybean DNA. Soybean leaf contains high levels of polysaccharides, polyphenols and secondary metabolites. This protocol when used on soybean results to smearing and degraded DNA. To overcome these problems, a protocol has been developed, availing on the combination of Polyvinyl pyrrolidone (PVP) and Cetyl trimethylammonium bromide (CTAB) in the extraction buffer, to prevent the solubilisation of polysaccharides and polyphenols during the DNA extraction method. It also involves successive chloroform: isoamyl alcohol extractions, addition of sodium acetate and successive nucleic acid precipitation with ethanol. Using this method, DNA was extracted from young leaves of different soybean genotypes. Modifications resulted to relatively high yield and good quality DNA. The yield of DNA ranged from 135.81 - 846.80 ng/µL and the purity ratio was between 1.76-1.95 indicating minimum level of contaminating metabolites. The present protocol provides a convenient DNA isolation method for soybean leaves that yields large amounts of pure and intact DNA.*

***Keywords*—** *DNA extraction, Doyle and Doyle, soybean.*

I. INTRODUCTION

Soybeans (*Glycine max* L. Merr) are one of the most valuable crops in the world not only as an oil seed crop and feed for livestock and aquaculture, but also as a good source of protein for the human diet and as a biofuel feedstock. Soybean seeds typically contain 30-45% protein (moisture-free basis) to 55% protein (moisture-free basis), while most legumes contain only 20-25% protein (Hammond et al., 2003; Hoffman and Falvo, 2004). Soybean's valuable properties have made it part of agricultural mixes in many parts of the world. Global demand for soybeans is increasing, largely due to consumer interest in alternatives to animal and vegetable proteins (Fraanje and Garnett, 2020).

Despite the economic importance, the genetic base of soybean cultivars is extremely narrow. The application of DNA technology in agricultural research has developed rapidly during the last twenty years, especially in the characterization of cultivars and in determining the diversity of many plant species (Lei et al., 2006; Chen and Yang, 2004; Nan et al. et al., 2003, Ipek and Madison, 2001, Cardoso et al.). Molecular marker analysis in genome studies has greatly enhanced the speed and efficiency of crop improvement and breeding programme (Song et al., 2023). With the development of polymerase chain reaction (PCR) technology, molecular markers based on PCR soon found a wide application in plant genetics and breeding. The use of these methods requires the ability to isolate high-quality and high-quantity genomic DNA for PCR analysis. The application of this powerful tool has however been constrained by lack of efficient nucleic acid isolation techniques (Ali et al., 2017). Since DNA extraction is an important step in molecular assay and plays a vital role in obtaining high resolution results in gel-based systems (Shillito et al., 2022; Ahmadikhah, 2009) , a reliable method is highly preferred in the isolation of genomic DNA. Large variations in size, content and organization of genome and contents of metabolites have been reported in different plant species. A single DNA isolation protocol is probably not suitable for all plant systems (Loomis, 1974). It is important to isolate good quality DNA that is relatively free of many contaminants found in plant cells. Chemotypic heterogeneity between species may not allow optimal DNA recovery with a single protocol, so even closely related species may require different isolation protocols (Porbski et al., 1995). The Doyle and Doyle (1990) method on rapid DNA isolation procedure for small quantities of fresh leaf tissue has been used to other crops including soybean. Hence, this study aims to optimize the Doyle and Doyle (1990) protocol on rapid total DNA isolation suitable for soybean leaf.

II. MATERIALS AND METHODS

Soybean Genotypes

The 16 soybean genotypes EGSy 01-30-25, Macs 54, Macs 54-a, LGSy 08-2a-2-1, LGSy 09-7b-1, G 84161, MTD 63, Con Khuong, GC30187-10, G 0073-47-1, G 84040-16-1, GC 87025-25-6, EGSy 98-31-4, GC 87051-7-2-10, PSB Sy 1 and PSB Sy 3 were used in this research. Samples of fresh immature, unbruised leaves of soybean were harvested from pot grown seedlings, washed with sterile distilled water and used for the isolation of genomic DNA (gDNA).

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Solutions

The extraction buffer consisting of 2% (w/v) CTAB, 0.1 M Tris-HCl (pH 8), 1.4 M NaCl, 20 mM EDTA, 0.2% β-mercaptoethanol (v/v), and 1.5% polyvinylpyrrolidone (PVP) (w/v) was prepared. In addition, chloroform: isoamylalcohol (24:1, v/v) solution was also prepared and stored.

DNA Isolation and Purification

The protocol of Doyle and Doyle (1990) used 5-7.5 ml of CTAB isolation buffer per 0.5-1 g leaf sample incubated at 60 °C for 30 minutes for complete isolation of DNA from fresh plant tissue for a rapid preparation procedure, and centrifuged at 6,000 rpm min g -1 10 min. The supernatant was extracted once with chloroform-isoamyl (24:1) and centrifuged at 6000 rpm g-1 for 10 minutes. The supernatant was transferred to new, clean tubes and was added with 2/3 volume cold isopropanol and let it stand for several hours to overnight at room temperature. Nucleic acid was precipitated with 10-20mL wash buffer (76% EtOH, 10µM ammonium acetate) and centrifuged at 6000 rpm $g^{-1}$for 10 min. The pellet formed was air dried, resuspended in 1 mL TE + RNAse and was incubated at 37°C water bath for 30 min before storing for further use. When this protocol was used on soybean leaves, results of electrophoretogram showed degraded DNA which prompted to modification of the protocol.

Modifications on the Doyle and Doyle (1990) protocol on rapid total DNA isolation preparation procedure for fresh plant tissue are presented in Table 1.

Young soybean leaves (0.25 g) were homogenized to a fine powder in a mortar and pestle, followed by the addition of liquid nitrogen. The lyophilized leaf samples were transferred to conical tubes with 2 mL pre-heat CTAB extraction buffer and 1.5%PVP (0.03 g) and were mixed until evenly suspended. The mixture was incubated in a water bath for one hour at 65 ºC with occasional shaking every 15 minutes. After incubation, supernatant about 650µL was transferred to 1.5 ml eppendorf tubes. An equal volume of chloroform:isoamyl (24:1) was added and was mixed thoroughly by inverting the tubes 15-20 times to form an emulsion. After mixing, tubes were centrifuged at 10,000 rpm for 5 min at 4°C to separate the phases. Only the upper aqueous phase was transferred to clean eppendorf tubes and re-extracted with equal volumes of chloroform and isoamyl alcohol (24:1) by centrifuging at 10,000 rpm for 5 min at 4°C. Again, the supernatant was transferred to new tubes and addition of equal volume of cold 2-propanol, 100% ethanol at twice the volume of the supernatant and sodium acetate at 10% of the volume of the supernatant were done to precipitate the nucleic acid. Tubes were incubated at -20ºC for at least one hr. Following the precipitation, the tubes were centrifuged at 10,000 rpm for 5 min. The supernatant was removed, and the pellet was washed with 70% cold ethyl alcohol and was centrifuged for 5 min at 10,000 rpm. This process was done twice. After the removal of the supernatant, the DNA pellet was allowed to dry (approximately 45 min to 1 hr) but not over-dried so the DNA will not be hard to re-dissolve. DNA was then resuspended by the addition of 200 µL of sterile nanopure water. When the DNA was completely dissolved, 0.5 µl (1/100 vol) of RNase A (10 mg ml 1) was added. The samples were incubated at 37 ºC for one hr and were stored prior to use.

TABLE 1. Modifications done on the CTAB protocol for isolation of plant DNA by Doyle and Doyle (1990).

Doyle and Doyle (1990)	Modifications
Preheat 5-7.5 ml Cetyl trimethylammonium bromide (CTAB) at 60°C	Preheat 2 ml Cetyl trimethylammonium bromide (CTAB) at 65°C
0.5 -1.0 g Leaf samples ground in preheat CTAB	0.25 g Leaf samples ground in liquid N Ground leaf samples added with CTAB and polyvinylpyrrolidone (PVPP)
Water bath incubation at 60°C for 30 min	Water bath incubation at 65°C for 1 hr
Centrifugation 6000 rpm x g/10 min	Centrifugation 10,000 rpm /5 min
Supernatant added with chloroform-isoamyl (24:1) Centrifugation 6000 rpm x g/10 min	Supernatant added with equal volume chloroform-isoamyl (24:1) Centrifugation 10,000 rpm for 5 min (procedure was done twice)
Supernatant added with 2/3 volume cold isopropanol	Supernatant added with equal volume cold isopropanol + 10% EtOH at twice the volume of supernatant) + Sodium acetate at 10% of the volume of supernatant
Let it stand for several hours to overnight at room temperature	Let it stand for 1 hr at -20°C
Precipitate nucleic acid with 10-20mL wash buffer (76% EtOH, 10µM ammonium acetate Centrifugation 6000 rpm x g/10 min	Precipitate nucleic acid with 0.5 mL 70% EtOH Centrifugation 10,000 rpm for 5 min (procedure was done twice)
Air dry	Air dry
Resuspension at 1 mL TE + RNAse	Resuspension at 200 µL snp water + RNAse
Incubate at 37°C water bath for 30 min	Incubate at 37°C water bath for 1 hr

DNA Quality Confirmation

The integrity of the DNA was determined through agarose gel electrophoresis. A 1% solution of agarose gel was prepared by dissolving agarose gel in 0.5X TAE buffer and was warmed in a microwave to completely dissolve the agarose. The solution was set aside to cool. The gel was casted in a supplied tray and comb and was allowed to set for a minimum of 20 min at room temperature on a flat surface. Prior to loading, 1.0 µL sample was mixed with 2.0 µL of BluJuice (25% sucrose, 50 mM Tris pH 8.0, 0.5 mM EDTA pH 8.0 and 2 mg mL^{-1} bromphenol blue). The tip was placed under the surface of the electrophoresis buffer and above the sample well opening. The sample was delivered slowly, allowing it to sink to the bottom of the well. During loading, it is very important not to place the tip into the well or touch the edge of the well with it. This can damage the well and cause unevenness or spots. DNA size standards should be loaded in the first well from left because many computer programs that are used for calculating DNA fragment size require the size standard in this position (Surzycki, 2000). The gel was run at 110 volts until 70-80% of the gel length was travelled by the tracking dye. After the run, the gel was stained for 1 min using 1.0 % ethidium bromide and was destained in

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distilled water for 15 to 20 min before placing it on an UV illuminator. The gel was photographed using the Quantity One (BioRad) Gel Photo documentation System to record results.

Quantification of extracted DNA and purity checking

DNA content was confirmed by measuring DNA purity and concentration using a NanoDrop™ spectrophotometer. The purity of a DNA solution can be determined by comparing the optical density values of the solution at different wavelengths (Clark, 2000). The 260/280 nm ratio observed for pure DNA is close to 1.8. Spectrophotometrically, 1.0 µL of the sample was mixed with 499 µL of distilled water and the dilution factor was taken. A 260/280 ratio below 1.8 often indicates the presence of contaminating protein or phenol.

III. Results and Discussion

Morphological characterization is the traditional and easiest way to differentiate genetic variation in a germplasm. In many accessions, the utilization of morphological characterization can still be considered useful for preliminary evaluation and a practical tool for studying genetic diversity, but this should be complemented by assessment of genetic diversity through molecular techniques which can provide a true representation of the entire genome (Dayaman, et al., 2009). A simple, reliable and fast determination of genetic diversity in plant varieties is essential for the proper varietal identification, classification and conservation and helpful in plant improvement and could be cost effective.

The use of Doyle and Doyle (1990) protocol for genomic DNA extraction resulted to degraded DNA as shown on the electrophoretogram (Figure 1). Smears usually indicate a contamination with DNA degrading enzymes (Valentin et.al, 2005) or there was too much salt in the DNA (http://www.bio.davidson.edu). Such DNA will likely be difficult to amplify by PCR. Good DNA should produce a sharp band of equal size and no smear should be visible.

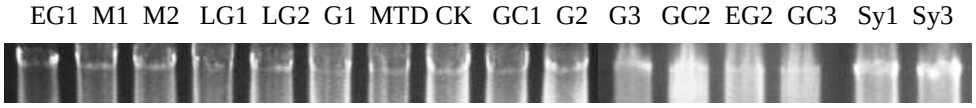

Figure 1. Agarose (1% gel) electrophoretograms of genomic DNA from leaves of 16 soybean genotypes using the Doyle and Doyle (1990) protocol[1].
[1]EG1-EGSy 01-30-25; M1-Macs 54; M2- Macs 54a; LG1-LGSy 08-2a-2-1; LG2-LGSy-09-7b-1; G1-G84161; MTD-MTD 63; CK-Con Khuong; GC1-GC 30187-10; G2-G 0073-47-1; G3-G84040-16-1; GC2-GC 8702502506; EG2-EGSy 98-31-4; GC3-GC87051-7-2-10; Sy1- PSB Sy 1; Sy3- PSB Sy 3

To overcome these problems, DNA extraction was standardized by modifying some of the steps in the original Doyle and Doyle (1990) DNA isolation protocol. Modifications on the Doyle and Doyle (1990) protocol were made to improve the quality of DNA. Immature soybean leaves were used to minimize the presence of high polyphenols and polysaccharide. Polyphenols, which are strong oxidants present in many plant species, can reduce yield and purity by covalently binding to extracted DNA, rendering it useless for most research applications (Padmalatha and Prasad (2006). The purpose of PVP in CTAB buffer was to inhibit polyphenol oxidase activity by forming a hydrogen-bonded complex and help remove impurities. The additional step of chloroform: isoamyl alcohol application was done so that proteins and lipids were effectively removed from the solution. Chloroform: isoamyl alcohol acts as a type of detergent by binding to proteins and lipids of cell membrane and dissolving them. It forms complexes with lipids and proteins causing them to precipitate out of the solution. The precipitation of ethanol was done twice to remove excess salts which were also the cause of the smears found on the electrophoretogram. We found that these modified steps are necessary to standardize genomic DNA and increase quality and quantity.

Intact DNA with less smearing was observed after the modifications were done on the Doyle and Doyle (1990) protocol for DNA isolation (Figure 2). Spectrophotometry confirmed that all the isolates have relatively high genomic DNA yield and were of good quality (Table 2). Fourteen out of the 16 genotypes have ~1.8 purity ratio which means that the modifications on the Doyle and Doyle (1990) protocol had resulted to high yield and "pure" DNA. A ratio of ~1.8 is generally accepted as "pure" for DNA (http://www.nanodrop.com).

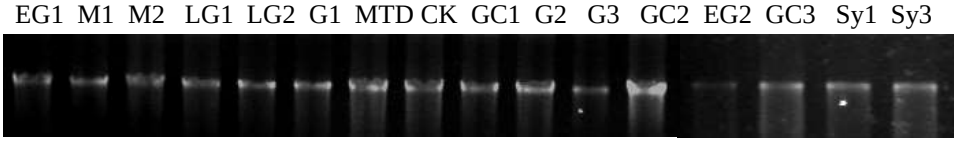

Figure 2. Agarose (1% gel) electrophoretograms of genomic DNA from leaves of 16 soybean genotypes using the modified Doyle and Doyle (1990) protocol[1].
[1]EG1-EGSy 01-30-25; M1-Macs 54; M2- Macs 54a; LG1-LGSy 08-2a-2-1; LG2-LGSy-09-7b-1; G1-G84161; MTD-MTD 63; CK-Con Khuong; GC1-GC 30187-10; G2-G 0073-47-1; G3-G84040-16-1; GC2-GC 8702502506; EG2-EGSy 98-31-4; GC3-GC87051-7-2-10; Sy1- PSB Sy 1; Sy3- PSB Sy 3

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TABLE 2. Soybean genotypes purity ratio and concentration values of DNA obtained after taking absorbance at 260/280.

Genotype	Purity Ratio	Concentration (ng/µL)
EGSy 01-30-25	1.95	726.58
Macs 54	1.93	236.62
Macs 54-a	1.93	516.81
LGSy 08-2a-2-1	1.92	846.80
LGSy 09-7b-1	1.93	372.72
G 84161	1.88	250.37
MTD 63	1.82	257.68
Con Khuong	1.91	376.29
GC30187-10	1.76	396.09
G 0073-47-1	1.90	376.29
G 84040-16-1	1.81	135.81
GC 87025-25-6	1.88	342.98
EGSy 98-31-4	1.91	230.00
GC 87051-7-2-10	1.83	206.79
PSB Sy 1	1.88	214.27
PSB Sy 3	1.78	203.48

IV. CONCLUSION

Modifications made on the Doyle and Doyle (1990) protocol for genomic DNA isolation was suitable for soybean leaf resulting to high concentration and good quality DNA. Modifications were cost effective by using 50% less plant tissue, 30% less chemicals and 50% less time. The modified protocol could be of broad significance in assessing the wide diversity of soybean germplasm and will be useful to molecular downstream applications.

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