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Rapid genomic DNA extraction for Soybean (*Glycine max* L. Merr) using modified CTAB protocol to obtain high-quality DNA

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Molecular and genetic analysis is one of the critical primary stages, starting with DNA extraction. DNA extraction is an essential preliminary step in molecular research. Extracting plant genomic DNA with CTAB buffer takes approximately three days to obtain high-quality DNA. There have yet to be reports regarding effective and efficient soybean genomic DNA extraction methods. This study aimed to obtain a rapid extraction method for soybean with high-quality DNA. This study used a modification of the CTAB method by shortening the duration its stages. The treatments tested were washing soybean leaf and callus organs with ethanol one and two times. The results showed that one-time washing using ethanol absolute obtained the highest average DNA concentrations in leaf and callus organs 458.72 ± 155 ng/µL and 363.87 ± 205 ng/µL, respectively. In addition, DNA purity was obtained 1.99 ± 0.16 and 1.86 ± 0.07, respectively, on A260/280 ratio. Good DNA quality was indicated by the absence of smears on the 1.5% electrophoresis gel. This study demonstrated that extracting plant DNA completed in 70 min to obtain the highest quality DNA.

Keywords: CTAB, Genomic, Isolation, Purity, Quality

Soybean (*Glycine max* L. Merr) is one of the primary foods besides rice and wheat. Soybean seeds are the main source of protein up to 40%, seed oil 20% and fatty acids up to 85%[1]. Moreover, soybeans contain good nutrients such as carbohydrates, vitamins, minerals, folic acid, and calcium[2]. The quality of soybean seeds is essential to note because it affects economic value. Seeds are the main source of material consumed and as planting material[3].

Until now, the development of soybean plants continues to be carried out through conventional breeding and with a biotechnological approach. Molecular analysis is one of the critical primary stages, starting with DNA extraction. The main problem that often occurs in DNA extraction is that there are still low-quality DNA and the presence of contaminants[4]. High-quality genomic DNA is needed as a basis for molecular analysis[5]. Various types of markers such as RAPD[6], SSR[7,8], AFLP[9,10], RLFP[11], IRAP[12], and SNPs[13] can be used in genetic diversity and relationship analysis of soybean in molecular biology studies[14]. Furthermore, genetic modified and molecular detection of specific genes[15] and selection of lines of superior from a population were reported on soybean[16].

Deoxyribonucleic acid (DNA) is a biopolymer double-stranded polysaccharide composed of sugar, phosphate, and nucleated adenine (A), thymine (T), guanine (G), and cytosine (C), which are linked together. Genomic DNA is a template for carrying genetic material for metabolic processes such as replication, transcription, and translation[17]. Pure DNA is obtained by extracting the DNA of plant organs. The basic principle of DNA extraction is to separate genomic DNA from other cell components such us polysaccharides, proteins, organic acids, and RNA. The success of DNA extraction is indicated by the high quality and quantity of DNA without contamination of these components[5]. DNA extraction can be done using several methods *i.e.* Alkaline extraction, Salting-out method, CTAB, phenol-chloroform method, sodium dodecyl sulphate (SDS), Solution and Solvent, and a commercial kit[5,18]. Commercial kits are available used for high-quality DNA extraction from various plant tissues. However, the resulting DNA is usually in low concentrations. Moreover, the costs required are also more expensive[19].

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Cetyl trimethyl ammonium bromide (CTAB) is often used for plant DNA extraction[20]. This method was first reported to be developed by Doyle and Doyle[21], who subsequently made many modifications to various plants. The CTAB method has several main stages: lysis, precipitation, washing, and purification. The advantages of this method are that it is effective for use on plants containing high polysaccharides and phenols, has a high ability to lysis DNA, has a high concentration of DNA, and is easy to do. However, the drawback of the CTAB method is that it takes a long time, around 48 to 72 h, to obtain high-quality DNA[22].

The CTAB basic protocol of sample incubation was carried out at 65°C for 60min[22,23]. Furthermore, the water bath incubation time was often modified to shorten the extraction time to 20-30 min[18,24]. In addition, the precipitation stage which requires overnight time[25], is also modified to be faster[26]. Then, modification at repeated purification and washing stages with ethanol was also reported to obtain optimal DNA quality[4,27]. Genomic DNA extraction using the CTAB method in molecular analysis of soybean (*G. max* L. Merr) has been reported[8,28]. In addition, extraction of genomic DNA using the CTAB method has been reported in samples of kepel leaves (*Stelechocarpusburahol*)[24] and yellow cosmos leaves (*Cosmos sulphureus* Cav.)[25], Maize[29], *Triticum aestivum*[30], *Anacardium occidentale*[5], *Vigna mungo*[31], *Arabidopsis thaliana*[32],*Oryza sativa*, *Solanum lycopersicum*, *Solanum tuberosum, Capsicum annum, Cicer arietinum,* and *Phaseulus vulgaris*[22]. However, this method still takes 48-72 h to obtain pure DNA, making it less efficient for obtaining genomic DNA quickly. Until now, studies on the modified CTAB method for obtaining high-quality DNA of soybeans have not been widely reported. Studies related to rapid extraction methods have been reported on soybean seeds and cotyledons[18]. However, the DNA quality is still low and not optimal. Therefore, modifying the CTAB protocol on soybean leaf and callus samples is necessary. This study aims to obtain an efficient and high-quality modified CTAB method for extracting DNA from soybean plant.

Materials and Methods

Soybean plants were grown in polybags on soil and manure media (1:1). Plants were maintained until leaves were obtained four weeks after planting. Callus was obtained by inoculating soybean cotyledons on Murashige and Skoog media. Incubation lasts two months at the Laboratory of In Vitro Culture, Faculty of Agriculture, Universitas Muhammadiyah Yogyakarta, Yogyakarta, Indonesia. The materials used were leaves and callus of soybean, CTAB buffer (2% CTAB, 2% polyvinyl pyrrolidone (PVP), 2% β-mercaptoethanol, 0.02 M EDTA, 1.4 M NaCl, 0.1M Tris-HCl pH 8.0 and ddH_2O), chloroform isoamyl alcohol (CIA) (24:1), Sodium acetate, cold isopropanol, ethanol absolute, 70% ethanol, agarose gel, and 0.5x TE buffer.

Genomic DNA extraction 1x washing

The DNA extraction of modified CTAB by Purwantoro *et al*.[25] consisted of nine stages. The flowchart in (Fig. 1). 1) DNA lysis stage, 100 mg of sample was grinded using a cold mortar and pestle, and added 700 µL CTAB buffer. 2) The solution was transferred to a new 1.5 mL tube, and added 700 µL CTAB buffer. 3) Samples were incubated in a water bath at 65°C for 20 min. Sample were inverted to homogenize in every 5 min. The next step was centrifuging the samples at 14,000 rpm for 3 min

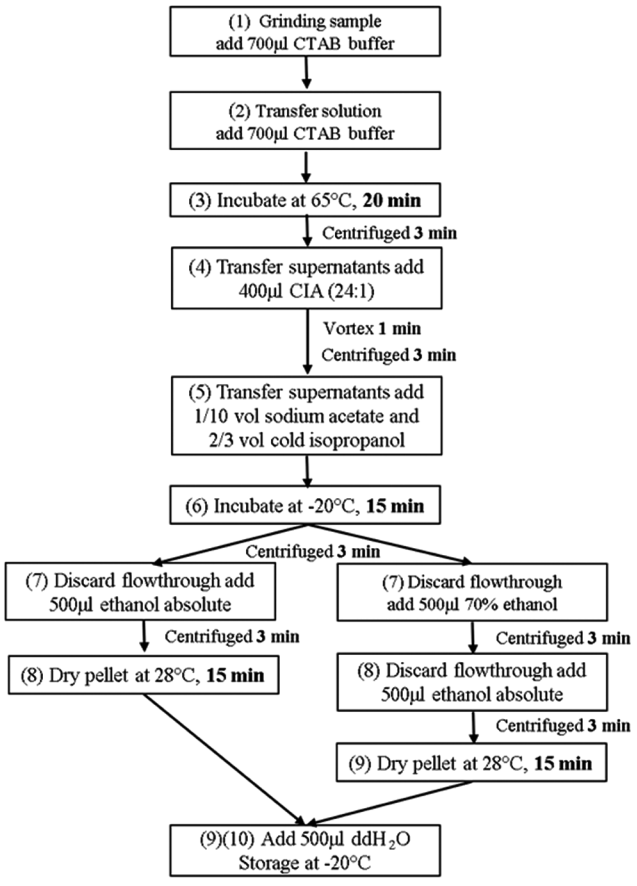

Fig. 1 — Flowchart of DNA extraction modified CTAB method protocols

using microcentrifuge EBA 21 Hettich®, USA. 4) The supernatant was transferred to a new 1.5mL tube and added 400µL CIA (24:1). Then the sample was vortexed using V-1plus, Biosan®, Latvia for 1 minute until the color of the solution changed to yellowish-white, then centrifuged at 14,000 rpm for 3 min. 5) Precipitation stage, the supernatant was transferred into a new 1.5 mL tube and added 1/10 of the volume of sodium acetate and 2/3 of the total volume of coldisopropanol. 6) The solution is homogenized and incubated at -20°C for 15 min. Next, the samples were centrifuged at 14,000 rpm for 3 min. 7) In the washing step, the flowthrough was discarded, and the DNA pellet was added with 500 µL ethanol absolute; then, the sample was centrifuged at 14,000 rpm for 3 min. 8) The flowthrough was discarded, and the pellet was dried for 15 min. 9) In the elution stage, the pellet was added 50µL ddH_2O and then stored at -20°C.

Genomic DNA extraction 2x washing

The 2x washing modified genomic DNA extraction protocol in steps 1 – 6 was carried out as above and continued with the 2x washing step. 7) The flowthrough was discarded, and the DNA pellet was added with 500 µL 70% ethanol, then centrifuged at 14,000 rpm for 3 min. 8) The flowthrough was discarded, and the DNA pellet was added with 500 µL of ethanol absolute, then centrifuged at 14,000 rpm for 3 min. 10) At the elution stage, the pellet is dried for 15 min. Then, the pellet was added 50 µL ddH_2O and stored at –20°C.

Qualitative and Quantitative DNA Analysis

The quality and quantity of DNA were carried out to determine the level of concentration and purity of DNA using a Nanophotometer® N60 (Implen, Germany). The test was performed by dropping 1 µL of DNA solution at the sample point. Analysis was carried out at absorbance wavelengths of 230 nm, 260 nm, and 280 nm.

Visualizing DNA genome

Genomic DNA visualization was performed by electrophoresis (Mupid-EXu®, Japan) on 1.5% agarose gel. The cocktail composition used was 5 µL Sybr safe® as DNA stain, 1 µL Loading dye, 2 µL Nuclease free water, and 2 µL DNA sample. The process was running at 100 volts for 25 min in 0.5 x TE solution. Next, a blue light transilluminator BluPAD (Bio-Helix®, Taiwan) visualized the genomic DNA band on blue light.

Data analysis

This research was carried out by experiments of single-factor, namely using leaf and callus with modifications of 1 and 2 washing times using 70% ethanol and ethanol absolute. Each treatment was repeated six times. Quantitative DNA concentration and purity data were analyzed with an average value with standard deviation. Quality data was obtained by visualizing DNA on agarose gel electrophoresis.

Results

Quantitative DNA Concentration

The results (Table 1) showed that the concentration of young leaf DNA with 1x washing using ethanol absolute obtained DNA concentration rate of 252.6 to 660.95 ng/µL with the highest average gain (458.72 ± 155 ng/µL) compared to 2x washing using 70 % ethanol and ethanol absolute on rate of 101.15 to 680.5 ng/µL with an average (369.7 ± 242 ng/µL). In addition, in the callus organ that was carried out with 1x washing using ethanol absolute, a DNA concentration rate of 115.7 to 697.45 ng/µL was obtained with a higher average concentration (363.87 ± 205 ng/µL) compared to 2x washing which only obtained a rate of 61.5 to 437.7 ng/µL with average (205.7 ± 147 ng/µL). These results indicate that the concentration of high-quality DNA can be used for molecular analysis.

Table 1 — Genomic DNA concentration on young leaf and callus of soybean (ng/µL)

Treatments	Young leaf		Callus	
	1x washing	2x washing	1x washing	2x washing
Sample 1	309.75	460.2	115.7	84.90
Sample 2	512.9	598.05	441.35	61.50
Sample 3	569.85	210.30	250.65	253.10
Sample 4	446.3	168.05	433.35	437.70
Sample 5	660.95	680.50	244.75	104.90
Sample 6	252.6	101.15	697.45	292.10
Average ± SD	458.72 ± 155	369.7 ± 242	363.87 ± 205	205.7 ± 147

Table 2 — DNA purity based on ratio A260 nm/A280 nm and A260 nm/A230 nm on young leaf and callus of soybean

Treatments	Young leaf				Callus			
	1x washing		2x washing		1x washing		2x washing	
Wavelength Ratio	A260/A280	A260/A230	A260/A280	A260/A230	A260/A280	A260/A230	A260/A280	A260/A230
Sample 1	2.00	1.27	1.72	2.21	1.79	0.73	1.69	1.96
Sample 2	2.09	1.47	2.06	2.23	1.95	0.77	1.97	1.24
Sample 3	1.66	1.93	1.79	1.26	1.88	1.23	1.95	2.46
Sample 4	2.11	2.24	2.11	2.16	1.94	0.79	2.01	0.70
Sample 5	2.05	1.65	2.16	2.31	1.78	1.05	1.65	0.52
Sample 6	2.04	2.08	2.12	2.43	1.85	0.86	1.75	1.17
Average ± SD	1.99 ± 0.16	1.77 ± 0.37	1.99 ± 0.18	2.1 ± 0.42	1.86 ± 0.07	0.90 ± 0.19	1.83 ± 0.15	1.34 ± 0.74

Quantitative DNA purity

The level of DNA purity to the nucleotide acid content is measured based on the wavelength A260/A280 nm ratio. Based on the study (Table 2), DNA purification from samples of young leaf and callus with one and two times ethanol washing obtained an average purification level of 1.83 ± 0.15 to 1.99 ± 0.18. The purity of DNA in young leaf samples 1x washing was obtained at a rateof 1.66 to 2.11. DNA purification was best in callus treatment with 1x washing, with a rate of 1.78 to 1.95 compared to 2x washing. From eachtreatment, a good purity has been obtained for molecular analysis.

Detection of the presence of contamination of organic compounds in DNA is known through the purity of the DNA at a wavelength ratio of A260/A230 nm. Table 2 showed that the average DNA purity in young leaves with 2x washing was higher than 1x ethanol washing about 2.1 ± 0.42 and 1.77 ± 0.37, respectively. In callus DNA, the level of DNA purity for organic compounds obtained low values from the two methods (0.90 ± 0.19 and 1.34 ± 0.74), respectively. This value indicates that the purity of leaf DNA at A260/A230 nm ratio was sufficient than callus DNA.

High-quality DNA is characterized by the appearance of single absorbance peak at a wavelength of 260 nm on the nano-photometer profile (Fig. 2). In the young leaf with 1x washing, the absorbance value at wavelength A260 nm was obtained between 5.02 to 15.48 (Fig. 2A). In contrast, in 2x washing the absorbance value was obtained between 2.02 to 13.62 (Fig. 2B). In the soybean callus sample with 1x washing, the absorbance value was obtained at a wavelength of A260 between 3.06 to 18.51 (Fig. 2C). In addition, on 2x washing, the absorbance value was obtained between 3.03 to 14.79 (Fig. 2D).

Qualitative DNA by agarose gel electrophoresis

Based on the results of DNA qualitative analysis on the Visualization of 1.5% agarose gel electrophoresis. It showed that there was a single band as a sign of the molecular weight of DNA at a size of more than 10 kb (Fig. 3). Visualization results on young leaf soybean DNA showed that the DNA bands were sufficiently free of contaminants in 1x washing, namely line 1, 2, 4, 5 and 6, while in 2x washing in line 2, 4 and 5 (Fig. 3A & B). In addition, the visual quality of callus DNA with 1x washing was quite good in each sample (Fig. 3C). In contrast, in 2x washing, it was obtained in line 1, 2, 3, and 5 (Fig. 3D). This indicates that the quality of the samples DNA was sufficient for DNA amplification.

The visual results of genomic DNA still have smearing under the DNA bands in several DNA samples, namely line 3 (Fig. 3A), line 1, 3, and 6 (Fig. 3B), lines 4 (Fig. 3C), and line 4 and 6 (Fig. 3D). This showed that there was still contamination of organic compounds and RNA that was still carried by DNA which is affected by the level of purity of the DNA sample (Table 2).

Discussion

Rapid modification of DNA extraction using CTAB in this study has been successfully carried out by reducing the duration of each stage. In this study, the extraction duration only required 70 min to obtain high-quality DNA. It showed that reducing the incubation duration to 20 min and precipitation to 15 min effectively extracted soybean DNA. This modification method is expected to be applied to other plants. Doyle and Doyle[21]. reported that the basic CTAB protocol takes 48-72 h to obtain high-quality DNA. In addition, the long duration required for the incubation stage is up to 60 min and precipitation lasts overnight[22]. CTAB is often used as a DNA extraction method in various

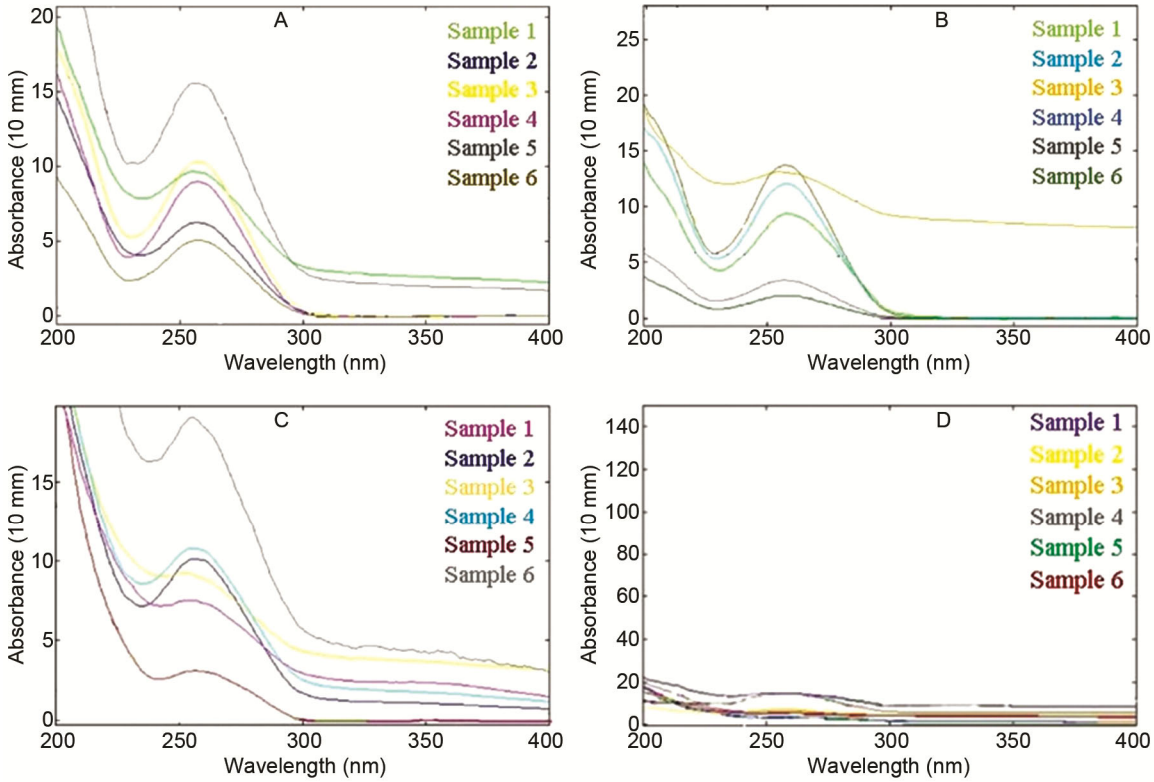

Fig. 2 — Nanophotometer measurement profile of genomic DNA extractions from soybean tissue using modified CTAB protocol. (A) Soybean leaves sample 1x washing; (B) Soybean leaves sample 2x washing; (C) soybean callus sample 1x washing; and (D) soybean callus 2x washing

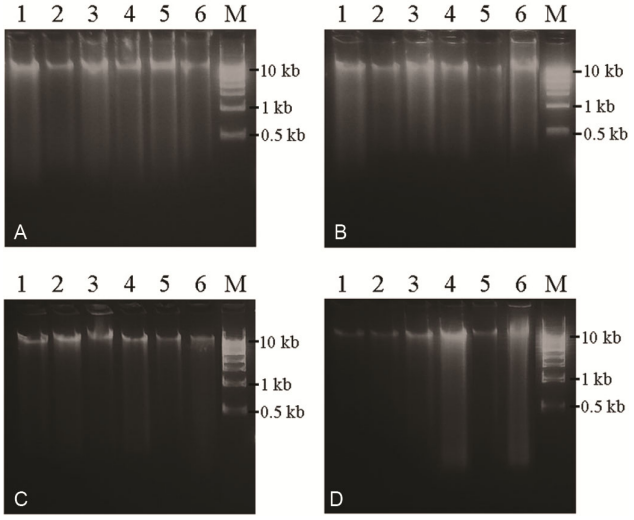

Fig. 3 — Visualize genomic DNA on 1.5% agarose gel electrophoresis of soybean (A) young leaf with 1x washing; (B) young leaf with 2x washing; (C) callus with 1x washing; and (D) callus with 2x washing), lines 1-6: number of samples, M: ladder 1 kb

plants because it is easy to do and costs less than the expensive commercial kits[19,30]. Modification of 65°C incubation time at 15-30 min and precipitation between 15-20 min has been reported with the acquisition of good DNA quality in cashew leaves (*Anacardium occidentale*)[27] and low-quality in soybean seeds[18]. Furthermore, modification of rapid DNA extraction takes 5-6 h in *A. thaliana* and *Z. mays* L.[33].

The ethanol washing step, which was only carried out once, was influential in obtaining high-quality DNA. The pellet washing step using ethanol is generally carried out twice[27]. The washing step removes residual sodium, polysaccharides, and organic compounds still attached to the DNA pellet. Ethanol absolute and 70% ethanol are effective for washing DNA pellets. Ethanol works to induce changes in the structure of the DNA molecule, which causes it to aggregate and precipitate to the bottom of the solution to form pellets[30].

The DNA lysis stage using mortar and cold pestle without liquid nitrogen is sufficient to maintain DNA quality from leaves or calluses. Cold mortar aims to avoid damage to DNA which is quickly degraded. Sample grinding that has not been maximized is directly proportional to the results of DNA concentration. Nugroho *et al*.[4] reported that DNA extraction without liquid nitrogen with two times

ethanol washing was effectively used to obtain high-quality DNA in Jatropha spp. Leaves. In addition, Sahu *et al*.[26] reported that extraction without liquid nitrogen has been successfully carried out on mangrove leaves (*Rhizophora* spp.), which contain high polysaccharides and phenolics.

The use of chemical material is needed to optimize the quantity and quality of DNA. The addition of EDTA damages the cell wall by binding to magnesium ions to maintain cell integrity and DNA integrity[34]. In addition, NaCl serves to remove proteins and carbohydrates from DNA. Then, NaCl effectively inhibits the co-precipitation of polysaccharides and DNA[35]. In addition, anti-browning compounds such as PVP and β-mercaptoethanol can be used to reduce the content of organic compounds and phenols[30].

Soybean leaves contain high polysaccharides[26]. Using the right concentration of PVP can reduce phenolic oxidation and the level of phenolic contamination in DNA. PVP forms complex compounds through hydrogen bonds with phenolics which will be deposited on debris, thus preventing integration between phenol and DNA[36]. Adding 2% PVP is effective for obtaining high-quality DNA in *Cosmos sulphureus* Cav., which contains high phenolic compounds[25,37]. Abdel-Latif and Osman[29] reported that adding 1%, PVP resulted in low-quality DNA of *Z. mays* L.

Genomic DNA can be obtained from all plant organs, such as leaves, stems, flowers, roots, seeds, and calluses[38]. Leaves are often used as DNA extraction samples because they are available in large quantities and are easier to obtain than other plant organs. Young leaves are the optimal sample because they still contain low phenolic compounds, so they can minimize polysaccharide and phenolic contamination to obtain good DNA quality compared to mature leaves[24]. In addition, callus results from cell division in plant explants in tissue culture[39]. It is used as a proxy for *in vitro* propagation and micropropagation.

The concentration and purity of DNA on a nano-photometer were analyzed by comparing the absorbance of nucleic acids and proteins at a wavelength of 260 nm and 280 nm, respectively. DNA concentration was measured to determine the amount of DNA molecule content in the elution solution. In addition, DNA concentration was measured based on the principle of absorption of ultraviolet light at a wavelength of 260 nm[40]. The UV absorbance method is easily interfered with by contaminants and fails to distinguish dsDNA from single-stranded DNA and RNA; however, the ratio of different wavelengths of UV absorption could be used to determine the quality of a sample[38,41]. Abdel-Latif and Osman[29] reported that good DNA purity was obtained at the A260 nm /A280 nm ratio between 1.8-2.0. Tiwari *et al*.[30] added that if the purity is more than 2.0, it indicates that RNA is still present, whereas if it is less than 1.7 indicates there is contamination of protein, phenol, and polysaccharides[42]. The A260/A230 nm ratio is used to detect the presence of organic compounds that DNA still carries with a good ratio between 2.0-2.2[43]. AL-Amery *et al*.[18] reported that polysaccharide contamination could inhibit primary amplification and annealing. In addition, a good quantity of DNA must have a DNA concentration of more than 100 ng/μL[44].

The results of genomic DNA visualization on agarose gel electrophoresis sometimes differ from those of spectrophotometer quantification. The spectrophotometer results are strongly influenced by the solvent components present in the stock DNA solution, such as phenol, polysaccharides, and proteins. At the wavelengths A260/A280 nm ratio, the concentration of DNA will be read, but in these conditions, the dissolved DNA contaminants significantly affect the stability of the resulting DNA concentration[44]. These contaminants cause the absorption of the 280 nm wavelength to increase so that the absorbance ratio gets lower, which causes the absorbance value to be below the set number. In gel electrophoresis, contaminants cause the appearance of a white mist or smear under the DNA band. In addition, smearing under the band can separate the DNA fragment from other components that dissolve in the DNA sample[24,42]. In addition, the appearance of a smear can be caused by an inaccurate agarose gel concentration. However, visualization of DNA band is generally effective on 1% agarose gel[45].

Conclusion

Rapid modification of DNA extraction using the CTAB method with 1x washing ethanol absolute on tissue organ of young leaves and callus obtained high-quality DNA with DNA concentrations of 458.72 ± 155 ng/μL and 363.87 ± 205 ng/μL, respectively. DNA purity ratio A260/280 obtained 1.99 ± 0.16 and 1.86 ± 0.07, respectively. Good DNA quality was

indicated by the absence of smears on the 1.5% agarose gel electrophoresis. This study demonstrated that the process of extracting DNA from soybean plants only takes 70 min to obtain high-quality DNA.

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Conflict of interest

All authors declare no conflict of interest.

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