

## AN EFFECTIVE METHOD FOR DNA EXTRACTION OF MATURE LEAF OF SAPODILLA (*Manilkara zapota* (L.) van Royen)

Vega Kartika Sari and Rudi Hari Murti<sup>\*)</sup>

Faculty of Agriculture University of Gadjah Mada Jl. Flora no 1 Bulaksumur Yogyakarta Indonesia

<sup>\*)</sup> Corresponding author Phone: +62-274 563062 E-mail: rhmurti@ugm.ac.id

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### ABSTRACT

Perennial crop leaves contain polysaccharides, polyphenols, and other secondary metabolites in high concentration. The presence of those compounds inhibit enzymatic activities and amplification of DNA. The existing extraction methods were not able to dissociate the metabolites contaminants of sapodilla (*Manilkara zapota* (L.) van Royen) leaves and thus resulting in low quality of extracted DNA. The aim of this experiment was to develop an effective method to extract DNA from mature leaf samples of sapodilla (*Manilkara zapota* (L.) van Royen). Fifth modification of Doyle & Doyle DNA extraction protocol with modified concentration of buffer reagent (consisted of: CTAB 2.8%; NaCl 2.5M; mercaptoethanol 3%, and PVP 2.5%) and repetition of some phase purification (liquid nitrogen; three times CIAA; two times ethanol 70%, RNase 1µl) generated high quality DNA and clear band of PCR amplification using RAPD primers.

Keywords: buffer modifications, DNA extraction, sapodilla

### INTRODUCTION

Sapodilla (*Manilkara zapota* (L.) van Royen), like most of perennial crops, contains high levels of polysaccharides, sap, polyphenols, several kinds of pigments, and other secondary metabolites. The presence of these compounds making sapodilla can be used as medicine for cough, diarrhea, fever, antibiotics, and antimicrobial (Chanda and Nagani, 2010). On the contrary, the mentioned metabolites of sapodilla lead to difficulty in DNA extraction and consequently resulted in limitation in the studies of molecular biology.

Existing DNA (*Deoxyribonucleic acid*) extraction protocol failed to separate the high amount of secondary metabolites in the leaves of fruit trees, medicinal plants, and some shrubs. Another difficulty was the polysaccharides compounds inhibited DNA amplification because the DNA was more more viscous (Sahu *et al.*, 2012) and it had glue-like consistency, which could inhibit Taq enzymatic activity and interfere the accuracy of restriction enzyme activities (Dehestani and Tabar, 2007). The presence of polyphenols gave brown color due to oxidation of DNA and made it useless for further testing in molecular studies (Sahu *et al.*, 2012).

High quality and uncontaminated DNA samples are taken as the first important step that affects successfulness of any further molecular analysis activities. DNA with high quality is shown by electrophoresis resulting in high intensity of DNA and low smear intensity (Utami *et al.*, 2012). However, the use of PCR process may enable amplified DNA to result in the desirable pattern of bands (Syafaruddin and Santoso, 2011).

There are various methods for DNA extraction such as Doyle and Doyle (1990). Utilization of DNA extraction kit becomes preferable even though high cost of the kit product itself is becoming the main concern (Amani *et al.*, 2011). Several DNA extraction protocol used phenol to separate cellular molecules and debris from the DNA. It is toxic, hazardous, and expensive (Sahu *et al.*, 2012). *Cetyl Trimethyl Ammonium Bromide* (CTAB) buffer method developed by Doyle and Doyle (1990) is preferable and frequently used in DNA extraction (Ribeiro and Lovato, 2007) of plant that contains polysaccharides and polyphenolic compounds (Jose and Usha, 2000). Unfortunately, the standard composition buffer used in this protocol is not suitable to be applied in sapodilla.

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Table 1. Buffer component standard of Doyle and Doyle (1990) and its modifications for DNA extraction from sapodilla

Treatment	Component of Buffer
Doyle and Doyle (1990)	2% CTAB; 1.4M NaCl; 0.1M Tris-HCl; 0.02M EDTA; 1% mercaptoethanol; 1% PVP
Modification 1	2.8% CTAB; 2M NaCl; 0.1M Tris-HCl; 0.02M EDTA; 1% mercaptoethanol; 2% PVP
Modification 2	2.8% CTAB; 3M NaCl; 0.1M Tris-HCl; 0.02M EDTA; 2% mercaptoethanol; 2.5% PVP
Modification 3	2.8% CTAB; 2.8M NaCl; 0.1M Tris-HCl; 0.02M EDTA; 2% mercaptoethanol; 2.5% PVP
Modification 4	3% CTAB; 2.5M NaCl; 0.1M Tris-HCl; 0.02M EDTA; 2.5% mercaptoethanol; 2.5% PVP
Modification 5	2.8% CTAB; 2.5M NaCl; 0.1M Tris-HCl; 0.02M EDTA; 3% mercaptoethanol; 2.5% PVP

Some modifications of DNA extraction techniques to obtain high quality of DNA from rich secondary metabolites leaves have been carried out. Modifications with the addition of antioxidants PVP (*Polivinilpolipirolidone*),  $\beta$ -mercaptoethanol, and utilization of liquid nitrogen facilitate the destruction of leaf tissue (Syafaruddin dan Santoso, 2011). Others modifications in the extraction stages were done by repetition or by modifying the volume, temperature, and duration of incubation (Chen *et al.*, 2010). Other modifications directed to different extraction DNA plant were performed by Dehestani and Tabar (2007) on the tea; Syafaruddin and Santoso (2011) on hazelnut; Utami *et al.* (2012) on the ginger. However, these methods are not suitable for sapodilla.

Therefore, this study aimed to obtain suitable DNA extraction method for sapodilla. In this study, buffer component modification, treatment, and repetition phases in the extraction of DNA with Doyle and Doyle (1990) CTAB method were applied to produce high quality DNA and clear DNA bands from the PCR process.

## MATERIALS AND METHODS

The experiment was conducted in the Laboratory of Genetics and Plant Breeding, Faculty of Agriculture University of Gadjah Mada in December 2013-April 2014. Genetic material taken as samples was sapodilla with fully open leaves taken from several areas in Yogyakarta. Experimental procedure by Doyle and Doyle (1990) method:

### a. Extraction and purification of DNA:

1. Preheat CTAB isolation buffer in a 30 ml glass centrifuge tube to 60°C in water bath. Modification of buffer components is shown in Table 1.

2. Powder 0.1 g fresh leaf tissue in liquid nitrogen in a chilled mortar and pestle. Scrape powder directly into preheated buffer and swirl gently to mix. If preferred, fresh tissue may be ground in 60°C CTAB isolation buffer in a preheated mortar.
3. Incubate sample at 60°C for 30 (15-60) minutes with optional occasional gentle swirling.
4. Extract the sample with chloroform-isoamyl alcohol (24:1) mixing gently but thoroughly. Modification of extraction phase is in Table 2.

Table 2. Treatment and repetition stages of DNA extraction Doyle and Doyle (1990) and its modifications

Treatment	Stages of DNA Extraction
Doyle and Doyle (1990)	Without liquid nitrogen; 1x CIAA; 1x etanol 70%
Modification 1	Without liquid nitrogen; 1x CIAA; 2x etanol 70%
Modification 2	Liquid nitrogen; 3x CIAA; 2x etanol 70%
Modification 3	Liquid nitrogen; 3x CIAA + phenol; 2x etanol 70%
Modification 4	Liquid nitrogen; 3x CIAA; 2x etanol 70%
Modification 5	Liquid nitrogen; 3x CIAA; 2x etanol 70%, RNase 1 $\mu$ l

5. Spin in clinical centrifuge (swinging bucket rotor) at room temperature to concentrate phases. The speed set for this experiment was 12000 rpm for 15 min.
6. Remove aqueous phase with wide bore pipet, transfer to clean glass centrifuge tube, add sodium acetat 1/10 volumes

- and then add cold isopropanol 2/3 volumes, and mix gently to precipitate DNA pellet.
7. Mixture was put in refrigerator (-20°C) for 1-24 hours to precipitate the DNA pellet.
  8. Pour off supernatant, add 500 µl ethanol, and mix gently to purification DNA pellet.
  9. Spin the supernatant in clinical centrifuge at room temperature, pour off ethanol, and allow it to quickly air dry at room temperature.
  10. Resuspend DNA pellet in 1 ml TE or aquabides.
  11. Add RNase and incubate at the temperature ranging from 30-90°C (min at 37°C).
  12. Dilute sample with distilled water or TE.
  13. DNA obtained was stored in the refrigerator and ready to be used.

#### b. Checking DNA quality and quantity

Checking quality of genomic DNA was performed by adding some loading dye in DNA mixture (1:5) for electrophoresis. Checking quantity of DNA including the purity and concentration was performed using a spectrophotometer at wavelengths of 260 and 280 nm.

#### c. Amplification of DNA

DNA amplification was carried out by filling the samples into PCR tube, for 1x PCR reaction mix consisting of 5 mL, NFW 3.25 mL, 0.25 mL RAPD primer and 3 mL DNA. Then, the samples were inserted into the PCR thermocycler with the following setting: one cycle for 4 minutes at 94°C followed by 45 cycles for 1 minute at 94°C (denaturation), 1 minute at 37°C (annealing), 1 minute 30 s at 72°C (extension), and it was completed with 7 minutes at 72°C and 1 minute at 4°C. Amplification product was visualized by electrophoresis.

#### d. Visualisation of DNA

Genomic DNA resulting from extraction or amplification of PCR was put in agarose well (1%) after added with DNA staining 1 µl, and it was performed in electrophoresis device for approximately 1 hour in 80 V using TBE 1x reagent. The following result was checked in

UV transilluminator light and photographed by digital camera.

## RESULTS AND DISCUSSION

Various methods of DNA extraction were applied by Dehestani and Tabar (2007), Chen *et al.* (2010), Syafaruddin and Santoso (2011), Utami *et al.* (2012), and Chathrath *et al.* (2013). Fruitful DNA extraction in producing a high quality of DNA depends on plant type or which plant tissue is used. Sapodilla leaf could not produce high quality DNA when it was extracted by using the Doyle and Doyle's method (1990) commonly used for leaves containing many polysaccharides and polyphenols (Jose and Usha, 2000).

The result of extraction using young leaves by using the method of Doyle and Doyle (1990) without modification produced very thin DNA and there were contaminants (RNA) and smear (Figure 1). It indicated that the method was not suitable for the extraction of the young leaf of sapodilla. Unavailability of young leaves is another weakness of young leaf sample, so the mature leaf is one recommended solution though it needed modifications in DNA extraction protocol.

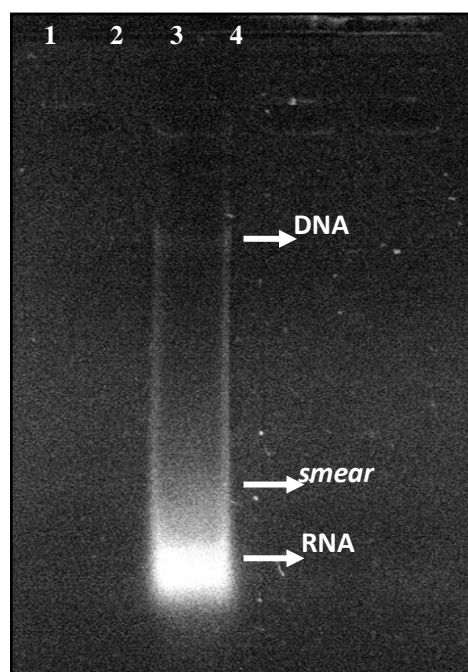


Figure 1. Result of DNA electrophoresis by Doyle and Doyle's method (1990)

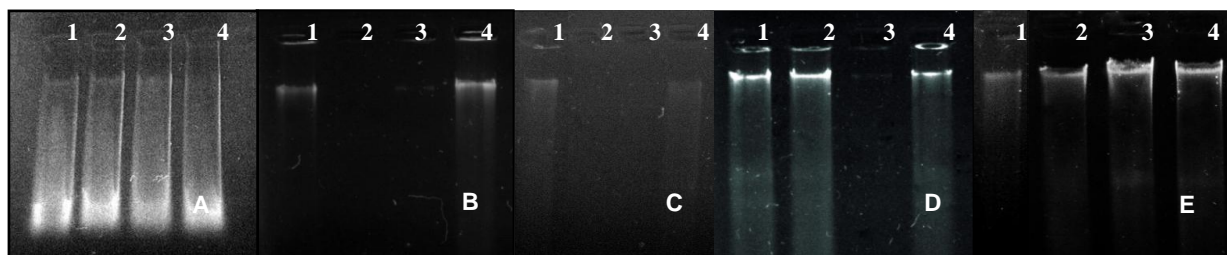


Figure 2. Electrophoresis result with modifications  
A) Modified-1; B) Modified-2; C) Modified-3; D) Modified-4; E) Modified-5

Table 3. Quantified results from modified Doyle and Doyle method (1990)

Sample	Modified-2		Modified-3		Modified-4		Modified-5	
	$\lambda 260/280$	[DNA]	$\lambda 260/280$	[DNA]	$\lambda 260/280$	[DNA]	$\lambda 260/280$	[DNA]
1	2,000	100	1,182	650	1,500	150	2,000	100
2	1,000	100	1,111	500	1,556	700	2,000	400
3	1,000	100	1,111	500	2,000	100	1,750	350
4	1,500	150	1,000	500	1,667	250	1,750	350

Remarks: Results in modified 1 were not quantified

Buffer modification, treatment, and extraction were performed to obtain high quality of DNA from mature leaves. Five modified Doyle and Doyle Methods (DDM) were applied and DNA electrophoresis results were shown in Figure 2. In the modified-1 DDM, the sample used consisted of two samples of fresh and dried leaves. Increasing buffer components in modified-1 DDM could not produce a significant improvement and the results of fresh and dried leaves were indistinguishable. The result of DNA electrophoresis contained many contaminants like RNA and smears. In modified-2, 3, 4, and 5 DDM, mature fresh leaves and liquid nitrogen were used to facilitate the destruction of thick and hard texture of sapodilla leaves into powder.

Increasing level of NaCl, percentage  $\beta$ -mercaptoethanol and repetition phases of CIAA in modified-2 was better than modified-1 DDM in generate DNA, but it was still very thin and a lot of residual DNA in the well that indicated the high levels of polysaccharides. Modified-3 DDM used phenol and there was no significant progress in the result. Phenol reagent was known as dangerous material and too costly so it was not involved in subsequent modifications. Modified-4 DDM produced thicker DNA but still a quite long smear, while the DNA in modified-5 DDM was also thick and the smear was diminished.

The purity or quality of DNA could be quantified by spectrophotometer in range of 1.8 to 2.0 at  $\lambda 260/280$  nm (Sambrook *et al.*, 1989). The DNA quantification of modified DDM showed an improvement of DNA quality and the best one was modified-5 DDM (Table 3). The purity of the best DNA ranging from 1.75 to 2.00 was equivalent to standard. Based on the results of this experiment, the mature leaf can be an alternative to extract DNA although it was known to contain many secondary metabolites and polysaccharides. This result was in accordance with that of Small *et al.* (2004), Chathrath *et al.* (2013) and Anuradha *et al.* (2013).

Thus, the increasing of the buffer components such as CTAB, NaCl, and PVP and repetition mercaptoethanol-CIAA treatment significantly improved the DNA quality of Sapodila. This result was linear with that of Chathrath *et al.* (2013). Modified CTAB, PVP, and mercaptoethanol were capable of producing high quality DNA, while other components were the same as the standar method. CTAB is detergent that could separate the polysaccharides and nucleic acids. RNA and DNA were dissolved in 0.7 M CTAB and NaCl, but in the conditions under 0.4 M NaCl, it will precipitate. The function of mercaptoethanol is to remove polyphenols, in line with PVP which serves as to bind phenolic components

and to suppress oxidation (Utami *et al.*, 2012). CIAA (*Chloroform isoamylalcohol*) repeated up to three times was aimed to optimize the extraction of DNA of contaminants such as mango extraction studies (Azmat *et al.* 2012). According to Syafaruddin and Santoso (2011), chloroform is organic solvent that can dissolve proteins, lipids and other molecules such as polysaccharides. Ethanol is made up of two replications as it aims to optimize the washing of DNA pellet as in the study of hazelnut 'sunan' extraction by Syafaruddin and Santoso (2011) and *Pennisetum gluacum* by Chathrath *et al.* (2013).

To proof the high quality DNA obtained in modified-5 DDM, the DNA was used as template for PCR analysis using RAPD marker. The results of PCR amplification using primer OPB 6 and OPC 19 are shown in Figure 3. The pattern of amplified DNA bands were clearly visible.

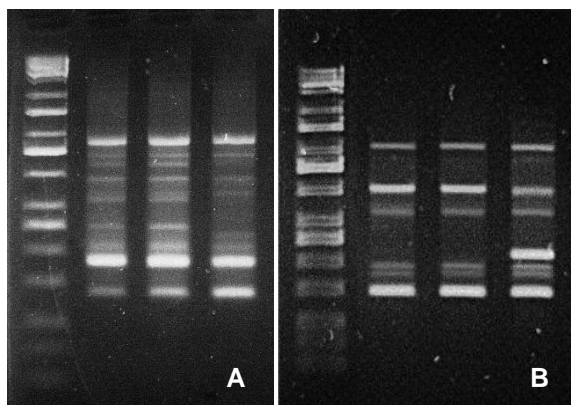


Figure 3. Results of PCR using RAPD  
A) OPC19 and B) OPB 6

DNA extraction with modified-5 DDM was recommended for DNA extraction of sapodilla. Modified-5 DDM has also been tested for DNA extraction in durian (*Durio* sp.), and it has resulted in clear bands of PCR amplification (data not attached).

### CONCLUSION

Sapodilla DNA extraction method was applied by modifying the components of CTAB buffer, repeating several stages, and using liquid nitrogen which produced high quality of DNA and banding pattern. Extraction method of modified 5 DDM was considered the most

effective for extraction of sapodilla mature leaf samples and recommended for extraction of DNA of other perennial crops.

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